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Two Distinct Cell Types Identified in the Ascidian Notochord

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ABSTRACT—We established a monoclonal antibody, designated CiNot-1, that recognizes ascidian *Ciona* notochordal cells in the early tailbud embryo. It distinguished two types of cells in the notochord, according to whether or not they expressed CiNot-1 antigen. Only A-line notochord cells expressed CiNot-1 antigen in cleavage-arrested embryos. Since A- and the B-line notochord precursor cells respond differently to inductive signals, CiNot-1 distinguished two specification mechanisms in the notochord. After the middle tailbud stage, CiNot-1 recognized the nerve cord and endodermal strand in addition to the notochord. In these tissues, several cells in the distal tip of the tail did not express CiNot-1 antigen. This revealed the distinct feature of unity in the distal tip of the larval tail and suggests the specification mechanism of this region.

INTRODUCTION

The eggs of ascidians (subphylum Urochordata, class Ascidiacea) offer an ideal experimental system with which to study the cellular and molecular mechanisms involved in the specification of embryonic cells (reviewed by Jeffery, 1985; Uzman and Jeffery, 1986; Satoh *et al.*, 1990; Swalla, 1990; Nishida, 1992a; Satoh, 1994). Eggs develop rapidly into tadpole larva that consist of comparatively few cells (about 2500), and only a few distinct types of differentiated cells. The cleavage pattern is quite determinate and the cell lineage has been well documented (Nishida and Satoh, 1985; Nishida, 1986, 1987). Since the presumptive cells of various tissues are distinguishable from a very early stage of embryogenesis, unique experimental and molecular approaches can be applied to analyze the mechanisms of cell differentiation.

The molecular and cellular mechanisms underlying muscle cell differentiation (Jeffery and Swalla, 1992; Tanaka and Nishikata, 1994), the epidermal cell differentiation (Nishida, 1993; Ueki and Satoh, 1995), the neural cell differentiation (Nishida, 1991; Okamura *et al.*, 1994), endodermal cell differentiation (Nishida, 1993), trunk lateral cell differentiation (Nishikata and Satoh, 1991) and notochord cell differentiation (Yasuo and Satoh, 1993; Nakatani and Nishida, 1994) have been studied in detail. The notochord offers a good experimental system for two reasons. In principle, the notochord is one of the major features that characterize the chordata (urochordates, cephalochordates and vertebrates) and is an important organ from an evolutionary perspective. *As-T*, which is the ascidian homolog of the mouse *Brachyury* (*T*) gene, has been cloned and it is expressed exclusively in fate-restricted notochord cells (Yasuo and Satoh, 1993, 1994). This offers a key advantage for molecular biological analyses of notochord cell development.

In addition, inductive interaction is necessary for notochord cell differentiation (Nishida, 1992b; Nakatani and Nishida, 1994). Two mechanisms may be involved in notochord development (Nakatani and Nishida, 1994). Precise analysis of the notochord differentiation process is very important and molecular probes are required to distinguish differences in the notochord.

We established hybridoma clone (1G9) producing a monoclonal antibody (CiNot-1) that recognizes the anterior portion of the notochord but not its posterior tip. Detailed observations of CiNot-1 expression in normal and cleavage-arrested embryos revealed that CiNot-1 was expressed only in the A-line (lineage) notochord cells indicating that there are two cell types in the notochord. This antibody will be a good marker with which to distinguish two cell types related to the origin of notochord cells. Moreover, CiNot-1 was also expressed in the nerve cord and endodermal strand adjacent to A- but not the B-line notochord cells. This staining profile revealed a distinct feature of the posterior tail tip region.

MATERIALS AND METHODS

Eggs and embryos

The ascidian *Ciona intestinalis* was collected near the Onagawa Fisheries Laboratory (Tohoku University, Onagawa Bay, Japan), Ushimado Marine Biological Laboratory (Okayama University, Ushimado, Japan), Mukaishima Marine Biological Laboratory (Hiroshima University, Mukaishima island, Japan) and Kobe University of Mercantile Marine (Kobe, Japan). Adults were maintained in aquaria (13°C) under constant light to induce oocyte maturation. Eggs were removed surgically from the gonoducts and washed with Millipore-filtered natural seawater. Eggs were inseminated with a diluted sperm suspension from other individuals activated by NaOH. Fertilized eggs were reared at 16°C. At this temperature, the first cleavage occured

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within about 60 min and tadpole larvae hatched about 20 hr after insemination.

Production of monoclonal antibody

Monoclonal antibodies were produced as described by Mita-Miyazawa *et al.* (1987) with minor modifications. Swimming tadpole larvae were collected and sonicated in ice-cold physiological saline (0.9% NaCl). After centrifugation at 10,000 × g for 10 min, the supernatant was injected intraperitoneally into BALB/c mice four times. Three days after the last immunization, mouse spleen cells were fused with myeloma cells (Sp2) using polyethylene glycol #4000 (Nacalai Tesque). The hybridoma cells were cultured in HAT medium, samples of which were assayed by immunofluorescence microscopy. Hybridoma cells producing the desired antibodies were cloned twice by selecting a single cell with a small pipette under an inverted microscope.

Fixation and immunofluorescence staining

Ciona eggs and embryos at the desired stages were fixed with a mixture of cold methanol:acetic acid (8:2) for 20 min followed by cold absolute ethanol. To screen the hybridoma supernatant, large quantities of fixed specimens of eggs and embryos were embedded in polyester wax (Steedman, 1957; BDH Chem. Ltd.), sectioned at 4 μm and mounted on small coverslips. To prepare whole mount samples, fixed specimens were extensively washed with phosphate-buffered saline (PBS). Both sectioned and whole mount specimens were incubated with CiNot-1 monoclonal antibody diluted (1:1) with PBS containing 0.1% blocking reagent (Boehringer). The BODIPY-FL goat anti-mouse IgG(H+L) conjugate (Molecular Probes, Inc.) was used as the secondary antibody. After immunohistological staining, specimens were clarified with methyl salicylate and mounted in methyl salicylate containing DABCO (1,4-diazabicyclo-[2.2.2]octane, Sigma).

Microscopy and observation

Cells were screened and analyzed using epifluorescence (Olympus: BSH-RFC) and laser scanning confocal microscopes respectively (Meridian: model INSIGHTplus-IQ system). Using the SFP procedure of INSIGHT-IQ analysis software (Meridian), whole mounted specimens were reconstructed from the images of each optical section.

Inhibition of cleavage

Cleavage of embryos was permanently arrested with 2 μg/ml of cytochalasin B (Sigma). At this concentration, cytochalasin B completely blocks the cytokinesis of ascidian embryos but nuclear division continues in cleavage-arrested blastomeres (Satoh and Ikegami, 1981).

Glycopeptidase F digestion

Early and middle tailbud embryos were homogenized in 20 mM phosphate buffer (pH 7.2) containing 50 mM EDTA. The homogenate was incubated at 37°C overnight with or without glycopeptidase F (Wako). The same volume of sample buffer containing SDS (0.0625 M Tris-HCl, pH 6.8; 5% 2-mercaptoethanol; 10% glycerol; 0.5 μM PMSF and 2.3% SDS) was added to both homogenates, which were then separated by electrophoresis according to Laemmli (1970). The separated proteins were electrophoretically blotted onto a nitrocellulose membrane, that was exposed to CiNot-1 containing hybridoma culture medium. The membrane was then soaked with alkaline phosphatase conjugated goat anti-mouse IgG+A+M (Zymed), then reacted with X-phosphate (5-bromo-4-chloro-3-indolyl phosphate, Wako) and NBT (nitroblue tetrazolium, Wako).

RESULTS

Notochord cell lineage

The notochord of an ascidian tadpole larva is composed of 40 cells aligned longitudinally in single file along the center of the tail. A filamentous basal lamina termed the "notochordal sheath" covers the entire notochord. The notochord is surrounded dorsally by the nerve cord, ventrally by the endodermal strand and laterally by the muscle cells. Cell-lineage studies have revealed that 32 notochord cells in the anterior and middle parts of the tail are derived from the A4.1 pair, while eight notochord cells in the posterior region of the tail are derived from the B4.1 pair (Nishida and Satoh, 1985; Nishida, 1987). The A-line notochord potential is asymmetrically inherited by four blastomeres of the 16-cell embryo (A5.1 and A5.2 pairs), the 32-cell embryo (A6.2 and A6.4 pairs) and the 64-cell embryo (A7.3 and A7.7 pairs) and also by eight blastomeres of the 110-cell embryo (A8.5, A8.6, A8.13 and A8.14 pairs). Four notochord precursor cells of the 64-cell embryo are restricted to the notochord and divide three times to form 32 notochord cells. On the other hand, the B-line notochord potential is inherited by a single blastomere pair of the 16-cell embryo (B5.1 pair) through the 110-cell embryo (B8.6 pair). The B8.6 pair became restricted to the notochord, giving rise to eight notochord cells (Fig. 1).

CiNot-1 recognizes notochord cells

We established a hybridoma clone that secretes a monoclonal antibody, designated CiNot-1. This antibody recognized notochord cells in *Ciona intestinalis* embryos. The antigen recognized by CiNot-1 first appeared exclusively in the notochord cells at the initial tailbud stage (Fig. 2a). At this stage, notochord cells were starting to align in the center of the embryo and intercalate. The entire notochord was covered with the notochordal sheath. Notochord cells and the notochordal sheath were stained except for the distal tip region of both. In the middle tailbud stage, CiNot-1 stained the cell surface of a wide variety of tissues and cells including the brain, nerve cord, endodermal strand and some neural cells, in addition to the notochord cells and notochordal sheath (Fig. 2b). The distal tip region of the notochord, nerve cord and endodermal strand was still negative for the CiNot-1 antigen expression. The posterior limit of antigen-positive cells in these tissues seemed to be at the same position. The distal tip region of these tissues in the swimming tadpole did not yet express the antigen (data not shown).

The CiNot-1 antibody stained high molecular weight smear bands on Western blots and migrated in electrophoresis gels with the early and the middle tailbud embryo total proteins. Furthermore, glycopeptidase F digestion of these proteins reduced the molecular weight of the stained bands (data not shown). These results suggested that CiNot-1 antigen is a glycoprotein.

Two phenotypes were found in the notochord

In the middle tailbud embryo, as the antigen positive notochord cells numbered no more than 32, the estimated number of antigen-negative notochord cells was eight (Fig. 2b). These numbers of antigen positive and negative notochord cells were in good accordance with the numbers of the A- and

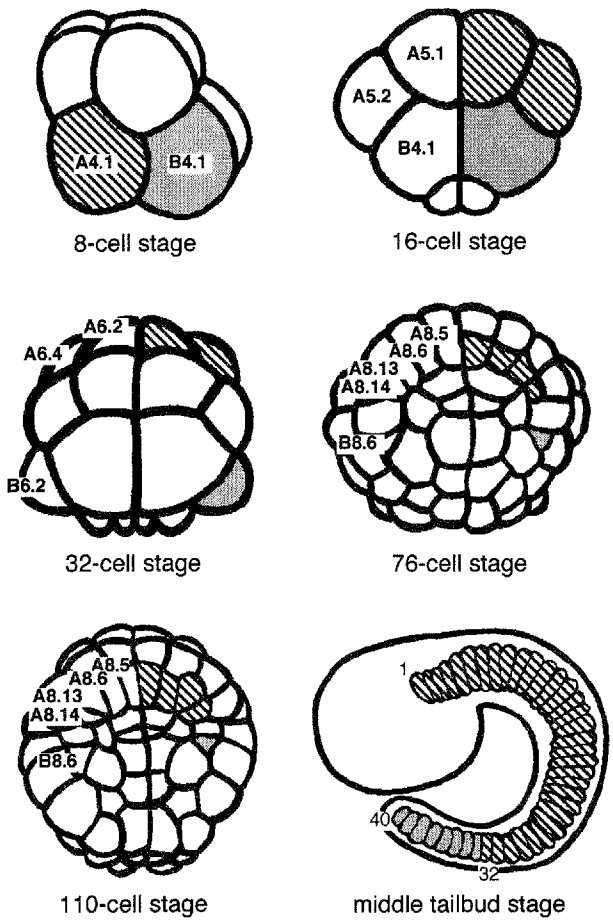


Fig. 1. Cell lineage of the notochord. Presumptive notochord blastomeres in the 8- (right side view), 16-, 32-, 76- and 110- (ventral view) cell stage embryos and notochord cells in the middle tailbud embryo (left side view) are indicated. As the cleavage of the ascidian embryo is bilaterally symmetrical, only the right side of the A-line notochord cells are stippled and B-line cells are hatched. The name of each notochord precursor blastomere is shown on left side of the embryo. A-line blastomeres are anterior-ventrally situated. B-line notochord precursor blastomeres are posterior-ventrally situated apart from A-line precursor cells in the 32-, 76- and 110-cell stage embryos.

B-line notochord cells, respectively. This suggested that CiNot-1 antigen was expressed only in A-line notochord cells. At the initial tailbud stage, although it is difficult to precisely count the antigen positive cells, more than five antigen-negative cells were found in the tip of the notochord (Fig. 2a). At the swimming tadpole stage, though it was also difficult to identify antigen negative cells because of being surrounded by positive cells, several cells situated at the posterior tip of the notochord did not express the antigen (data not shown). These results suggested that only A-line cells expressed CiNot-1 antigen in the notochord throughout early development. In other words, A- and B-line notochord cells differ in terms of CiNot-1 antigen expression.

CiNot-1 expression in cleavage-arrested embryos

To obtain more insight, we identified CiNot-1 expressing cells in cleavage-arrested embryos. Embryos were arrested at desired stages in seawater containing cytochalasin B and reared until the control embryos reached the middle tailbud stage. CiNot-1 expression was examined in these cleavage-arrested embryos. The results are shown as a graph (Fig. 3). The number of the antigen positive blastomeres increased with the developmental stage. Most of the embryos arrested at the 8-cell stage expressed the CiNot-1 antigen in two blastomeres. Most of the embryos arrested at the 16-, 32- and 64-cell stages developed CiNot-1 antigen in four or five blastomeres. Most cleavage-arrested 110-cell embryos expressed CiNot-1 antigen in eight blastomeres. These numbers were in good accordance with those of the A-line notochord precursor cells at each stage. Judging from their size and position in the cleavage-arrested embryos, these antigen positive cells seemed to be A-line blastomeres. These were the A4.1 pair of the arrested 8-cell embryo; the A5.1 and A5.2, A6.2 and A6.4, A7.3 and A7.7 pairs of the arrested 16-, 32- and 64-cell embryos, respectively; A8.5, A8.6, A8.13 and A8.14 pairs of the arrested 110-cell embryo (Fig. 2c; compare with Fig. 1). However, blastomeres other than A-line precursors expressed CiNot-1 antigen in some specimens. In all these embryos, extra CiNot-1 positive blastomeres always appeared in contact with A-line notochord precursor cells. According to a cell lineage analysis (Nishida, 1987), the B-line presumptive-notochord blastomeres are situated in the posterior half of the embryo apart from the A-line notochord precursors. These blastomeres were identified as nerve cord, and not B-line notochord precursors. We concluded that within the notochord, CiNot-1 was expressed only in the A-line cells.

CiNot-1 expression in other tissues

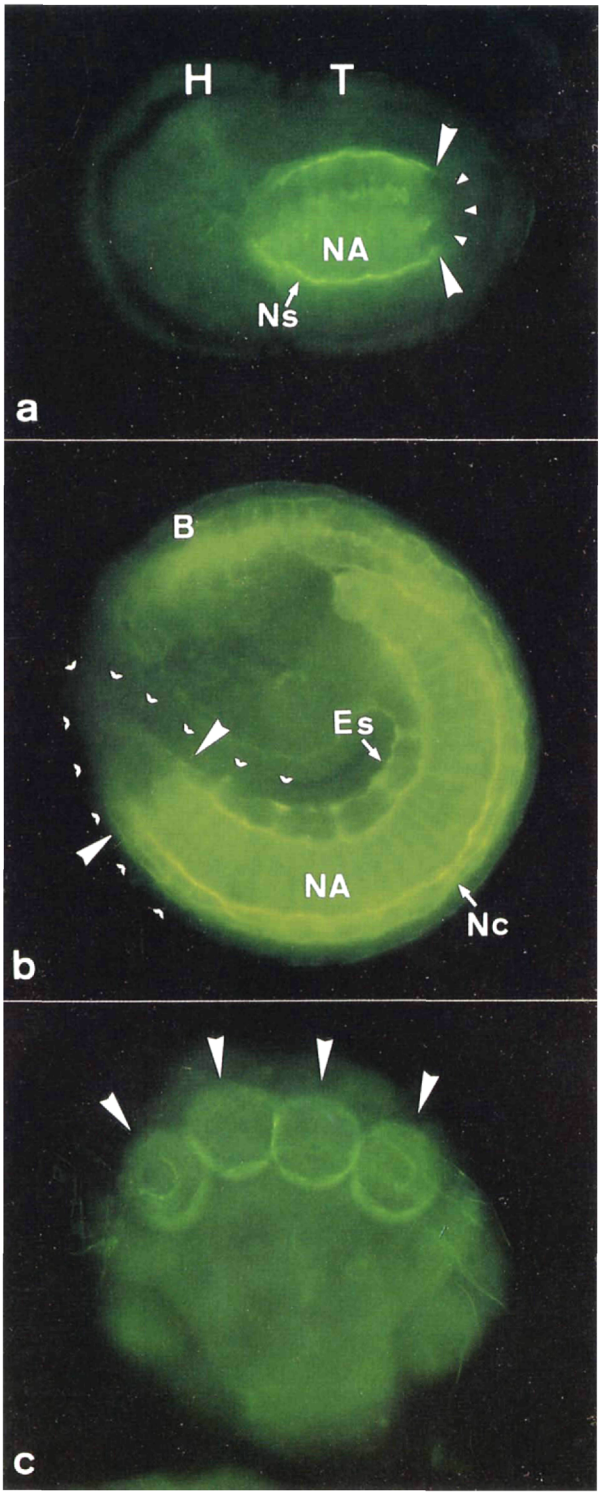
From the middle tailbud stage, CiNot-1 is expressed in several tissues besides the notochord. One feature of this antigen expression was noted in the distal tip region of the larval tail. The CiNot-1 antigen was expressed in the anterior and the middle portion of both the endodermal strand and the nerve cord, but not in the posterior tip region of either. The position of the posterior border of the antigen expression in these tissues was the same as that in the notochord cells. Moreover, the notochordal sheath surrounded the B-line notochord cells, but it was not stained with CiNot-1 antibody. Epidermal cells did not express CiNot-1 antigen. Thus, the internal structures of the larval tail were divided into the CiNot-1 positive anterior portion and the CiNot-1 negative posterior tip. This showed that the internal distal tip region of the tail differed from the other region of the tail in the ability to express CiNot-1 antigen.

DISCUSSION

Using a monoclonal antibody we showed that *Ciona* notochord cells consisted of two phenotypes. This difference might originate from their lineage. Moreover, a small region of

the posterior tip of the larval tail had a distinctive feature. As this small region consisted of at least three tissues, the mechanisms that make and maintain the different phenotypes may influence these tissues at the same time.

CiNot-1 antigen was not a differentiation marker of the notochord, as it recognized some other tissues at later stages. SDS-PAGE indicated that CiNot-1 antigen is a glycoprotein.



However, CiNot-1 revealed that the notochord cells could be divided into two types according to whether or not they expressed the antigen. This also suggested that the notochord cells differentially control their own gene expression according to their position.

Moreover, these two phenotypes in the notochord coincided with their cell lineages, in that the only A-line cells expressed CiNot-1 antigen. These results were supported by the findings that the B-line posterior tip notochord cells of *Ciona* and *Styela* express alkaline phosphatase enzyme activity (Whittaker, 1977, 1990; Bates and Jeffery, 1987). These results predict lineage-specific cell specification mechanisms in notochord development. A study of the cleavage-arrested and partial embryos also supports this notion (Nishikata and Satoh, 1990). Moreover, Nakatani and Nishida (1994) suggested that A-line notochord cell development requires inductive signals that differ from those that induce B-line presumptive notochord blastomeres. Notochord-precursor cells of the B-line give rise to only 20% of all notochord cells that are located at the tip of the larval tail (Nishida, 1987). During the specification of muscle cells, the mechanism of determination of secondary muscle, which is located at the tip of the tail, is different from that of primary muscle, which is derived from the major muscle-lineage cells and located in the anterior and middle part of the tail (Nishida, 1990). These findings suggested that the posterior tip of the larval tail develops in a manner distinct from the rest of the tail.

CiNot-1 expression also indicated a distinct region in the posterior tip of the larval tail, that is, the CiNot-1 negative region of the notochord, the nerve cord and the endodermal strand. The biological meaning of this region is yet to be understood. The fact that the secondary muscle cells are also situated at the posterior tip reinforced the notion that the posterior tip of the tail has a key role in the ascidian development.

Both the endodermal strand and the nerve cord, like the notochord, were composed of two cell lineages. Notochord

Fig. 2. Whole mount specimens of *Ciona* normal embryos were stained with CiNot-1 monoclonal antibody and observed under a confocal microscope. (a) Initial tailbud stage. Notochord cells (NA) and notochordal sheath (Ns) situated in the center of the tail region were stained. Several cells and the notochordal sheath located at the posterior tip of the notochord were not stained (small arrowheads). The border between the stained and the unstained notochord cells is indicated by large arrowheads. H and T indicate the trunk (head) region and tail, respectively. (b) Middle tailbud stage. The staining profile of the notochord aligned in a row at the center of the tail, was the same as that during the initial tailbud stage. The outline of the caudal tip of the tail is shown by thin arrowheads. The large arrowheads indicate the posterior border of the A-line notochord cells. In addition to the notochord, endodermal strand (Es), nerve cord (Nc), brain (B) and several neural cells were also stained. Part of the endodermal strand and nerve cord that were adjacent to the CiNot-1 negative notochord cells also did not express CiNot-1 antigen. (c) Cleavage-arrested 32-cell stage embryo. Four presumptive notochord blastomeres were brightly stained (arrowheads). Since they were in contact and made a row, they were supposed to be A6.4 and A6.2 pairs.

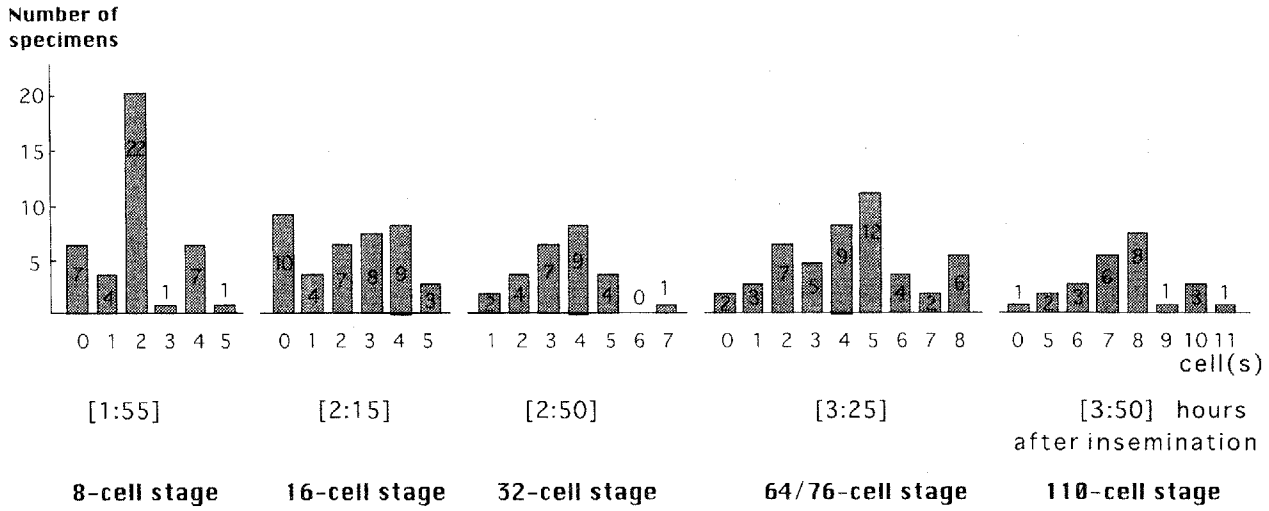


Fig. 3. CiNot-1 antigen expression in cleavage-arrested blastomeres. These graphs show the number of the specimens bearing the indicated number of CiNot-1 antigen positive blastomeres. Several batches of embryos were cleavage-arrested from the indicated time points after insemination. Each time point corresponds to the indicated developmental stage. Each graph was obtained from several clutches of cleavage-arrested embryos. Since the real 64-cell stage is about 5 min long, the specimens cleavage-arrested from 3:25 after insemination contain 64- and 76-cell stage embryos.

cells are derived from A4.1 and B4.1, endodermal strand cells from B4.1 and b4.2 and nerve cord cells from A4.1 and b4.2. A-line nerve cord precursor cells give rise to the ventral and lateral part of the entire nerve cord, while b-line nerve cord precursor cells give rise to the dorsal part (Nishida, 1987). This suggested that the lineage difference in each tissue did not coincide with a difference in CiNot-1 expression. The mechanism that generates a cell type difference at specific positions in the endodermal strand, the nerve cord and the notochord, remains unknown. However, there could be a regulatory mechanism that controls CiNot-1 expression throughout several tissues.

CiNot-1 was first expressed in the A-line notochord cells, then in the nerve cord and the endodermal strand neighboring them. The posterior border of the CiNot-1 expression in these tissues was the same. Although, the possibility cannot be denied that a separate mechanism is involved in the specification of each tissue, A-line notochord cells and the neighboring CiNot-1 positive nerve cord and endodermal strand may share a common mechanism. The coincidence of the expressed region and the sequential expression of CiNot-1 antigen supports this notion. Since CiNot-1 antigen was expressed on the cell surface and the basement membrane, it may be a membrane protein that mediates this common specification mechanism.

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