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# The Recently-Described Ascidian Species *Molgula tectiformis* Is a Direct Developer

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**ABSTRACT**—*Molgula tectiformis* is a new ascidian species recently described by Nishikawa (1991). In Otsuchi Bay, Iwate, Japan, they are easily obtainable from cages for culturing scallops. We report here that *M. tectiformis* is another example of a direct developer: their embryonic development is lacking the tadpole larva. The fertilized egg is orange and about 150 μm in diameter. At 18°C, the egg cleaves at about 20 min intervals and gastrulation occurs about 5 hr after fertilization. In contrast to conventionally-developing ascidians, *M. tectiformis* does not form a tadpole larva. Immediately before hatching, three stolons or ampullae begin to extend from the tailless embryo. After hatching the stolons mediate the attachment of the juvenile body to the substratum. Histochemistry for tissue-specific enzyme activity did not detect muscle-specific acetylcholinesterase, endoderm-specific alkaline phosphatase, and pigment cell-specific tyrosinase. In addition, *in situ* hybridization could not prove the presence of muscle actin gene transcripts in the embryo. These results suggest that these larval tissues do not differentiate in *M. tectiformis* embryos. Because *M. tectiformis* is common and gravid year-around in Otsuchi Bay, this direct developer provides the opportunity for further analysis of molecular changes during evolution that cause an alternative mode of development.

## INTRODUCTION

In addition to the usual developmental processes involved in forming a tailed (tadpole or urodele) larva, a dozen or so ascidian species, exhibit other modes of embryonic development or the formation of tailless (anural) larvae (Lacaze-Duthiers, 1874; Berrill, 1931; Millar, 1954; Whittaker, 1979; Swalla and Jeffery, 1990, 1992; Bates and Mallett, 1991; Bates, 1995). These tailless (or anural) species belong to the families Molgulidae and Styelidae and include *Molgula bleizi*, *M. occulta*, *M. arenata*, *M. pacifica*, *M. provisionalis*, *M. robusta*, *M. retortiformis*, *Bostrichobranchus pilularis*, *B. digonas*, *Eugyra arenosa*, *Pelonaia corrugata* and *Polycarpa tinctor* (reviewed by Jeffery and Swalla, 1990, 1992; Bates, 1993). Anural species may be subdivided into those that exhibit indirect (incomplete) or direct (complete) development. In species with indirect anural development, such as *M. arenata* and *M. occulta*, a tailless, slug-like larva hatches from the chorion before metamorphosis (Whittaker, 1979; Swalla and Jeffery, 1990). Species with direct anural development, such as *M. provisionalis* and *M. pacifica* (Bates and Mallett, 1991; Bates, 1995), have completely eliminated the larval stage. In these species, hatching occurs after the beginning of metamorphosis, which is initiated within the chorion. A recent molecular phylogenetic analysis suggests that anural species have been derived at least five different times from ancestors with urodele larvae (Hadfield *et al.*, 1995).

The tadpole larva has a head containing a dorsal brain and pigmented sensory organs (otolith and ocellus) and a tail containing a notochord, spinal cord, and striated muscle cells. These chordate features are lacking in anural larvae, although undifferentiated precursor cells are frequently observed (Whittaker, 1979; Swalla and Jeffery, 1990). Therefore, ascidian species with these two modes of development provide an opportunity to study molecular mechanisms involved in an evolutionary change in development (Swalla and Jeffery, 1990, 1996; Swalla *et al.*, 1993; Satoh and Jeffery, 1995; Kusakabe *et al.*, 1996).

No ascidian species with anural development have been previously reported from the coast of Japan. Recently, an ascidian species belonging to the family Molgulidae has appeared in large numbers off the coast of northern Japan. This new species has been described by Nishikawa (1991) as *Molgula tectiformis*. Here we report that *M. tectiformis*

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develops without a larval stage and is another example of an anural (direct) developer.

## MATERIALS AND METHODS

*Biological materials*

*Molgula tectiformis* (Nishikawa, 1991) was collected in Otsuchi Bay near the Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Otsuchi, Iwate, Japan. A large number of *M. tectiformis* are found at a depth of 5~10 m in this location. The animals are attached to fixed substrates, such as cages for culturing scallops and ropes.

Gametes can be obtained by controlling the light and dark periods (cf. Satoh, 1994). The animals spawn by light stimulation after long exposure of light followed by dark adaptation. In this study, however, we obtained gametes by dissecting the gonad. After exposure to seawater, oocytes matured by breaking down the germinal vesicle. Eggs were fertilized by mixing them with a suspension of non-self sperm. Fertilized eggs were cultured in filtered seawater at about 18°C. At this temperature, first cleavage took place about 1 hr after insemination, and the embryos divided at approximately 20 min intervals. Juveniles hatched out of the chorion about 13 hr after insemination. Photomicrographs of *M. tectiformis* embryos and juveniles were taken through a Zeiss Axioplan microscope.

*M. tectiformis* embryos at appropriate stages and juveniles were collected by low-speed centrifugation and fixed for histochemistry and *in situ* hybridization. Embryos and larvae of *Ciona savignyi* or *Molgula occidentalis*, which form conventional tadpole larvae, were used as controls.

*Histochemistry for enzymatic activity specific to larval tissues*

Acetylcholinesterase (AChE): AChE is a marker of muscle cell differentiation in ascidian embryos (cf. Satoh, 1994). Histochemical detection of AChE was carried out according to the method described by Karnovsky and Roots (1964) after specimens were fixed for 10 min in 5% formalin seawater at room temperature.

Alkaline phosphatase (AP): AP is predominantly expressed in endoderm cells of ascidian tadpole larvae, and is a marker of endoderm differentiation (cf. Satoh, 1994). AP activity was detected histochemically as described by Whittaker and Meedel (1989).

Tyrosinase: Tyrosinase (dopa oxidase) is a key enzyme for the production of melanin. In ascidian embryos, tyrosinase is produced by the otolith and ocellus pigment cell lineages (cf. Satoh, 1994). Tyrosinase activity was detected according to the method described by Whittaker (1973).

In situ *hybridization*

An antisense RNA probe containing most of the coding region of the *MocuMA1* gene, which hybridizes to muscle actin mRNA from a variety of ascidian species (Kusakabe *et al.*, 1996), was used to determine whether *M. tectiformis* embryos express muscle actin. The procedures used for *MocuMA1* probe preparation and *in situ* hybridization of sectioned embryos were as described previously (Kusakabe *et al.*, 1996).

## RESULTS

*Embryogenesis of* M. tectiformis

Figure 1 shows the embryonic development of *M. tectiformis*. The egg was orange in color and about 150 μm in diameter (Fig. 1A). It is surrounded by numerous, large test cells which vigorously interact with the surface of the embryo during development. *M. tectiformis* is self-fertile: eggs fertilized with sperm of the same individual develop normally. In this study, however, we followed embryogenesis of eggs fertilized with sperm of another individual. Fertilization initiates ooplasmic segregation. After ooplasmic segregation, the animal hemisphere became transparent whereas the vegetal hemisphere became orange-brown (compare Fig. 1B with 1A). The transparent, animal cytoplasm was segregated mainly into the epidermis of the embryo, while the orange-brown vegetal cytoplasm was partitioned mainly into the endoderm of the embryo (Fig. 1). Whether the myoplasm, an egg cytoplasmic region containing tail-muscle cell determinants (cf. Satoh, 1994), was present in *M. tectiformis* eggs could not be determined.

As in the case of ascidians with urodele larvae, cleavage of *M. tectiformis* eggs was bilaterally symmetrical. The first and second cleavages were meridional, dividing the egg into two (Fig. 1B) or four cells (Fig. 1C), usually of equal sizes. The third cleavage was latitudinal, and it usually produced animal and vegetal cells of unequal sizes. In 8-cell embryos, the four animal cells were transparent and small, whereas the four vegetal cells were intensely in orange color and large (Fig. 1D). Therefore, the future dorsal (vegetal)-ventral (animal) axis could be discerned by the position of colored blastomeres at the 8-cell stage. At the fourth cleavage of ascidian eggs, the division pattern of the animal tier cells is reversed relative to the vegetal tier cells (cf. Satoh, 1994). This division pattern reversal was also observed at the fourth cleavage in *M. tectiformis* embryos (Fig. 1E). The fifth cleavage occurred about 20 min later to form a 32-cell embryo. The subsequent cleavages increased the number of embryonic cells, although precise division pattern could not been followed (Fig. 1F, G). Gastrulation occurred at about 4.5 hr after fertilization (18°C). At first, the vegetal side of the embryo flattened (Fig. 1G). Then the vegetal cells invaginated (Fig. 1H). Gastrulation appeared to be accompanied by epibolic movement of the animal cells. At about 11 hr after insemination and after the completion of gastrulation, a transparent stripe became visible on the posterior-vegetal side of the embryo. This transparent stripe may correspond to the position of the neural tube, which forms on the dorsal side of ascidian embryos during neurulation (cf. Satoh, 1994). The morphogenetic processes of gastrulation and neurulation were completed within a few hours.

Later during embryogenesis, the outer surface of the embryo became transparent, indicating the formation of the epidermis (Fig. 1J). No other conspicuous changes in the embryonic morphology were evident during later phases of embryogenesis. About 13 hr after fertilization, *M. tectiformis* embryos hatched from the chorion. A portion of the chorion became thin, a hole was eventually formed in this region, and the juvenile passed through the hole in the chorion (Fig. 1J). The hatched larva was ovoid. Three transparent ampullae appeared while the embryo was still in the chorion (Fig. 1J, K). Following hatching and settlement, they form a firm attachment to the substratum (Fig. 1L).

Therefore, *M. tectiformis* does not develop a conventional tadpole larva. Because juvenile formation took place during late embryogenesis, this species is also a direct developer.

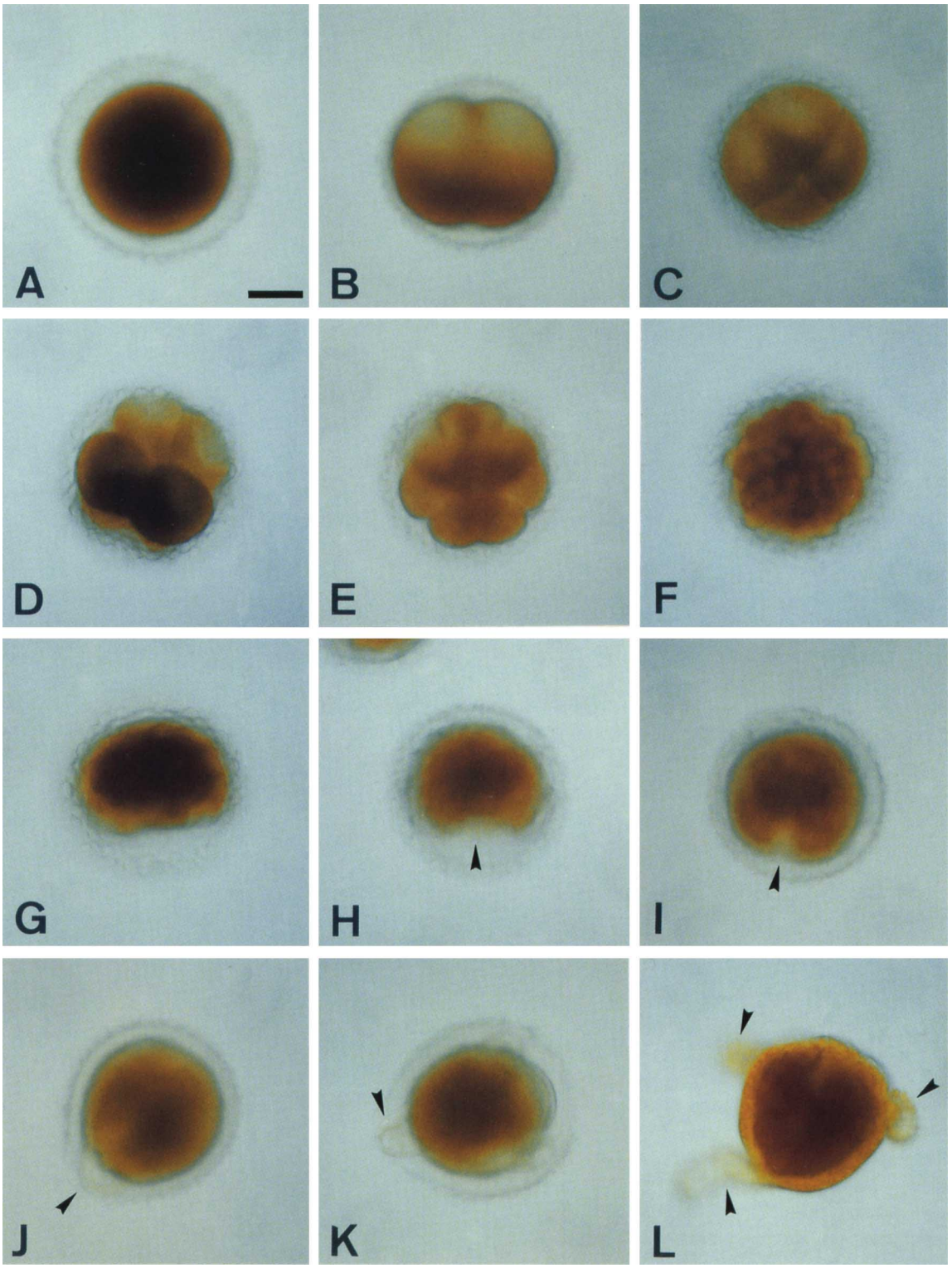


Fig. 1. Normal embryogenesis of the ascidian *Molgula tectiformis*. (**A**) An unfertilized egg. (**B**) A 2-cell embryo, lateral view. The animal pole is up. (**C**) A 4-cell embryo. (**D**) An 8-cell embryo, posterior view. (**E**) A 16-cell embryo, vegetal pole view. The anterior is up. (**F**) An embryo at about 64-cell stage, vegetal pole view. (**G**) An embryo at about 100-cell stage, lateral view. The vegetal side is flattened. (**H**) A middle gastrula (5-hr embryo), lateral view. The animal pole is up. Invagination of the vegetal cells is seen (arrowhead). (**I**) An 11-hr embryo. Neurulation is evident by an appearance of a slit (arrowhead). (**J**) A 12-hr embryo. Formation of a stolon is evident (arrowhead). (**K**) A 13-hr embryo immediately before hatching. Arrowheads indicate stolons. (**L**) A hatched juvenile. Three adhesive stolons develop (arrowheads). Scale bar represents 50 μm for all panels.

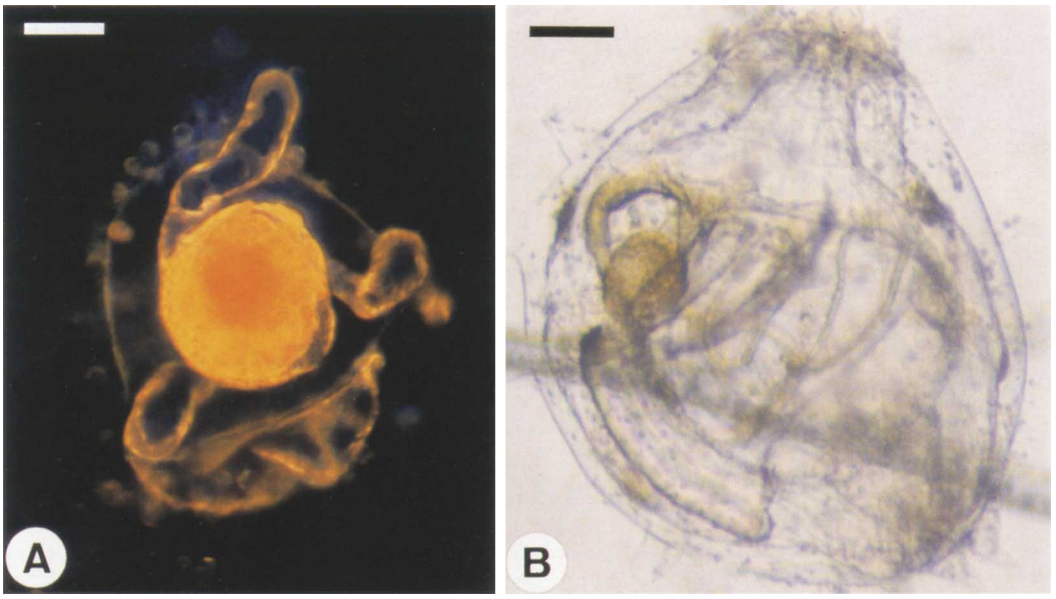


Fig. 2. Development of *Molgula tectiformis* juveniles. (**A**) A juvenile at several hr after hatching showing three ampullae. (**B**) A young adult 4 days after hatching, showing the development of various internal organs. Scale bar, 50 μm for **A** and 200 μm for **B**.

Other direct-developing ascidian species are *B. digonas* (Swalla and Jeffery, 1992) and *M. pacifica* (Young *et al.*, 1988; Bates and Mallett, 1991): there was no tail region with apparent differentiation of the notochord and muscle (Fig. 1). No otolith and ocellus pigment cells were observable in the embryo (Fig. 1). In addition, no larval adhesive organ was evident (Fig. 1).

After hatching, the formation of young adults proceeded rapidly (Fig. 2). Juveniles soon lost the ampullae. About 3 or 4 days after hatching, the development of various internal organs was evident in the young adult (Fig. 2B).

*Muscle cell differentiation in* M. tectiformis *embryos*

During indirect anural development in *M. occulta*, neither the larval muscle actin nor the myosin heavy chain genes are expressed but the muscle-lineage cells develop the muscle-specific enzyme AChE (Jeffery and Swalla, 1991; Kusakabe *et al.*, 1996). As described above, *M. tectiformis* is a direct developer, suggesting that differentiation of muscle cells is suppressed. We examined this issue by detection of AChE by histochemistry and muscle actin gene expression by *in situ* hybridization.

As shown in Fig. 3A, the control *C. savignyi* larva developed AChE activity in the tail muscle cells. However, AChE activity was not detected in *M. tectiformis* embryos (Fig. 3B). This was confirmed by examining more than 30 embryos at different stages of later embryogenesis (at 10 and 12 hr of development). AChE activity, however, was detected in the body-wall muscle cells of juveniles 4 days after hatching (data not shown). The results show that *M. tectiformis* embryos do not express AChE.

Sections of *M. tectiformis* embryos at 12 hr of development were hybridized *in situ* with the antisense *MocuMA1* probe to determine whether muscle actin is expressed. The *MocuMA1* probe, which contains most of the coding region of an *M. oculata* larval-muscle actin gene, has been shown to detect muscle actin transcripts in urodele ascidian embryos (Kusakabe *et al.*, 1996). As shown in Fig. 4A, the *MocuMA1* probe detected transcripts in tail muscle cells of *M. occidentalis*, the urodele developer used as a control in our experiments. In contrast, muscle actin transcripts could not be detected in *M. tectiformis* embryos, although small cells visible in the posterior region may be vestigial-muscle cell precursors (Fig. 4B). The results suggest that *M. tectiformis* embryos do not express muscle actin.

In summary, the lack of AChE and muscle actin expression suggests that *M. tectiformis* embryos lack differentiated muscle cells.

*Development of alkaline phosphatase in* M. tectiformis *embryos*

Alkaline phosphatase (AP) activity is used to monitor endoderm differentiation in ascidian embryos (cf. Satoh, 1994). We examined development of AP activity in *M. tectiformis* embryos. As a control, *C. savignyi* larvae clearly showed the AP activity in endodermal cells (Fig. 3D). However, AP activity was not detected in *M. tectiformis* embryos and newly hatched juveniles (Fig. 3C), although AP activity became detectable in juveniles 4 days after hatching (Fig. 3D). The results suggest that endoderm cell differentiation has also been modified in *M. tectiformis* embryos.

*Development of tyrosinase activity in* M. tectiformis *embryos*

Ascidian tadpole larvae usually develop two pigment cells in the brain vesicle: one is the otolith and the other is the ocellus (cf. Satoh, 1994). The stage at which these pigment cells differentiate varies with species (cf. Satoh, 1994). During

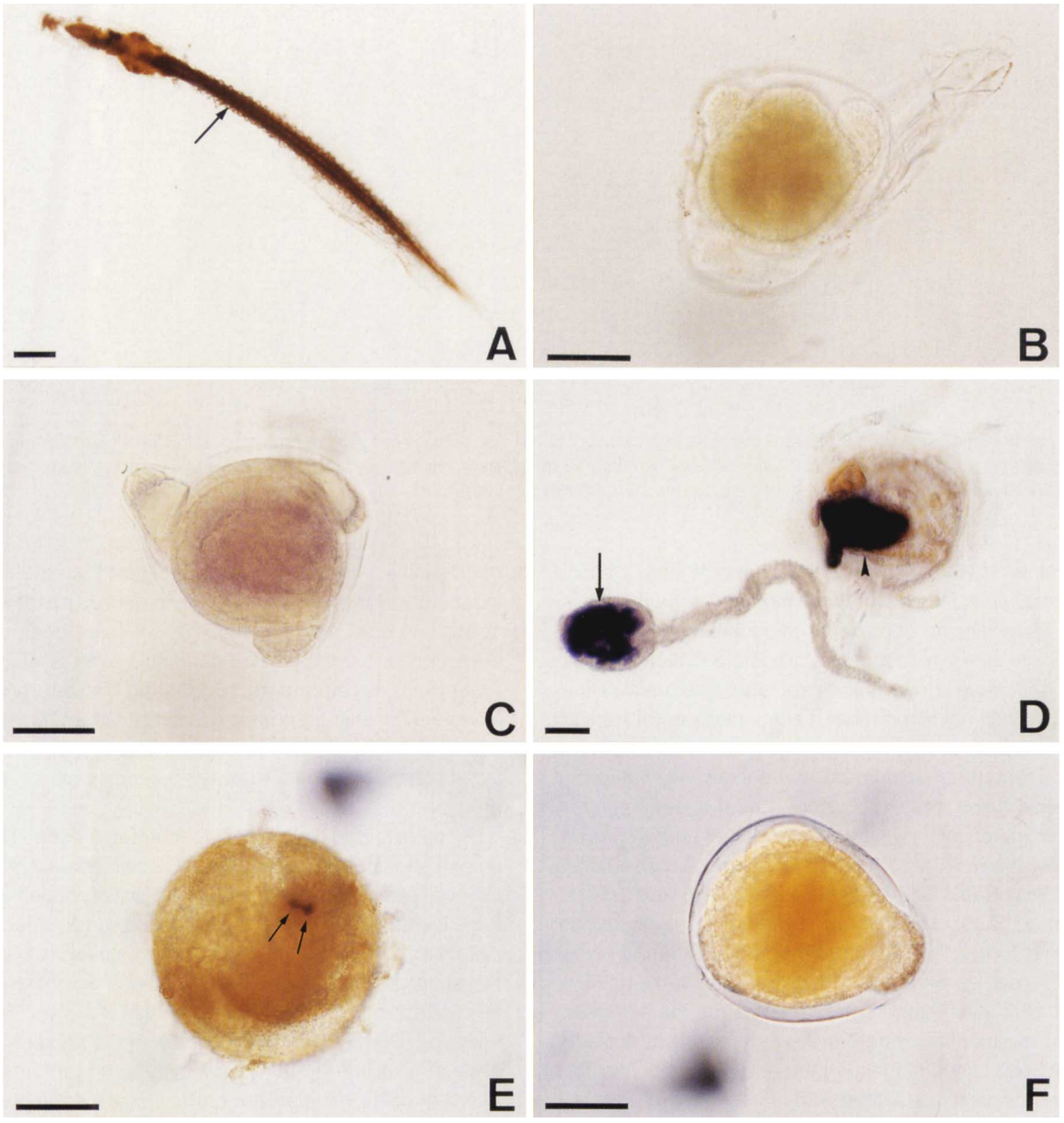


Fig. 3. Histochemical detection of tissue-specific enzymatic activities. (**A**, **B**) Detection of muscle-specific AChE. (**A**) *Ciona savignyi* larva (control) showing AChE activity in muscle cells of the tail (arrow). (**B**) AChE activity is not detected in *Molgula tectiformis* embryo at 13.5 hr after fertilization. (**C**, **D**) Histochemistry of endoderm-specific AP. (**C**) *M. tectiformis* juvenile at 13.5 hr of development does not show AP activity. (**D**) AP activity is evident in the endoderm of a *M. tectiformis* juvenile at 4 days after hatching (arrowhead) as well as *C. savignyi* larva (arrow; control). (**E**, **F**) Histochemical detection of pigment cell-specific tyrosinase. (**E**) *C. savignyi* tailbud embryo showing tyrosinase activity in two pigment cells (arrows). (**F**) Tyrosinase activity is not detected in *M. tectiformis* embryo at 13.5 hr of development. Scale bars represent 50 μm.

embryogenesis of most direct developers, pigment cells do not differentiate (cf. Jeffery and Swalla, 1990). The direct developer *B. digonas*, which develops one or two pigment cells (Swalla and Jeffery, 1992), is a notable exception. As described above, no pigment cells were formed in *M. tectiformis* embryos. We performed histochemistry to determine whether tyrosinase expression is also lacking in *M. tectiformis* embryos. Although *C. savignyi* embryos showed tyrosinase activity in two pigment cells (Fig. 3E), none of the *M. tectiformis* embryos examined (more than 30 specimens) developed enzymatic activity (Fig. 3F). The results show that the melanin synthesis pathway has been eliminated in *M. tectiformis* embryos.

## DISCUSSION

In this investigation, we have shown that *M. tectiformis* is an anural (direct) developer. Therefore, similar to other anural

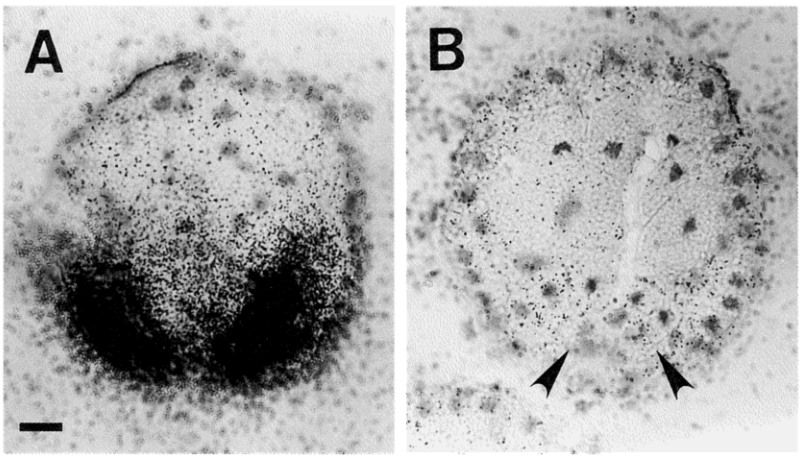


Fig. 4. Detection of muscle actin transcripts by *in situ* hybridization. (**A**) *M. occidentalis* embryo (control) at early tailbud stage showing transcripts in the tail muscle cells. (**B**) Transcripts are not detected in a 12 hr. *M. tectiformis* embryo, although small unlabeled cells are present in the posterior region (arrowheads). Scale bar represents 15 μm for all panels.

species, *M. tectiformis* does not exhibit a tadpole larva. Despite this modification in the conventional mode of embryogenesis, fertilized *M. tectiformis* eggs undergo ooplasmic segregation and cleavage similarly to those of urodele ascidian species, and they gastrulate and form a neural tube. After neurulation, however, they do not differentiate a pigmented neural sensory organ or exhibit the morphogenetic movements resulting in notochord and tail development. In addition, *M. tectiformis* embryos do not express AP, suggesting a deficiency in endoderm differentiation. Similar to a few other anural species, including *M. bleizi*, *M. pacifica*, *M. retortiformis*, and *B. digonas* (Berrill, 1931; Bates and Mallett, 1991; Swalla and Jeffery, 1992; Bates, 1995), *M. tectiformis* embryos begin metamorphosis prior to hatching. In most urodele ascidian species, metamorphosis, as marked by the extension of the ampullae, is initiated after hatching (cf. Satoh, 1994). Thus, there appears to be two major differences in development between *M. tectiformis* and conventional urodele ascidian embryos. The first difference, which is shared with all other anural species, is that *M. tectiformis* embryos lack the urodele neural sensory and tail structures. The second difference is that metamorphosis begins precociously before hatching. The form of anural development characteristic of *M. tectiformis* may have evolved through a combination of elimination and heterochronic acceleration of developmental processes.

The anural species that has been most thoroughly investigated is *M. occulta*, an indirect developer (Swalla and Jeffery, 1990, 1996; Jeffery and Swalla, 1991, 1992; Kusakabe *et al.*, 1996). Although no tail appears during embryogenesis, *M. occulta* embryos contain vestigial notochord and muscle cells, which fail to undergo terminal differentiation. The vestigial muscle cells of *M. occulta* embryos express AChE but they do not contain muscle actin transcripts (Swalla and Jeffery, 1990; Kusakabe *et al.*, 1996). AChE is also expressed in the vestigial muscle cells of another anural indirect developer, *M. arenata* (Whittaker, 1979). A recent study showed that lack of muscle actin expression in *M. occulta* was caused by structural deficiencies in the coding regions rather than the regulatory machinery of the larval muscle actin genes (Kusakabe *et al.*, 1996). In *M. tectiformis* embryos, we have been unable to detect either the expression of muscle actin or AChE during embryonic development, suggesting that the program of muscle differentiation has degenerated even further than in *M. occulta* and *M. arenata*. It will be interesting to determine if larval muscle actin genes are still present in the *M. tectiformis* genome.

The extent to which urodele features are modified differs among anural ascidian species. All known anural species, with the notable exception of *B. digonas* (Swalla and Jeffery, 1992), have lost the pigmented neural sensory organ (Berrill, 1931; Jeffery and Swalla, 1990). The lack of pigment cell differentiation stems from a deficiency in the melanin synthesis pathway, as shown by the absence of tyrosinase activity in *M. occulta* embryos (Swalla and Jeffery, 1990). In the present study, we have shown that tyrosinase activity and pigmented neural sensory cells are also absent in *M. tectiformis* embryos. Differences in AChE expression have also been reported in anural ascidian species. The indirect anural developers *M. arenata* (Whittaker, 1979) and *M. occulta* (Jeffery and Swalla, 1991) express AChE, whereas the activity of this enzyme is reduced to very low levels or absent in the anural direct developers *M. provisionalis*, *M. pacifica*, and *B. digonas* (Bates and Mallett, 1991; Swalla and Jeffery, 1992; Bates, 1995). These comparisons suggest that loss of AChE expression is correlated with the direct mode of anural development, consistent with the suggestion that these anural species are older, in an evolutionary sense, than species with indirect anural development (Bates, 1993).

There is one feature of anural development we have observed in *M. tectiformis* eggs and embryos that is distinct from other anural species: the fact that they are surrounded by large and active test cells. It has been shown previously that *M. pacifica* and *M. occulta* embryos either lack test cells or contain reduced numbers of smaller test cells (Young *et*

*al.*, 1988; Bates and Mallett, 1991). The test cells have a secretory function in tunic synthesis and assembly during larval development (cf. Satoh, 1994). The reason why *M. tectiformis* has retained the test cells while other anural ascidian species have eliminated them is unclear, but may be related to fundamental differences in the structure of the larval tunic.

Many anural ascidian species, including *M. arenata*, *M. occulta*, *B. pilularis*, and *B. digonas*, are restricted to rather homogeneous habitats at the ocean bottom, where they lie partially buried in sand or mud. Berrill (1931) suggested that these anural species evolved from urodele ancestors because there was no selective advantage for a larval dispersal stage in these habitats. More recently, Hadfield *et al.* (1995) proposed that the tadpole larva may be under negative selective pressure in patchy bottom habitats because extensive larval dispersal would interfere with settling and feeding of the rapidly-developing juveniles. It is difficult to adapt either hypothesis to *M. tectiformis*, which are attached to firm substrates in Otsuchi Bay. Other anural ascidian species, including *M. bleizi*, *M. pacifica*, and *M. provisionalis* (Berrill, 1931; Young *et al.*, 1988; Bates and Mallett, 1991; Bates, 1995), are also attached to substrates rather than buried in sand or mud flats. It is possible that anural developers initially evolved in homogeneous bottom habitats, then speciated and invaded the shoreline, as first suggested by Berrill (1931). If this is the case, the presumed bottom-dwelling ancestor of *M. tectiformis* must have become extinct because no other anural *Molgula* species have been reported in the coastal waters of northern Japan.

Anural species have been useful as models for elucidating the molecular basis of urodele ascidian development, the evolution of developmental mechanisms, and the regulatory genes that may have operated in the ancestral chordate (Jeffery and Swalla, 1992; Swalla *et al.*, 1993; Satoh and Jeffery, 1995; Kusakabe *et al.*, 1996; Swalla and Jeffery, 1996). The Japanese ascidian *M. tectiformis* has several features that are unique among anural species, including year round availability of gametes, relatively large eggs and embryos, and existence of colored cytoplasmic regions. These features will be important assets in future studies of the mechanisms of anural development.

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