Muscle cell differentiation in ascidian embryos analysed with a tissue-specific monoclonal antibody

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Summary

Utilizing a muscle-specific monoclonal antibody (Mu-2) as a probe, we analysed developmental mechanisms involved in muscle cell differentiation in ascidian embryos. The antigen recognized by Mu-2 was a single polypeptide with a relative molecular mass of about 220\times10^3. It first appeared at the early tailbud stage and continued to be expressed until the swimming larva stage. There were distinct and separate puromycin and actinomycin D sensitivity periods during the occurrence of the antigen, suggesting the new synthesis of the polypeptide by developing muscle cells. Embryos that had been permanently arrested with aphidicolin in the early cleavage stages up to the 32-cell stage did not express the antigen. DNA replications may be required for the antigen expression. Embryos that had been arrested with cytochalasin B in the 8-cell and later stages developed the antigen, and the number and position of the arrested blastomeres exhibiting the differentiation marker almost corresponded to those of the B4.1-line muscle lineage. Furthermore, in quarter embryos developed from each blastomere pair isolated from the 8-cell embryo, all the B4.1 as well as a part of b4.2 partial embryos expressed the antigen, while the a4.2 and A4.1 partial embryos did not show the antigen expression. These results may provide further support for the existence of cytoplasmic determinants for muscle cell differentiation in this mosaic egg.

Key words: ascidian embryo, muscle differentiation, monoclonal antibody, determinants, DNA replication.

Introduction

The embryological development of ascidians is characterized by its typical mosaic pattern of differentiation. Due to the accumulating evidence for the presence of cytoplasmic determinants responsible for cell differentiation (reviews by Whittaker, 1979; Jeffery, 1984; Uzman & Jeffery, 1986) and due to the supposed existence of a DNA replication cycle during which such determinants may interact with the appropriate genes responsible for the specification of embryonic cells (Satoh & Ikiyama, 1981a; Mita-Miyazawa, Ikiyama & Satoh, 1985), the cellular and molecular mechanisms underlying muscle cell differentiation in ascidian embryos seem likely to become major subjects of current developmental biology.

Differentiation of muscle cells in ascidian embryos has hitherto been analysed mainly by assessing the development of histochemically detectable muscle-specific acetylcholinesterase (AChE) (e.g. Whittaker, 1973; Satoh, 1979), although this differentiation has also been investigated by observing the occurrence of myofibrils (e.g. Pucci-Minafra & Ortolani, 1968; Crowther & Whittaker, 1983) and muscle-specific membrane excitability (e.g. Hirano, Takahashi & Yamashita, 1984). However, these markers are not always used directly in further molecular studies on the cell specification. In a previous study, in order to obtain more-specific immunological probes for studying cellular and molecular mechanisms involved in muscle cell differentiation in ascidian embryos, we produced several monoclonal antibodies that specifically recognized muscle cells of developing Halocynthia roretzi embryos (Mita-Miyazawa, Nishikata & Satoh, 1987).

In the present study, we first observed that the antigen recognized by one of the muscle-specific monoclonal antibodies, Mu-2, is a single and newly synthesized polypeptide and that its expression is
restricted to developing muscle cells, i.e. the Mu-2 antigen can be used as a muscle differentiation marker of ascidian embryos. Thereafter, utilizing this immunological probe, we analysed the developmental mechanisms involved in muscle cell differentiation in ascidian embryos by asking the following questions. (1) Do cleavage-arrested embryos with cytochalasin B synthesize the antigenic polypeptide, similar to the case of AChE (Whittaker, 1973; Satoh, 1979) and myofibrils (Crowther & Whittaker, 1983) and, if so, which blastomeres express the antigen? (2) Do quarter embryos derived from each blastomere pair of an 8-cell embryo produce the antigen and, if so, which quarter embryos? (3) Is a certain number of DNA replications required for development of the muscle-specific antigen?

**Materials and methods**

**Embryos**

Adults of the ascidian Halocynthia roretzi were collected in the vicinity of Asamushi Marine Biological Station, Mutsu Bay, Aomori, Japan. Naturally spawned eggs were fertilized with a suspension of nonself sperm and raised in filtered seawater at 13-15°C. At this temperature, they developed to gastrulae about 8 h after fertilization, early tailbud embryos at 15 h of development and hatched out about 33 h after fertilization.

**Muscle-specific monoclonal antibody and immunofluorescence staining**

Details of production of a hybridoma clone (3M3A7), producing a muscle-specific monoclonal antibody (Mu-2), and methods for fixation and preparation of sectioned embryos have been described in the accompanying paper (Mita-Miyazawa et al. 1987).

For preparation of whole-mounted samples, eggs and embryos were also fixed for 10 min in methanol (−20°C) then in ethanol (−20°C). After washing with PBS, the samples were surgically dechorionated with sharpened tungsten needles and then treated following the same procedure as for the sectioned specimens.

**Polyacrylamide gel electrophoresis and immunobLOTS**

Homogenates of Halocynthia swimming larvae in SDS (sodium dodecylsulphate)-containing sample buffer (0-0625 M-Tris–HCl, pH 6-8; 5 % 2-mercaptoethanol; 10 % glycerol; 0-5 μM-PMSF and 2-3 % SDS) were separated by electrophoresis in a 10 % polyacrylamide gel (PAGE) presence of 2 % SDS. The separated proteins were electrophoretically blotted onto nitrocellulose paper, which was then soaked in 2-5 % skimmed milk, cut into strips according to the individual lanes, exposed to hybridoma culture medium and washed three times with PBS containing 0-1 % Tween 20 and then three times with PBS without Tween 20. The paper was exposed to 125I-labelled protein A (Amersham) diluted in skimmed-milk-containing PBS. Finally, the nitrocellulose blots were rinsed and washed extensively in PBS, dried and exposed to Fuji RX X-ray film overnight at −80°C.

As a control, rabbit actomyosin purchased from Sigma was similarly electrophoresed.

**Inhibition of protein, RNA and DNA synthesis**

Puromycin di-HCl (Sigma) at 200 μg ml⁻¹ was used as an inhibitor of protein synthesis. At this concentration puromycin blocks 99 % of labelled valine incorporation into the acid-soluble fraction in Ciona embryos (Whittaker, 1966).

Actinomycin D at 20 μg ml⁻¹ causes the maximal (70 %) inhibition of labelled uridine incorporation in ascidian embryos (Smith, 1967), thus preventing the occurrence of histochemically detectable enzyme activity (Whittaker, 1973; Satoh, 1979). We used actinomycin D at this concentration as an inhibitor of RNA synthesis.

Aphidicolin (10 μg ml⁻¹), a specific inhibitor of DNA polymerase, blocks over 85 % of labelled thymidine incorporation, while the incorporation of labelled uridine and valine are unaffected by aphidicolin treatment (Satoh & Ikegami, 1981b). We used aphidicolin at this concentration in order to examine whether DNA synthesis was a prerequisite for the occurrence of muscle-specific antigen. Aphidicolin was a gift from Dr S. Ikegami (Hiroshima University).

**Inhibition of cleavages**

Cytochalasin B (Aldrich) at 2 μg ml⁻¹ was used as a cleavage inhibitor. Cytochalasin B at this concentration completely blocks cytogenesis of ascidian embryonic cells but nuclear division continues (Satoh & Ikegami, 1981b). Cleavage-arrested embryos were cultured until the hatching time of normal control embryos and then fixed for immunocytochemical observations.

**Blastomere isolations**

Blastomere isolations were carried out according to Deno, Nishida & Satoh (1985). Eggs were dechorionated with sharpened tungsten needles between 10 and 30 min after fertilization, and were then cultured to the 8-cell stage in 0-9 % agar-coated Falcon Petri dishes. The four cell pairs of the 8-cell embryo (i.e. A4.2 + A4.2, B4.2 + B4.2, A4.1 + A4.1 and B4.1 + B4.1) were separated with a glass needle under a dissecting microscope. Isolated blastomeres were cultured separately in 0-9 % agar-coated Falcon 24-well multiwells. Millipore-filtered (pore size, 0-2 μm) seawater containing 50 μg ml⁻¹ streptomycin sulphate was used for culture of dechorionated embryos and isolated blastomeres. Isolates were reared until normal embryos hatched and then fixed for immunological examinations.

**Results**

**Occurrence of the muscle-specific antigen during normal development**

We first examined the occurrence and localization of the antigen recognized by a muscle-specific monoclonal antibody, Mu-2. Embryos at various developmental stages were fixed and stained with the Mu-2
antibody by indirect immunofluorescence. The staining pattern is shown in Fig. 1. The antigen recognized with Mu-2 was not detected in the neurula and earlier stages (Fig. 1A). At the early tailbud stage, the antigen became detectable in muscle cells of the elongating tail (Fig. 1B). From this stage to the end of the larval stage (i.e., prior to metamorphosis) the antigen existed exclusively in the tail muscle cells. Cells and tissues other than the muscle did not express the antigen. Fig. 1C,D shows sectioned muscle cells of the middle tailbud-stage embryo. At this stage the whole cytoplasm except for the nucleus stained uniformly and especially bright staining was seen on small granules in the peripheral cytoplasm.

A newly hatched swimming larva also stained with the antibody (Fig. 1E,F). By indirect immunofluorescence staining, the cross striation of myofibrillar components could be seen in the peripheral region of the muscle cells (Fig. 1F, arrowheads). During metamorphosis, the antigenicity disappeared concomitantly with absorption of the tail. However, the antibody intensely stained the muscle fibre of the adult body wall (Fig. 1G).

**Immunoblot analysis of the antigen**

In order to identify an antigenic polypeptide recognized by the Mu-2 antibody obtained, proteins of *Halocynthia* swimming larva as well as rabbit actomyosin (Sigma) were electrophoresed on SDS-polyacrylamide gel (Fig. 2, lanes A and B). Then the ascidian proteins were blotted onto nitrocellulose paper and reacted with the antibody. The Mu-2 antibody reacted with a single band with a relative molecular mass (Mr) of about 220×10^3 (lane C). This band had a similar mobility to the rabbit myosin heavy chain (lane A). Therefore, it is highly probable that the Mu-2 antigen is the myosin heavy chain.

**Requirement of protein and RNA syntheses for the antigen expression**

As the antigen first appears at the early tailbud stage, the antigenic polypeptide seems to be newly synthesized around that period. To determine whether the muscle-specific antigen develops throughout the translational and transcriptional activities, we examined the effects of puromycin and actinomycin D on the antigen expression. Embryos at various developmental stages were immersed in seawater containing each reagent. The results are summarized in Table 1.

When puromycin was added to the early or late gastrula stage embryos, they did not express the Mu-2 antigen. However, when the treatment was started at the neural plate and later stages, the expression of antigen could be clearly detected. A continuous treatment of actinomycin D from the 8-cell, 16-cell and 32-cell stages onward, prevented the development of this antigen. Embryos treated continuously with the inhibitor from the 64-cell and later stages, however, expressed the antigen.

This result suggests that both transcriptional and translational activities are required for the antigen expression, although we cannot exclude the possibility that the mRNA is present earlier and that genes encoding translational control factors are described at these times. When puromycin was added at cleavage stages, embryos stopped further development soon after puromycin treatment. However, gastrula and later stages treated with puromycin continued to develop for a while and then stopped. A similar time lag was noticed in cleavage-stage embryos treated with actinomycin D. This may be due to the slow penetration of the reagents into the cells. Therefore, the stages at which real transcriptional and translational machineries became functional, are likely to be later than those observed in the experiments.

**Expression of the antigen is prevented by aphidicolin**

It has been shown that neither cytokinesis nor nuclear division is required for the occurrence of histochemically detectable muscle AChE, but that a definite number of DNA replication cycles may be a prerequisite for its development, since aphidicolin, a specific inhibitor of DNA synthesis, prevents its occurrence when treated before certain rounds of DNA replications (Satoh & Ikegami, 1981a,b; Mita-Miyazawa et al. 1985). We examined whether several rounds of DNA replications are required for the antigen expression by immersing embryos in aphidicolin. When DNA synthesis was blocked with aphidicolin in the 8-cell, 16-cell and 32-cell stages, they did not express the antigen at all. However, when aphidicolin treatment started at the 64-cell stage, the arrested embryos expressed the antigen in a few blastomeres. In addition, aphidicolin-arrested early gastrula stages showed the antigen expression in more than ten blastomeres.

**Differentiation expression of the antigen in cleavage-arrested embryos**

Recent cell lineage analyses in ascidian embryo have revealed that 14 muscle cells on each side of the tail of a developing *Halocynthia* embryo are derived from the B4.1-cell pair (the posterior vegetal blastomeres) of the 8-cell embryo, whereas the A4.1-cell pair (the anterior vegetal blastomeres) and the b4.2-cell pair (the posterior animal blastomeres) give rise to two and five muscle cells, respectively (Nishida & Satoh, 1983, 1985). The a4.2-cell pair (the anterior animal blastomeres) does not contribute to the formation of muscle cells. In addition, it is well known that
ascidian embryos in which cleavages are permanently arrested with cytochalasin B eventually develop a tissue-specific enzyme, AChE (Whittaker, 1973; Satoh, 1979). Using an inhibitor of cytokinesis, cytochalasin B, we determined whether or not the Mu-2 antigen would be expressed in the cleavage-inhibited embryos.

![Fig. 2. Immunoblot analysis of an antigenic polypeptide recognized with the Mu-2 antigen. Proteins of *Halocynthia* larvae (lane B) and rabbit actomyosin (lane A) were separated in SDS-PAGE. The former was blotted onto nitrocellulose paper and reacted with the antibody (lane C). The protein separation pattern was visualized by Coomassie brilliant blue and the antibody reaction pattern was visualized by an $^{125}$I-labelled protein A. The Mu-2 monoclonal antibody recognized a single polypeptide with a relative molecular mass of about $220 \times 10^3$ (lane C, arrowhead). This band has a similar mobility to the rabbit myosin heavy chain.](image)

**Fig. 1.** Localization and expression of an antigen recognized with the Mu-2 monoclonal antibody on polyester wax sections (A–D,G) and whole mounts (E,F) of the ascidian *Halocynthia roretzi*. (A) Neurula; (B) early tailbud; (C) middle tailbud; (D) enlargement of C; (E) swimming tadpole larva; (F) higher magnification of larval tail; (G) cross section of adult body wall. Scale bar, 50 μm. The antigen expression is not detected in the neurula stage (A), but first appears at the early tailbud stage (B). The antibody stains only the tail muscle cells (C). Notochord (nc) and epidermal cells do not stain. The cytoplasm, especially peripheral small granules (arrows), of muscle cells stains, but the nucleus (n) does not (D). Muscle cells of swimming larval tail (t) are specifically stained, but there is no antigenicity in the head (h) region (E). The total number of muscle cells on the left side of the tail can be counted as 21 (E). In this stage, muscle striation pattern (arrows) is clearly stained at the periphery of the muscle cells (F). In the adult body wall, muscle cells are associated to make bundles within connective tissues (G). Each of the bright spots is a single muscle cell. In such muscle cells, the nucleus also does not stain.

![Fig. 3. Development of the antigen expression in quarter embryos](image)

**Development of the antigen expression in quarter embryos**

We also examined the developmental potential to express the antigen in quarter partial embryos derived from each blastomere pair of the 8-cell embryo.

The results are shown in Fig. 5. In quarter embryos developed from the B4.1 blastomere pair, from which most muscle cells are derived, the antigen expression was evident in all 18 specimens examined (Fig. 5D,H). In addition, 4 out of 27 b4.2 partial embryos, which have the potential to produce ten muscle cells, developed the antigen (Fig. 5B,F). However, the A4.1 partial embryos, which also have the potential to form four muscle cells, did not develop the immunocytochemically detectable antigen expression (0/18; Fig. 5C,G). None of the a4.2 partial embryos (0/16) expressed this antigen (Fig. 5A,E).
Table 1. Expression of the Mu-2 antigen in the presence of puromycin, actinomycin D and aphidicolin

<table>
<thead>
<tr>
<th>Reagents (µg·ml⁻¹)</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>eG</th>
<th>lG</th>
<th>NP</th>
<th>N</th>
<th>eT</th>
</tr>
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<tbody>
<tr>
<td>Puromycin (200)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Actinomycin D (20)</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>Aphidicolin (10)</td>
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The Mu-2 antigen expression was detected by indirect immunofluorescence in whole and sectioned specimens. Abbreviations of developmental stages; 8, 16, 32 and 64 mean 8-, 16-, 32- and 64-cell stages, respectively; eG and lG, early and late gastrula; NP, neural plate; N, neurula; eT, early tailbud.

Fig. 3. Differentiation expression of the Mu-2 antigen in cytochalasin B-arrested Halocynthia embryos. Scale bar, 100 µm. Divisions of embryos at the early cleavage stages up to 64-cell stage were permanently arrested with cytochalasin B. Arrested embryos were reared for more than 25 h, then fixed for indirect immunofluorescence staining. Here we show embryos arrested in the 8-cell (A), 16-cell (B), 32-cell (C) and 64-cell (D) stages. The antigen expression is seen in 2, 4, 6 and 6 cells of the arrested 8-cell, 16-cell, 32-cell and 64-cell stages, respectively. All of these blastomeres are the precursors of muscle cells.

Discussion

As the basis for studying the spatial and temporal regulation of gene expression during the development of ascidian embryos, we have established hybridoma clones which produce monoclonal antibodies specific to muscle cells of Halocynthia embryos (Mita-Miyazawa et al. 1987). As clearly shown in this study, an antigen recognized by the Mu-2 antibody is a single polypeptide of about $220 \times 10^3 \, M_\text{r}$. The antigen can first be detected at the early tailbud stage and exists specifically in the larval tail muscle cells. Although the antigenicity disappears during metamorphosis, the antibody recognizes body wall muscle cells of the adult animal. In addition, this antibody cross reacts with muscle components of another ascidian, Ciona intestinalis, and therefore may be a common component of ascidian muscle cells. Obinata, Ooi & Takano-Ohmuro (1983) have reported that ascidian myosin heavy chain has a similar mobility to that of vertebrates. As shown in the SDS gel electrophoresis, the similar mobility of the Mu-2 antigen to rabbit myosin heavy chain suggests that the Mu-2 antigen is a myosin heavy chain or its closely related component. Meedel (1983) has described the myosin-ATPase expression during the embryogenesis of Ciona intestinalis; the temporal expression pattern is comparable to that of the Mu-2 antigen shown in this study. It is also likely that the Mu-2 antigen is different from AChE, another muscle differentiation marker with a relative molecular mass of 65–70×10^3 on SDS-PAGE (Meedel, 1980). Moreover, the experiments with puromycin and actinomycin D suggest that the Mu-2 antigen is newly
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Fig. 5. Differentiation expression of the Mu-2 antigen in quarter embryos developed from the isolated a4.2 cell pair (A,E), b4.2 cell pair (B,F), A4.1 cell pair (C,G), and B4.1 cell pair (D,H) of the 8-cell Halocynthia embryo. Scale bar, 100 μm. (A–D) Photomicrographs of each quarter embryo. (E–H) Immunofluorescence micrographs of the same quarter embryos of A–D. The B4.1 quarter embryo (H) shows an obvious antigen expression, and the b4.2 quarter embryo (F) also expresses the antigen. The a4.2 and A4.1 quarter embryos, however, do not express the antigen (E,G).

Muscle differentiation in ascidian embryos has been studied intensively with respect to cytoplasmic determinants. Results of descriptive and experimental studies hitherto obtained are as follows. (1) Not only the B4.1 blastomere of an 8-cell embryo but also the b4.2 and A4.1 give rise to muscle cells. In the case of Halocynthia embryos, fourteen muscle cells of the anterior and middle part of each side of the tail are derived from the B4.1, whereas two muscle cells of the posterior tail region originate from the A4.1 and five (or two in the case of C. intestinalis) muscle cells of the caudal tip region from the b4.2 (Nishida & Satoh, 1983, 1985). (2) Cytochalasin-arrested embryos develop AChE and other differentiation markers (e.g. Whittaker, 1973; Crowther & Whittaker, 1983). However, the differentiation markers are expressed only in the arrested B4.1 blastomere but not in the b4.2 and A4.1. (3) Partial embryos originating from isolated B4.1 (the B4.1 quarter embryos) differentiate muscle cells (e.g. Whittaker,.
therefore, only epidermis differentiation markers are cleavage-arrested 1- to 4-cell stages is epidermis and, superior differentiation expression in blastomeres of the first and second questions is as follows: the most differentiation pathway being solely expressed. The answer to is an exclusive event, only one superior differentiation expression in cleavage-arrested Halocynthia blastomeres. We have also emphasized the significance of the pattern of muscle cell differentiation in ascidian embryos is mosaic or autonomous and that cytoplasmic determinants, their existence and accurate segregation, play important roles for the differentiation. However, there are several questions or discrepancies between the results mentioned above. First, why do cytochalasin-arrested 1- to 4-cell stages of H. roretzi fail to develop AChE, muscle-type membrane excitability and a specific antigen? Second, why do cytochalasin-arrested 8-cell and later stages develop the differentiation markers solely in the B4.1, but not in the b4.2 and A4.1? Third, why do A4.1 quarter embryos fail to express the differentiation markers? Fourth, why do the isolated and arrested b4.2 not express the specific antigen in spite of its expression when isolated b4.2 is allowed to develop to a quarter embryo? We have already shown that two elementary events are prerequisites for muscle cell differentiation in ascidian embryos: the existence of cytoplasmic determinants within the cell and a certain number of DNA replications (Mita-Miyazawa et al. 1985), i.e. cell–cell interaction or inductive control is not required, at least for muscle cell differentiation in ascidian embryos. We have also emphasized the significance of the quantity of cytoplasmic determinants within the cell (e.g. Nishida & Satoh, 1983), whereby, in order to accomplish the muscle differentiation, the cell has to have over a threshold concentration of determinants within the cell. Furthermore, differentiation expression in cleavage-arrested Halocynthia embryos is an exclusive event, only one superior differentiation pathway being solely expressed. The answer to the first and second questions is as follows: the most superior differentiation expression in blastomeres of cleavage-arrested 1- to 4-cell stages is epidermis and, therefore, only epidermis differentiation markers are expressed in the arrested cells while muscle differentiation is suppressed (Hirano et al. 1984; Mita-Miyazawa, Nishikata & Satoh, unpublished data). After the third cleavage, muscle determinants segregated into the B4.1 result in a high enough concentration in the cells, whereas small but insufficient amounts of determinants are partitioned into the b4.2, and the A4.1 receive a lesser amount of determinants. Thus, if cleavage is arrested in the 8-cell and later stages, only the B4.1 and its daughter cells express the differentiation markers, and not the b4.2 and A4.1. The third question is interpreted as being due to a lower amount of determinants segregated into the A4.1 and by further inadequate segregation of determinants during formation of the A4.1 quarter embryo. The answer to the fourth question is as follows: as mentioned earlier, the b4.2 receives only small amounts of determinants. Therefore, when the b4.2 is arrested with cytochalasin immediately after isolation, it does not differentiate muscle cells. However, if isolated b4.2 is allowed to divide, determinants may be segregated and concentrated into certain cells of the muscle lineage, which therefore may develop the muscle markers. Thus, all these experimental results may be explained in terms of the concentration of cytoplasmic determinants within the cell.

In this study, we have analysed muscle cell differentiation in ascidian embryos with a specific immunological probe. The immunological method is easier than electron microscopic observation on the myofibril development or the measuring of the membrane excitation properties. The specificity of this immunological probe for assessing muscle differentiation is higher than that of the histochemical detection of AChE activity, since AChE is also detected in nonmuscle tissues in postembryonic stages (Meedel & Whittaker, 1979; Deno et al. 1985). Immunological probes detect the antigen directly and thus make it possible to analyse the molecular identity of the differentiation marker. Furthermore, an immunological probe is a clue to the studies of molecular mechanisms involved in cell differentiation. Further analyses of the molecular identity of the Mu-2 antigen and of the molecular mechanism of this differentiation are now in progress.

We thank Dr T. Numakunai and all other members of Asamushi Marine Biological Station for facilitating our work there. Thanks are also due to K. Nishide and M. Matsunaga for technical assistance and Dr A. Rossiter for reading of the manuscript. This study was supported by Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (60105001).
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(Accepted 26 September 1986)