Monoclonal antibodies against components of the myoplasm of eggs of the ascidian *Ciona intestinalis* partially block the development of muscle-specific acetylcholinesterase

TAKAHITO NISHIKATA, IZUMI MITA-MIYAZAWA*, TAKUYA DENOt and NORIYUKI SATOH

*Present address: R & D Center, Nippon Meat Packers, Inc., Kakogawa 675-01, Hyogo, Japan
†Present address: Department of Biology, Osaka Kyoiku University, Tennoji, Osaka 543, Japan

Summary

The myoplasm of *Ciona intestinalis* eggs, believed to contain cytoplasmic determinants responsible for muscle cell differentiation in ascidian embryos, emits weak pale-blue autonomous fluorescence. Utilizing this feature as a marker, the cytoplasm was isolated according to the method described by Jeffery (1985ft). Electron microscopy showed that the isolated cytoplasm contained mitochondria, pigment granules, yolk particles and fine granular materials; these are ultrastructural components of the myoplasm of the intact egg. Monoclonal antibodies were prepared against the isolated cytoplasm. Twelve monoclonal antibodies, identified by indirect immunofluorescence, stained the myoplasmic region. When unfertilized eggs were centrifuged, stratifying their mitochondria and some other cytoplasmic components, components identified by several antibodies, for example IIG6B2, remained at the peripheral cytoplasm of the egg. Other antibodies recognized components stratified as the mitochondrial layer. Four representative antibodies were microinjected into fertilized eggs in order to examine their inhibitory effects on the muscle differentiation; the IIG6B2 antibody blocked the development of muscle-specific acetylcholinesterase in more than 80% of the embryos tested.

Key words: ascidian embryo, myoplasm, monoclonal antibodies, cytoplasmic determinants, muscle differentiation.

Introduction

Various descriptive and experimental studies since the turn of the century have documented the presence of cytoplasmic information or determinants responsible for specification of embryonic cell features (reviews by Wilson, 1925; Davidson, 1986). The determinants are thought to be localized in predetermined regions of the egg and are segregated by a determinate cleavage pattern into certain cells, where they appear to play a crucial role in programming differentiation. A well-known example of a cytoplasmic determinant is that responsible for muscle cell differentiation in ascidian embryos (reviews by Whitaker, 1979; Jeffery, 1985a; Uzman & Jeffery, 1986). The determinant is confined to the myoplasmic crescent formed after ooplasmic segregation and is eventually found within the muscle lineage cells of developing larvae (Conklin, 1905a,b). The best evidence for the factor is based on the results of experiments with cleavage-arrested embryos and modification of the distribution of cytoplasmic components. Ascidian embryos that have been permanently cleavage-arrested with cytochalasin B develop muscle-specific markers (Whittaker, 1973; Satoh, 1979; Crowther & Whittaker, 1983). These markers appear in the correct progenitor cells as defined by the cell lineage analyses. Furthermore, modification of the distribution of the myoplasm causes an occurrence of additional cells exhibiting the muscle differentiation marker (Whittaker, 1980, 1982).

Despite such extensive investigation, there is little known about the molecular nature of these determinants. We have already reported successful transplantation of the myoplasm (Deno & Satoh, 1984), which means the establishment of a bioassay system for
monitoring isolation of true determinants. In this study, we have attempted to produce monoclonal antibodies that specifically recognize myoplasmic components. Recently, Jeffery and his co-workers (Jeffery, Tomlinson & Brodeur, 1984; Jeffery, 1985b) have isolated the yellow (myoplasmic) crescent from Styela eggs using the yellow pigment granules as a marker of the myoplasm. We found recently that the so-called myoplasm of Ciona eggs emits an autonomous fluorescence (Deno, 1987) and, in this study, we have attempted to isolate the myoplasmic components using this characteristic feature. We have produced several monoclonal antibodies directed against the isolated cytoplasm. Among these, 12 monoclonal antibodies recognize antigenic components that behave as myoplasm. In unfertilized, centrifuged eggs, some of the antigenic components remain at the cortex region of the egg, suggesting that the antigenic components are not mitochondria. Effects of the monoclonal antibodies on the differentiation of muscle cells were examined by injecting the antibodies into fertilized eggs. These eggs were allowed to develop until the hatching stage and then examined histochemically for the muscle-specific acetylcholinesterase. We find that many of the injected eggs fail to develop the enzyme activity.

Materials and methods

Ascidian eggs and embryos

Adults of the ascidian Ciona intestinalis were collected in the vicinity of Maizuru Fisheries Laboratory of Kyoto University, Maizuru Bay and of Onagawa Fisheries Laboratory of Tohoku University, Onagawa Bay, Japan. They were maintained in temperature-controlled aquaria (18°C) under constant light to induce oocyte maturation. Eggs were removed surgically from the gonads and fertilized with diluted suspension of sperm of other individuals. Fertilized eggs were reared in filtered natural seawater at 18°C. At this temperature, first cleavage takes place about 1 hr after insemination and tadpole larvae hatch out at about 17 hr of development.

Fluorescent microscopy

To observe autonomous fluorescence of the myoplasm of Ciona eggs, unfixed whole specimens were mounted in seawater on microscope slides. Pieces of Scotch tape were put on the slides to hold a space, about 200 μm, between the slides and the coverslips. A Nikon Labophot equipped with an epifluorescence optic unit EFD was used. The EFD unit for u.v. illumination consisted of a mercury lamp house (Osram HBO-100w/2), Nikon CF Fluor objective lenses for epifluorescence microscopy and a u.v. excitation filter cassette consisting of a dichroic mirror DM400, an excitation filter UV330-380 and a barrier filter 420k, with a main wavelength of 365 nm. Photomicrographs were taken with Kodak Tri-X Pan film.

Isolation of myoplasmic components

Isolation of the myoplasmic components was carried out according to the method described by Jeffery et al. (1984) and Jeffery (1985b), except for the procedure of dechorionation. About 10 min after insemination, eggs were immersed in seawater containing 1% sodium thioglycolate (Wako Pure Chem. Ind., Ltd., Tokyo) and 0-05% actinase E (Kaken Pharma., Co., Ltd., Tokyo), with the pH of the solution adjusted to about 11-0 by addition of drops of 1N-NaOH (Mita-Miyazawa, Ikegami & Satoh, 1985). Gently pipetting the eggs removed the chorion within 13 min. The dechorionated eggs were washed several times with filtered seawater, transferred into Petri dishes coated with 2% agar and maintained until the myoplasmic crescent stage. Eggs at the crescent stage were collected by centrifugation and suspended in 10 vol. of an ice-cold isolation medium containing 50 mM-Tris-HCl, 500 mM-NaCl, 10 mM-MgCl₂, 5 mM-CaCl₂, and 10 μg ml⁻¹ leupeptin (pH 7-2). The suspension was homogenized by 20 strokes of a Teflon pestle fitted into a Potter–Elvehjem glass homogenizer and centrifuged at 500g for 3 min. The pellet was resuspended in 10 vol. of isolation medium, homogenized by five strokes of the pestle and centrifuged. Cycles of homogenization and centrifugation were repeated until the supernatants became clear. Then the pellet was resuspended in 0-2 ml of isolation medium and centrifuged through a step gradient consisting of 1-0 ml of 0-2 M-sucrose over 0-8 ml of 2-0 M-sucrose. After centrifugation of the gradient for 20 min at 7000 g (4°C), the cytoplasmic aggregates identified as myoplasm banded at the interface between the sucrose solutions.

Transmission electron microscopy

For examination of ultrastructure of the isolated cytoplasm, the aggregate was first fixed for 1 hr at room temperature in 2-5% glutaraldehyde in 0-2 M-sodium phosphate buffer (pH 7-4) containing 3-0% NaCl. After several rinses with the buffer, specimens were postfixed for 0-5 hr at room temperature in 1% osmium tetroxide in the same buffer. Fixed materials were then dehydrated with ethanol, cleared with propylene oxide and embedded in Epon 812 resin. Specimens were sectioned with an LKB 8800 Ultratome. Thin sections were double stained with 1% aqueous uranyl acetate and lead citrate and examined with a JEM 100-SX electron microscope operated at 100 kV.

Antigen preparation and immunization

The antigen was a homogenate of the isolated cytoplasm. It was suspended in 0-9% NaCl solution. A female BALB/c mouse was injected intraperitoneally with 0-5 ml cytoplasm suspension and the animal was boosted intraperitoneally 19, 24 and 27 days later with 0-5 ml cytoplasm suspension.

Preparation of monoclonal antibodies

Three days after the last intraperitoneal hyperimmunization, the mouse was killed, its spleen was removed and 1-7×10⁶ spleen cells were fused with 4-3×10⁵ P3U-1 myeloma cells (purchased from Flow Lab.) by using 50% (w/v) polyethylene glycol 4000 (Nakarai Chem. Co. Ltd., Kyoto). The fused cells were separated into 96 mult wells and, when cell growth was apparent (7–10 days after fusion), samples
of supernatant of hybridoma culture medium from each well were assayed by immunofluorescence microscopy. Hybrid cells producing antibodies of interest were cloned twice by picking up only one cell with a small pipette under an inverted microscope.

**Fixation and immunofluorescence staining of eggs and embryos**

For immunohistochemical staining to screen the hybridoma supernatant medium and also to examine the distribution pattern of antigenic components, large quantities of *Ciona* embryos were fixed for 10 min in methanol (-20°C), followed by ethanol (-20°C) and embedded in polyester wax (Steedman, 1957; BDH Chem. Ltd). Sectioned specimens were mounted on small coverslips. Immediately before immunofluorescence staining, polyester wax was removed with absolute ethanol. Specimens were then washed with phosphate-buffered saline (PBS) and incubated with 75 µl of hybridoma culture fluid for 1 h at room temperature. The coverslips were washed in PBS at room temperature for 0.5 h, and each coverslip was incubated for 0.5 h at room temperature with 6 µl of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG serum (Miles-Yeda Ltd) diluted 1:60 in PBS. The coverslips were washed in PBS at room temperature for 0.5 h, mounted in 80% glycerol and observed with Nikon Labophot equipped with an epifluorescence optic unit EFD.

**Centrifugation of unfertilized eggs**

Unfertilized eggs were laid upon a Percoll bed and were centrifuged at 9000 g for 30 min. Since the animal–vegetal axis of the egg was randomly oriented with respect to the direction of the centrifugal force, stratification of the cytoplasmic components occurred randomly with respect to the egg axis. After centrifugation, stratified eggs were divided into two groups. One group of eggs was directly observed with a fluorescent microscope under u.v. excitation, while the others were fixed for immunocytochemistry.

**Microinjection of monoclonal antibodies**

Monoclonal antibodies were prepared for examination as follows. About 1 x 10^6 hybridoma cells ml^-1 producing the myoplasm-specific monoclonal antibody were cultured for 2–3 days with FCS-free culture medium in a plastic dish. Before intracellular injection of monoclonal antibodies, eggs were mechanically dechorionated about 10 min after fertilization with sharpened tungsten needles. As the dechorionated ascidian eggs were sticky, the naked eggs were treated individually during the injection and culture procedures.

Microinjection of culture medium containing concentrated monoclonal antibody was carried out with a micro-manipulator (Model MP-1, Narishige Science Instruments Lab., Tokyo). Injection pipettes of tip diameter approx. 4 µm were prepared by pulling thin-walled capillary tubing using a microelectrode puller (Model PG-1, Narishige Science Instruments Lab., Tokyo). The tips of the micro-pipettes were filled with the medium and about 0.02–0.03 nl of the medium was forced into the fertilized egg by pressure. The injected eggs were then allowed to develop for 17 h until the hatching stage in a 1% agar-coated plastic dish filled with Millipore-filtered seawater containing 5 µg ml^-1 streptomycin, then fixed for histochemistry.

**Histochemistry for acetylcholinesterase**

Acetylcholinesterase is histospecific for embryonic muscle cells at prehatching developmental stages of ascidians (Durante, 1956). The enzyme activity is first detectable at the neurula stage and increases dramatically with development (Meedel & Whittaker, 1979). Recently, it has been shown that in the *in vivo* translation system of *Xenopus* oocytes, development of translationally active mRNA for the embryonic muscle acetylcholinesterase, begins around early gastrulation (Meedel & Whittaker, 1983; Perry & Melton, 1983). Histochemical detection of acetylcholinesterase was carried out by the method of Karnovsky & Roots (1964) after the experimental embryos were fixed for 30 min in cold 5% formalin in seawater. Stained embryos were dehydrated in ethanol and cleared in xylene for microscopic examination.

**Results**

**An autonomous fluorescence of the myoplasm of *Ciona* eggs**

Recently it was found that under a fluorescence microscope with u.v. excitation, living *Ciona* eggs emit specific autonomous fluorescence in the myoplasmic region of egg cytoplasm; fixed embryos do not exhibit the fluorescence. Although details of the finding have been published elsewhere (Deno, 1987), we briefly describe here the behaviour of fluorescent cytoplasm to facilitate understanding of the following results.

In unfertilized eggs, weak pale-blue-coloured autonomous fluorescence is seen at the peripheral cytoplasm except for about a quarter of the egg; this nonfluorescent peripheral cytoplasmic region appears to be the animal pole region (Fig. 1A). During ooplasmic segregation following fertilization, the fluorescence becomes condensed into a 1/8–1/4 part of the whole egg and appears as a crescent-like form in the vegetal–posterior region of the pre cleavage zygote (Fig. 1B). When the first cleavage begins, the furrow is formed at a right angle to the long axis of the
fluorescent region and divides the fluorescent region equally into the daughter blastomeres. At the 8-cell stage, the posterior–vegetal blastomere pair (B4.1 pair) inherits most of the fluorescent cytoplasm (Fig. 1C). The quantity of the fluorescent cytoplasm inherited by each blastomere pair is variable even within a single batch of embryos. In some embryos, almost all of the fluorescent cytoplasm is segregated into the B4.1 pair, whereas in others, portions of the fluorescent cytoplasm appear in others of the six blastomeres (Fig. 1C). During gastrulation and neurulation, presumptive muscle lineage blastomeres are recognizable by the fluorescence and can be followed through the ectodermal cell layer in living embryos. Finally, muscle cells that align in three rows on each side of the elongating tail exhibit distinct autonomous fluorescence (Fig. 1D).

Isolation of myoplasmic components

A method of isolation of the myoplasmic crescent in *Styela* eggs has recently been devised by Jeffery et al. (1984) and Jeffery (1985b). Utilizing the above-mentioned characteristic feature of *Ciona* myoplasm as a marker for monitoring isolation of the cytoplasm, we attempted to isolate the myoplasmic components from the crescent-forming-stage *Ciona* eggs. Prior to cytoplasmic isolation, we dechorionated eggs and removed accessory cells such as follicle cells and test cells. If lightly homogenized cytoplasm was centrifuged in a discontinuous sucrose gradient, cytoplasmic aggregates (Fig. 1E) were banded between the sucrose solutions. The isolated cytoplasms were less intact than the isolated *Styela* yellow crescent reported by Jeffery (1985b). However, under u.v. excitation, the cytoplasm aggregates emitted intense pale-blue autonomous fluorescence (Fig. 1F). No autonomous fluorescence was observed in the supernatant fraction.

Ultrastructure of the isolated cytoplasm

The myoplasm is known to contain a large aggregate of mitochondria, pigment granules, endoplasmic reticulum, yolk particles and fine granular materials (Berg & Humphreys, 1960; Mancuso, 1962). Thin sections of the isolated cytoplasm examined by transmission electron microscopy showed aggregates of mitochondria (Fig. 2A,B), pigment granules (Fig. 2A), yolk particles (Fig. 2B) and fine granular materials (Fig. 2B). This result, as well as the intense autonomous fluorescence of the isolated cytoplasm,

![Fig. 1. Autonomous fluorescence of whole eggs and embryos of the ascidian *Ciona intestinalis* (A–D) and isolated cytoplasm (E), and its autonomous fluorescence (F). Bars in A, 50 μm, and in E, 100 μm. (A) An unfertilized egg without the chorion under u.v. illumination showing that about three quarters of the peripheral cytoplasm (indicated by four arrowheads), except for the animal pole (ap) region, emit weak pale-blue-coloured autonomous fluorescence. (B) A fertilized egg after ooplasmic segregation. Fluorescent egg cytoplasm concentrates into a small region and forms a 'crescent' (arrowhead) in the vegetal–posterior region of the egg. (C) An 8-cell embryo. Most of the fluorescent cytoplasm is segregated into B4.1 blastomere pair, but a little of it is inherited by the other six blastomeres. (D) An early tailbud stage embryo showing that autonomous fluorescent muscle cells (mc) are clearly seen in its tail. (E,F) A micrograph of an aggregate of isolated cytoplasm (E) and a comparatively intense autonomous fluorescence of the isolated cytoplasm (F).](image-url)
Monoclonal antibodies against myoplasm of Ciona eggs

indicates that the isolated cytoplasm contains at least some components of the myoplasm of intact eggs.

Production of monoclonal antibodies directed against isolated cytoplasm

To obtain specific immunological probes for studying the molecular identity of the myoplasmic components responsible for muscle differentiation, we utilized the hybridoma technique of Galfre et al. (1977) to generate monoclonal antibodies against components included in the isolated cytoplasm. We immunized mice with a homogenate of the isolated cytoplasm, fused the mouse spleen cells to mouse myeloma cells to establish hybridoma cell lines and screened individual hybridoma culture fluid samples by immunofluorescence microscopy. As described in Materials and methods, more than 200 samples were tested, from which we identified and cloned 12 hybridoma cell lines that secrete antibodies directed against components that behave as the myoplasm.

Fig. 3 shows a spatial distribution pattern of such components identified by one of the 12 monoclonal antibodies (IIH10G4 line). Examination by indirect immunofluorescence revealed that, in an unfertilized egg, the fluorescence is restricted to the periphery of the vegetal hemisphere (Fig. 3A). During ooplasmic segregation, this fluorescent cytoplasmic region is condensed into the so-called myoplasmic crescent in the vegetal-posterior region of the precleavage zygote (Fig. 3B). The fluorescent crescent is divided equally between the two daughter blastomeres at first cleavage (Fig. 3C). In an 8-cell embryo, however, the fluorescent components are not always partitioned to only the B4.1-cell pair, since the six other cells also receive a considerable amount of fluorescent cytoplasm (Fig. 3D). This pattern continued until tailbud formation; most of the fluorescence was seen in presumptive muscle cells of gastrulae (Fig. 3E) and neurulae as well as in muscle cells of tailbud embryos (Fig. 3F), while less fluorescent cytoplasm was seen in cells of the presumptive nervous system (Fig. 3E,F). The spatial distribution pattern of cytoplasmic components recognized by the other 11 monoclonal antibodies was almost identical to that of IIH10G4, although the patterns differed in detail between the antibodies.

As mentioned earlier, living Ciona eggs emit pale-blue autonomous fluorescence only when observed under u.v. illumination. However, eggs lost the autonomous fluorescence when they were fixed. The pattern of fluorescence distribution observed is attributable to the FITC-labelled second antibody.

Distribution of the antigenic components in unfertilized, centrifuged eggs

The distribution pattern of the antigenic components and also of the pale-blue autonomous fluorescent cytoplasm resembled that of mitochondria traced by vital staining (Zaloker & Sardet, 1984). In order to clarify whether cytoplasmic components identified by the antibodies are mitochondria, we examined unfertilized eggs in which the cytoplasmic components had been stratified by centrifugation. We examined the distribution of cytoplasmic components recognized by three representative monoclonal antibodies, IIF9E9, IC5E1 and IIG6B2.

After centrifugation, an unfertilized egg was stratified into four layers (Fig. 4A,C,E). These four layers were designated by Bell & Holland (1974) as a
centripetal clear layer, yolk layer, mitochondrial layer and centrifugal clear layer. The mitochondrial layer was located between the yolk layer and the centrifugal clear layer (Fig. 4A). As shown in Fig. 4B, the autonomous fluorescent cytoplasm moved from the peripheral cytoplasmic region (Fig. 1A) to the mitochondrial layer, suggesting that the cytoplasmic components emitting the autonomous fluorescence are mitochondria. Cytoplasmic components recognized with IIF9E9 also moved to the layer between the yolk layer and centrifugal clear layer (Fig. 4E,F). This suggests that the IIF9E9 antibody recognizes mitochondrial components.

On the other hand, components identified by IIG6B2 (Fig. 4C,D) and by IC5E1 did not move even after stratification. The components remained underneath the surface of the egg as in intact unfertilized eggs. This may indicate that these antibodies recognize components other than mitochondria, but specific to the so-called myoplasm.

**Effects of microinjection of the monoclonal antibodies on muscle cell differentiation**

In order to examine the effects of the obtained monoclonal antibodies on muscle cell differentiation during Ciona embryogenesis, we injected the antibodies into fertilized and dechorionated eggs and observed the occurrence of muscle-specific acetylcholinesterase in the developing embryos. Four monoclonal antibodies (IC5E1, IIC4A7, IIF9E9 and IIG6B2) were concentrated from hybridoma-culturing medium and injected as described in Materials and methods. As a control, a monoclonal antibody (A7D5; Mita-Miyazawa et al. 1987) which specifically recognized epidermal cells of developing embryos of another ascidian species, Halocynthia roretzi, was injected. Two series of experiments were carried out and, in each case, eggs of the same batch were used for both experimental and control procedures. The results are summarized in Fig. 5 and Table 1.
Monoclonal antibodies against myoplasm of Ciona eggs

The control eggs injected with the epidermis-specific monoclonal antibody developed to tadpole larvae of normal appearance (Fig. 5A) and, with the exception of only three (a control of IIG6B2 in Series A and two of IC5E1 in Series B), all developed histochemically detectable muscle-specific acetylcholinesterase (Table 1). This clearly indicates that the inhibitory effects described below are caused by injection of the specific antibodies.

Conversely, microinjection of the obtained monoclonal antibodies caused various developmental abnormalities and a decrease in the frequency of embryos developing histochemical acetylcholinesterase activity. The effects of microinjection of IIIF9E9 caused severe damage to development. This antibody may recognize mitochondrial components. Injected eggs usually developed into only cell aggregates, as shown in Fig. 5D. Sometimes they ceased develop-

Fig. 4. Distribution of cytoplasmic components with autonomous fluorescence and of components recognized with two antibodies in unfertilized, centrifuged eggs. Bar, 50 μm. (A) Photomicrograph of an unfertilized, centrifuged egg within the chorion. A centripetal clear layer (cp), dark yolk layer (yl), mitochondrial layer (ml), and centrifugal clear layer (fl) are obvious. cp, centripetal side; cf, centrifugal side; tc, test cells. (B) A fluorescent micrograph under u.v. illumination of the same egg shown in A. Pale-blue autonomous fluorescence is observed at the mitochondrial layer between the yolk layer and centrifugal clear layer (arrow). Test cells (tc) emit straw-coloured autonomous fluorescence. (C,D) Distribution of cytoplasmic components recognized with one of the monoclonal antibodies (IIG6B2). Bright staining is found only at the peripheral cytoplasm (arrowheads) of the stratified egg except for the region of the supposed animal pole (ap). This pattern indicates that the components do not move against the centrifugal force which moves mitochondria into a certain region. (E,F) Distribution of the components identified by another antibody, IIIF9E9. Disc-shaped stainings are clear between the yolk layer and centrifugal clear layer (arrows). This layer coincides with the mitochondrial layer.
ment after several cleavages and also became less aggregated (Fig. 5E). In this case, we could not process them for histochemical reactions and therefore these embryos are not included in the data shown in Table 1. About 70% of the eggs injected with IIF9E9 that developed to advanced stages failed to develop the muscle-specific enzyme activity (Table 1).

Microinjection of IC5E1 also caused developmental damages as shown in Fig. 5B. About half of the eggs injected with IC5E1 failed to develop acetylcholinesterase (Table 1).

However, the effects of microinjection of IIC4A7 were rather minor; injected eggs developed abnormal tadpole larvae but they consisted of an oval head and elongated tail (Fig. 5C). The test materials surrounding the larva were obvious (Fig. 5C), suggesting the development of normal function of epidermal cells. Nearly all of them (about 95%) developed acetylcholinesterase activity (Table 1).

---

**Fig. 5.** Developmental abnormalities caused by injection of the monoclonal antibodies. Bar, 100 μm. (A) As a control, microinjection of A7D5 antibody, which specifically recognizes epidermis of developing *Halocynthia* embryos and does not cross react with *Ciona* tissues, does not affect *Ciona* embryogenesis. Tadpole larvae of normal appearance develop from the injected eggs. (B–F) Microinjection of the monoclonal antibodies (B, IC5E1; C, IIC4A7; D, E, IIF9E9; and F, IIG6B2) causes various developmental abnormalities. Eggs injected with IIC4A7 develop into abnormal tadpole larvae but they consist of an oval head and elongated tail (C), whereas eggs injected with IC5E1 (B) or with IIF9E9 (D) form cell aggregates. Some eggs injected with IIF9E9 cease development after several cleavages (E). (F) An abnormal tadpole larva developed from an egg injected with IIG6B2. The test materials (t) are obvious in C and F.
The result of microinjection of IIG6B2 is most interesting. As shown in the centrifuged eggs, this antibody recognizes components other than mitochondria. Injected eggs developed into abnormal tadpole larvae in which the head and tail regions were distinguishable (Fig. 5F). In addition, the larvae developed the test materials (Fig. 5F). However, the occurrence of acetylcholinesterase was very low; more than 80% of them failed to develop muscle-specific enzyme activity (Table 1).

### Discussion

In this study, we first isolated the myoplasmic components from Ciona eggs utilizing an autonomous fluorescence of the myoplasm as a marker. The isolation was carried out according to the method developed by Jeffery et al. (1984). The isolated cytoplasms were less intact than the isolated Styela yellow crescent reported by Jeffery et al. (1984) and Jeffery (1985b). However, a high level of autonomous fluorescence of the isolated cytoplasm and the presence of mitochondria and pigment granules in the isolated cytoplasm indicate that at least some components specific to the myoplasm were isolated. Then, we produced monoclonal antibodies directed against the isolated myoplasm and established 12 lines of hybridomas that produce monoclonal antibodies that recognize components of Ciona egg myoplasm. Indirect immunofluorescence revealed that the distribution pattern of the antigens in eggs and embryos of various stages, as shown in Fig. 3, very closely resembles that of the myoplasm demonstrated in Fig. 1. This distribution pattern also resembles that of mitochondria traced by vital staining (Zalokar & Sardet, 1984). As was shown in centrifuged eggs, the antibody IIF9E9 may recognize mitochondrial components. However, other antibodies such as IIG6B2 and IC5E1 may identify cytoplasmic components other than mitochondria, since the components remained at the cortical cytoplasm even after centrifugation. More precise determination of the antigenic components may be done by electron microscope studies coupled with an immunological probe.

Microinjection of four representative monoclonal antibodies caused various developmental abnormalities, particularly IIF9E9. In addition, the antibody failed to develop acetylcholinesterase, although the injected eggs accomplished the morphogenetic movements and developed into abnormal tadpole larvae. However, the processes by which the antibodies prevent the occurrence of histospecific enzyme are obscure. Therefore, inductive effects of antigens purified with the antibodies are also to be tested by injecting the antigens into non-muscle lineage blastomeres. Such studies are now in progress.

Production of monoclonal antibodies that recognize components that have the same distribution as myoplasm does not necessarily mean that these components are muscle-cell determinants or a part of them. However, the present study suggests a promising route to identify the molecular nature of the determinant(s). We hope to achieve this goal by repeated cycles of microinjection experiments of fractions of the isolated myoplasm, and production of monoclonal antibodies against the effective components.

We thank Dr William R. Jeffery for critical reading of the manuscript. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture, Japan (60105001, 61228007) and from Yamada Science Foundation.

### Table 1. Effects of microinjection of monoclonal antibodies against myoplasm on the development of muscle-specific acetylcholinesterase

<table>
<thead>
<tr>
<th>Monoclonal antibodies injected</th>
<th>Control embryos</th>
<th>Experimental embryos</th>
<th>Control embryos</th>
<th>Experimental embryos</th>
<th>Control embryos</th>
<th>Experimental embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC5E1</td>
<td>10/10</td>
<td>6/37</td>
<td>28/30</td>
<td>25/31</td>
<td>38/40 (95.0%)</td>
<td>31/68 (45.6%)</td>
</tr>
<tr>
<td>IC4A7</td>
<td>23/23</td>
<td>37/37</td>
<td>29/29</td>
<td>25/29</td>
<td>52/52 (100%)</td>
<td>62/66 (94.8%)</td>
</tr>
<tr>
<td>IIF9E9</td>
<td>8/8</td>
<td>7/20</td>
<td>16/16</td>
<td>8/26</td>
<td>24/24 (100%)</td>
<td>15/46 (32.6%)</td>
</tr>
<tr>
<td>IIG6B2</td>
<td>19/20</td>
<td>6/21</td>
<td>3/35</td>
<td>9/20 (95.0%)</td>
<td>9/26 (95.0%)</td>
<td>9/20 (95.0%)</td>
</tr>
</tbody>
</table>

Fertilized Ciona eggs injected with each of 4 myoplasm-specific monoclonal antibodies were allowed to develop for about 18 h and then fixed for histochemical reactions for muscle-specific acetylcholinesterase. As a control, eggs were injected with an epidermis-specific antibody of Halocynthia embryos, which does not cross react with Ciona tissues. Two series of experiments were carried out. In each case, eggs of the same batch were used for experimental and control procedures.
References


(Accepted 29 April 1987)