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Histospecific acetylcholinesterase development in the presumptive muscle cells isolated from 16-cell-stage ascidian embryos with respect to the number of DNA replications

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SUMMARY

The presumptive muscle cells (B5.1 blastomeres) were isolated from 16-cell-stage embryos of the ascidian, *Ciona intestinalis*. The isolated cells were allowed to divide either twice or three times thereafter. Then further divisions of the cells were continuously inhibited by a simultaneous treatment with aphidicolin (a specific inhibitor of DNA synthesis) and cytochalasin B (an inhibitor of cytokinesis). When development of muscle-specific acetylcholinesterase in these division-arrested progeny cells of B5.1 blastomeres was examined histochemically, the B5.1 blastomeres which had been allowed two further divisions did not produce any detectable acetylcholinesterase activity. Whereas those which had been allowed three further divisions showed the tissue-specific enzyme activity. These results provide further evidence for the presence of a quantal DNA replication cycle for the tissue-specific enzyme development, which is qualitatively different from the other DNA replication cycles.

INTRODUCTION

Muscle cells of the tail of developing ascidian embryos produce acetylcholinesterase, which is an excellent marker of cell differentiation. Acetylcholinesterase activity can be first detected histochemically in the muscle rudiments of the neurula, and the enzyme activity increases progressively with time of development (Durante, 1956; Meedel & Whittaker, 1979). Studies with puromycin and actinomycin D suggest that the histospecific protein is synthesized at the time it is first detected and that acetylcholinesterase development requires new RNA synthesis, which begins between the early and late gastrula stages (Whittaker, 1973; Meedel & Whittaker, 1979; Satoh, 1979). Recently Meedel & Whittaker (1983) and Perry & Melton (1983) have revealed that in the *in vivo* translation system of *Xenopus* oocytes, development of translationally active mRNA for the larval muscle acetylcholinesterase begins around early gastrulation.

Key words: ascidian embryos, isolated muscle lineage cells, tissue-specific enzyme development, quantal DNA replication cycle.

Neither cytokinesis nor nuclear division is required for development of the tissue-specific enzyme (Whittaker, 1973; Satoh & Ikegami, 1981b). However, a definite number of DNA replication cycles may be necessary for its development, suggesting that the activation of the appropriate genes responsible for the synthesis of the tissue-specific enzyme might take place during a particular DNA replication cycle (Satoh & Ikegami, 1981a, b). This is one of the features of muscle cell development in ascidian embryos, an experimental system for studying the cellular and molecular mechanisms involved in differentiation.

Another feature of muscle cell development in ascidian embryos is the autonomy of this cell differentiation. If the muscle lineage cells are isolated from 8-cell-stage embryos and allowed to develop in isolation, the progeny cells of these isolated blastomeres differentiate not only histospecific muscle acetylcholinesterase but also myofibrils (Whittaker, Ortolani & Farinella-Ferruzza, 1977; Whittaker, 1982; Crowther & Whittaker, 1983; Deno, Nishida & Satoh, 1984, 1985). This autonomy supports the theory that a localized and segregated egg cytoplasmic determinant is responsible for larval muscle development in ascidian embryos but interaction between the muscle cells and the other types of cells are not (see Whittaker, 1979; Jeffery, Tomlinson, Brodeur & Meier, 1984 for reviews; Deno & Satoh, 1984).

Coupled with these experimental results, it can be envisaged that muscle cell differentiation in ascidian embryos would take place only if two necessary conditions were met; (i) the cells were provided with the segregated cytoplasmic information, and (ii) the cells underwent a definite number of DNA replications. In

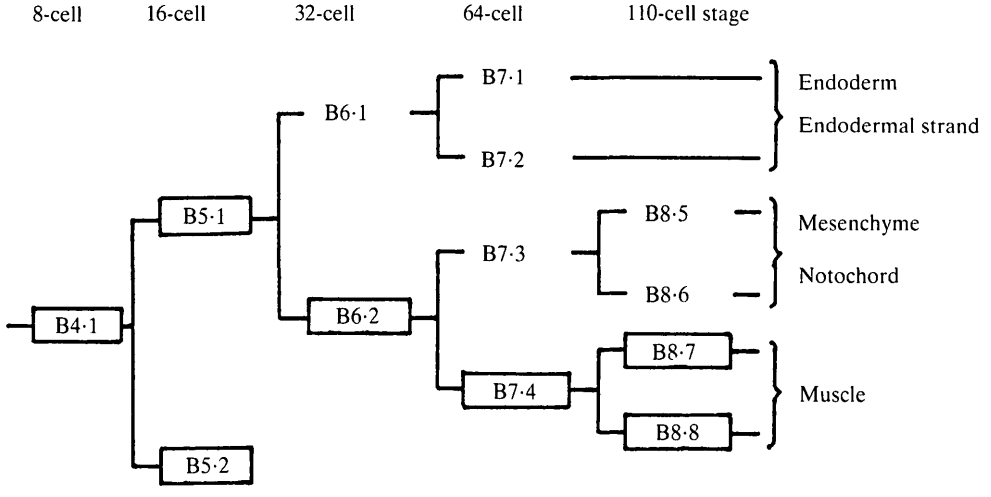

Fig. 1. A diagram illustrating cell lineages of the B5.1 blastomere. Muscle lineage cells are enclosed. Nomenclature is that of Conklin (1905). Illustrated according to studies of Conklin (1905) and Nishida & Satoh (1983, 1985). According to the most recent cell-lineage studies (Nishida & Satoh, 1983, 1985), the B5.1 blastomere eventually gives rise to eight muscle cells of the middle part of the larval tail. Besides, the B5.2 blastomere develops into six muscle cells of the anterior part of the tail, while the A5.2 blastomere gives rise to two cells of the posterior part and the b5.3 blastomere to two cells of the caudal tip region.

order to examine this hypothesis, we have in this study isolated the presumptive muscle cells (B5.1 blastomeres) from 16-cell-stage *Ciona* embryos and allowed them to divide either twice or three times thereafter. Further divisions of the blastomeres were then blocked with inhibitors of DNA replication and of cytokinesis. The cell lineage of the B5.1 blastomere is shown in Fig. 1. According to recent cell lineage analyses in ascidian embryos by intracellular injection of a tracer enzyme, eight muscle cells on each side of the middle part of the larval tail originate from the B5.1 blastomere (Nishida & Satoh, 1983, 1985). Among the progeny cells of the B5.1 blastomere, the B6.2, B7.4, and B8.7 and B8.8 cells are muscle lineage cells (Conklin, 1905; Nishida & Satoh, 1985). Since divisions of the B7.3 and B7.4 blastomeres take place earlier than those of the B7.1 and B7.2 cells, the isolated B5.1 blastomere forms six cells when normal embryos reach the 110-cell stage. The previous study with aphidicolin revealed that if the 64-cell-stage embryos of another species, *Halocynthia roretzi* were arrested by a simultaneous treatment with aphidicolin and cytochalasin B, these cleavage-arrested embryos did not show acetylcholinesterase activity (Satoh & Ikegami, 1981a). On the other hand, if the 110 cell-stage embryos were arrested with the inhibitors, a few of these embryos differentiated the histospecific enzyme. These previous results led us to believe that isolated B5.1 blastomeres which had divided twice then arrested would not develop acetylcholinesterase, while those which had divided three times then arrested would differentiate the histospecific enzyme.

MATERIALS AND METHODS

Embryos

Adults of the ascidian *Ciona intestinalis* (L.) were collected at Takahama, Wakasa Bay, Japan. Eggs and sperm were obtained by dissection of the gonoducts.

Blastomere isolation

An ascidian egg is enclosed within a chorion, which must be removed prior to blastomere isolation. Dechorionation was performed as follows; about 10 min after fertilization eggs were immersed in seawater containing 1% sodium thioglycolate (Wako Pure Chem. Ind., Ltd, Tokyo) and 0.05% pronase E (Kaken Co., Ltd, Tokyo) with the pH of the solution adjusted to about 10.0 by addition of drops of 1N-NaOH. As shown in Fig. 2, gently pipetting the eggs removed the chorion within 9–16 min at room temperature (18–23°C). The dechorionated eggs were washed several times with filtered seawater and were then transferred into plastic Petri dishes coated with 2% agar and maintained until the 4-cell stage. At the 4-cell stage, the culture medium was replaced with calcium- and magnesium-free seawater. When the embryos reached the 16-cell stage (Fig. 3A), they were chilled to about 10°C to prevent further cleavages. The 16-cell-stage embryos were collected and the blastomeres were isolated by vibrating the embryos with a Vortex mixer with the intensity dial set at 4 for 10–20 sec (Fig. 3B; Patricolo & Bozzaro, 1975). From the resulting isolated cells, the B5.1 blastomeres (the nomenclature of blastomeres is that of Conklin (1905)) were selected by their large size and characteristic pigment pattern, and were cultured in plastic Petri dishes coated with 2% agar containing Millipore-filtered seawater at 18–20°C.

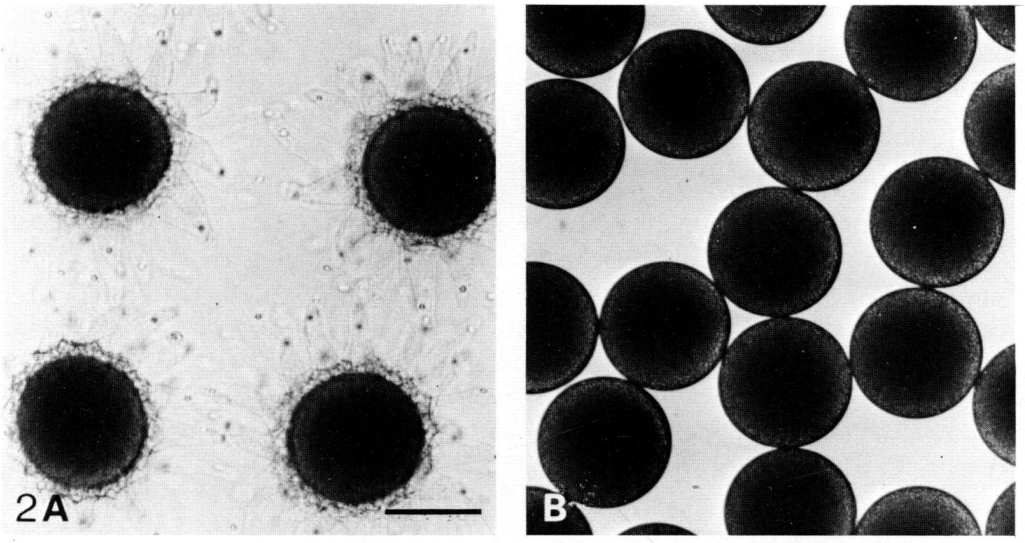

Fig. 2. (A) Normal unfertilized eggs of *Ciona intestinalis* with the chorion and follicle cells. (B) Dechorionated eggs. Scale bar, 100 μm.

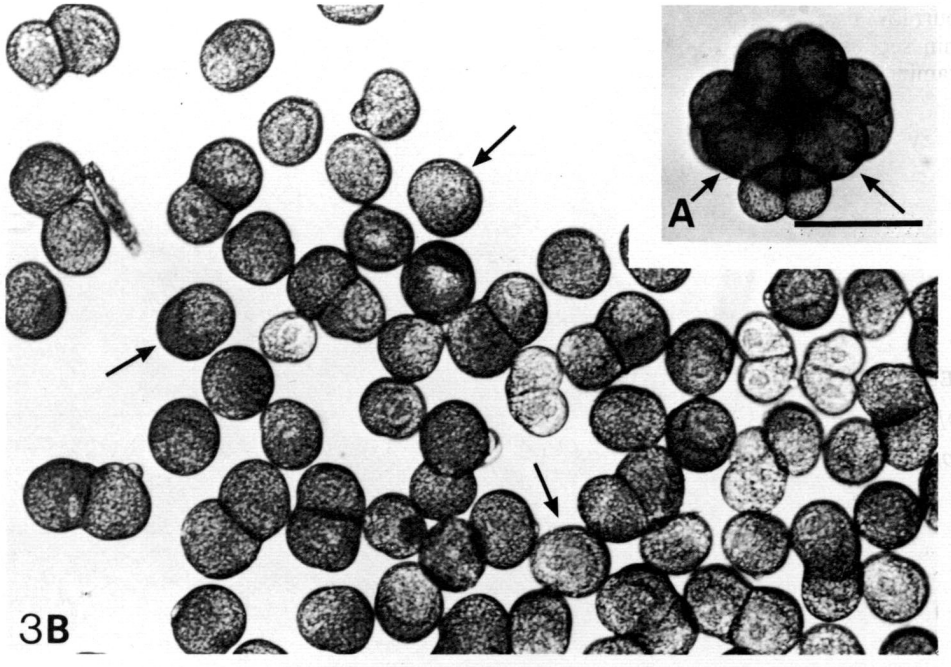

Fig. 3. (A) A 16-cell-stage embryo that developed from a dechorionated egg. Arrows indicate the pair of B5.1 blastomeres. (B) Isolated blastomeres from 16-cell-stage embryos. More than half of the cells are isolated but some of the cells are still paired. The B5.1 blastomeres (arrows) are selected by their large size and characteristic pigment pattern. Scale bar, 100 μm.

Cleavage inhibition and DNA synthesis in cleavage-arrested embryos

Isolated B5.1 blastomeres were allowed to divide either twice or three times, then further divisions of the cells were permanently arrested by a simultaneous treatment with aphidicolin ($10\,\mu g.ml^{-1}$) and cytochalasin B ($2\,\mu g.ml^{-1}$; Aldrich). Aphidicolin is a specific inhibitor of the activity of DNA-polymerase α (Ikegami *et al.* 1978). In order to examine the effects of a simultaneous treatment with aphidicolin and cytochalasin B on DNA synthesis in ascidian embryos, about 6000–8000 *Ciona* gastrulae were reared for 2 h in 10 ml of seawater containing $10\,\mu g.ml^{-1}$ aphidicolin, $2\,\mu g.ml^{-1}$ cytochalasin B, and [^{3}H] thymidine ($20\,\mu Ci$, $50\,Ci.mmol^{-1}$; Amersham). After incubation the embryos were washed three times with ice-cold seawater (pH 5·0) to remove the outer follicle cells, which are the principal source of possible bacterial contamination. After washing the embryos were gently homogenized, and 100 % trichloroacetic acid (TCA) was added to the homogenate at a final concentration of 10 %. The TCA-insoluble precipitate was washed four times with 5 % TCA, suspended in 1·0 ml of 5 % TCA and then boiled for 15 min. Two aliquots of 0·5 ml of the supernatant of centrifugation at 2700 r.p.m. for 10 min were counted in a liquid scintillation counter.

Transmission electron microscopy

Normal 16-cell-stage embryos as well as isolated blastomeres presumed to be the B5.1 cells were first fixed for 60 min 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 30 min 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 Spurr low viscosity resin (Spurr, 1969). Specimens were sectioned with an LKB 8800 Ultrotome. Thin sections were double stained with 1 % aqueous uranyl acetate and lead citrate, and examined with a Hitachi HU-11D-1 electron microscope operated at 75 kV.

Enzyme histochemistry

Acetylcholinesterase activity was detected histochemically by the direct-colouring thiocholine method of Karnovsky & Roots (1964) in the cleavage-arrested 16-cell-stage embryos as well as in isolated blastomeres at the time when normal control embryos reached the late tailbud stage. All specimens were fixed in 5 % formalin–seawater for 20 min and reacted to ascertain acetylcholinesterase activity as described.

RESULTS

DNA synthesis in embryos permanently arrested with a simultaneous treatment with aphidicolin and cytochalasin B

Aphidicolin is a specific inhibitor of DNA-polymerase α. In the previous study we reported that aphidicolin blocked more than 90 % of [^{3}H] thymidine incorporation into *Ciona* embryos but did not affect the incorporation of labelled uridine and leucine (Satoh & Ikegami, 1981b). However, aphidicolin allowed one more cleavage and stopped divisions thereafter, no matter how soon after a cleavage it was applied. In addition, sometimes, furrow-like cracks appeared in eggs kept in aphidicolin for more than 5–6 h. It has been reported that fertilized starfish eggs could continue cleavages for at least several times in the presence of aphidicolin in spite of almost complete inhibition of labelled thymidine incorporation into the eggs (Nagano, Hirai, Okano & Ikegami, 1981). Therefore, these phenomena do not always mean that the inhibitory effect of aphidicolin is too weak to block DNA

Table 1. *Effects of a simultaneous treatment with aphidicolin (10 μg.ml^{-1}) and cytochalasin B (2 μg.ml^{-1}) on [^{3}H]thymidine incorporation into ascidian embryos*

Experiment	[^{3}H]Thymidine incorporation (× 10^{-2} c.p.m./embryo)			
	Control	Cytochalasin B	Aphidicolin	Cytochalasin B + aphidicolin
A	291·2	581·5 (200%)*	49·7 (17%)*	102·9 (35%)* (18%)**
B	265·6	683·8 (258%)*	45·7 (17%)*	98·2 (37%)* (14%)**
C	294·0	779·7 (265%)*	48·7 (16%)*	130·9 (45%)* (17%)**

* Acceleration or inhibition compared to the control; ** Inhibition compared to cytochalasin-arrested embryos.

synthesis. But these conditions are not adequate for analysing the relationship between the number of DNA replication cycles and tissue-specific enzyme development. In this study, then, we used aphidicolin simultaneously with cytochalasin B, which blocks cytokinesis but not nuclear division or DNA synthesis.

We examined [^{3}H] thymidine incorporation in embryos permanently arrested with aphidicolin and cytochalasin B. The results are summarized in Table 1. When *Ciona* gastrulae were allowed to develop for 2 h in seawater containing 10 μg.ml^{-1} aphidicolin and labelled thymidine, the incorporation of radioisotope was blocked more than 80 %. Although the inhibitory degree of aphidicolin detected in this study was slightly lower than that of the previous study (Satoh & Ikegami, 1981b), this confirms that aphidicolin inhibits DNA synthesis of ascidian embryos. If embryos were treated with cytochalasin B for 2 h in the presence of [^{3}H] thymidine, however, the incorporation was accelerated to about 200–250 % (Table 1). Because the nuclei in cytochalasin-arrested cells continued to divide with a temporal pattern very similar to that of normal embryos (Satoh & Ikegami, 1981b), this acceleration is unlikely to be due to higher rates of DNA synthesis. Instead, cytochalasin B may affect the plasma membrane and/or cortical cytoplasmic composition, resulting in the higher incorporation of the radioisotope. When aphidicolin was applied with cytochalasin B, the [^{3}H] thymidine incorporation was inhibited about 60 % compared with normal control embryos. However, if the value was compared to cytochalasin-arrested embryos, the inhibition was more than 80 % (Table 1). Therefore, we concluded that a simultaneous treatment with aphidicolin and cytochalasin B affects primarily DNA synthesis in ascidian embryos, although other side effects cannot be discounted.

Confirmation that isolated and selected blastomeres were the muscle lineage cells

As shown in Fig. 3B, more than half of the blastomeres were isolated by the procedure described above, but some blastomeres remained in pairs, probably

because a cellular structure connected the blastomeres along the plane of bilateral symmetry, as has been shown in another ascidian species (Satoh & Deno, 1984). From among the isolated blastomeres the B5.1 blastomeres were selected by their large size and characteristic pigment pattern. Since the B5.2 blastomere is comparatively small, the possibility of contamination by B5.2-line muscle lineage cells in the selected cells seems low.

Whether the selected blastomeres were the presumptive muscle cells or not was examined by two different methods; ultrastructural observations of the cells and histochemical detection of acetylcholinesterase in the cleavage-arrested blastomeres. It has been verified that the muscle lineage cells of ascidian embryos possess in their cytoplasm a dense aggregate of mitochondria that is an excellent marker for these cells (Mancuso, 1969; Crowther & Whittaker, 1983). Fig. 4A illustrates the ultrastructural characteristics of the B5.1 blastomeres. A large number of yolk granules are present in the peripheral region of the cytoplasm and dense aggregations of mitochondria are distributed in the central region of the muscle lineage cell. When the selected blastomeres were examined with electron microscope, the central regions of the cells were occupied by a large number of mitochondria (Fig. 4B), suggesting that they were the muscle lineage cells.

To study acetylcholinesterase differentiation, first four batches of 58 cytochalasin-arrested, 16-cell embryos were examined. Forty of the arrested embryos (69%) produced acetylcholinesterase in the B5.1 blastomeres. This means that every B5.1 blastomere in cytochalasin-arrested 16-cell embryos does not always develop the enzyme activity, as was already noticed in previous studies (Whittaker, 1973; Satoh, 1979). Second, development of the enzyme was examined in the selected blastomeres. Isolated B5.1 blastomeres were continuously arrested with cytochalasin B for more than 8 h then histochemical detection of the enzyme activity was carried out. Forty-eight out of 131 blastomeres (37 %) in five batches developed acetylcholinesterase activity. Although the reason for the decrease in the frequency of acetylcholinesterase development in the isolated and division-arrested blastomeres compared to that of arrested whole embryos is obscure, this result as well as that of ultrastructural observations indicates that the isolated and selected blastomeres were the presumptive muscle cells, B5.1.

Acetylcholinesterase development in progeny cells of isolated B5.1 blastomeres

B5.1 blastomeres were allowed to divide either twice or three times after isolation, then DNA synthesis and cytokinesis were permanently blocked with aphidicolin and cytochalasin B. Histochemical reactions for acetylcholinesterase activity in the division-arrested blastomeres were carried out at the time when normal control embryos developed to the middle tailbud stage (beyond the time of normal acetylcholinesterase development). We examined seven batches of 42 cases in series A and 14 cases in series B in which the B5.1 blastomeres were allowed to divide twice then arrested. As shown in Table 2, none of them produced histochemically detectable acetylcholinesterase activity (Fig. 5A). On the other hand,

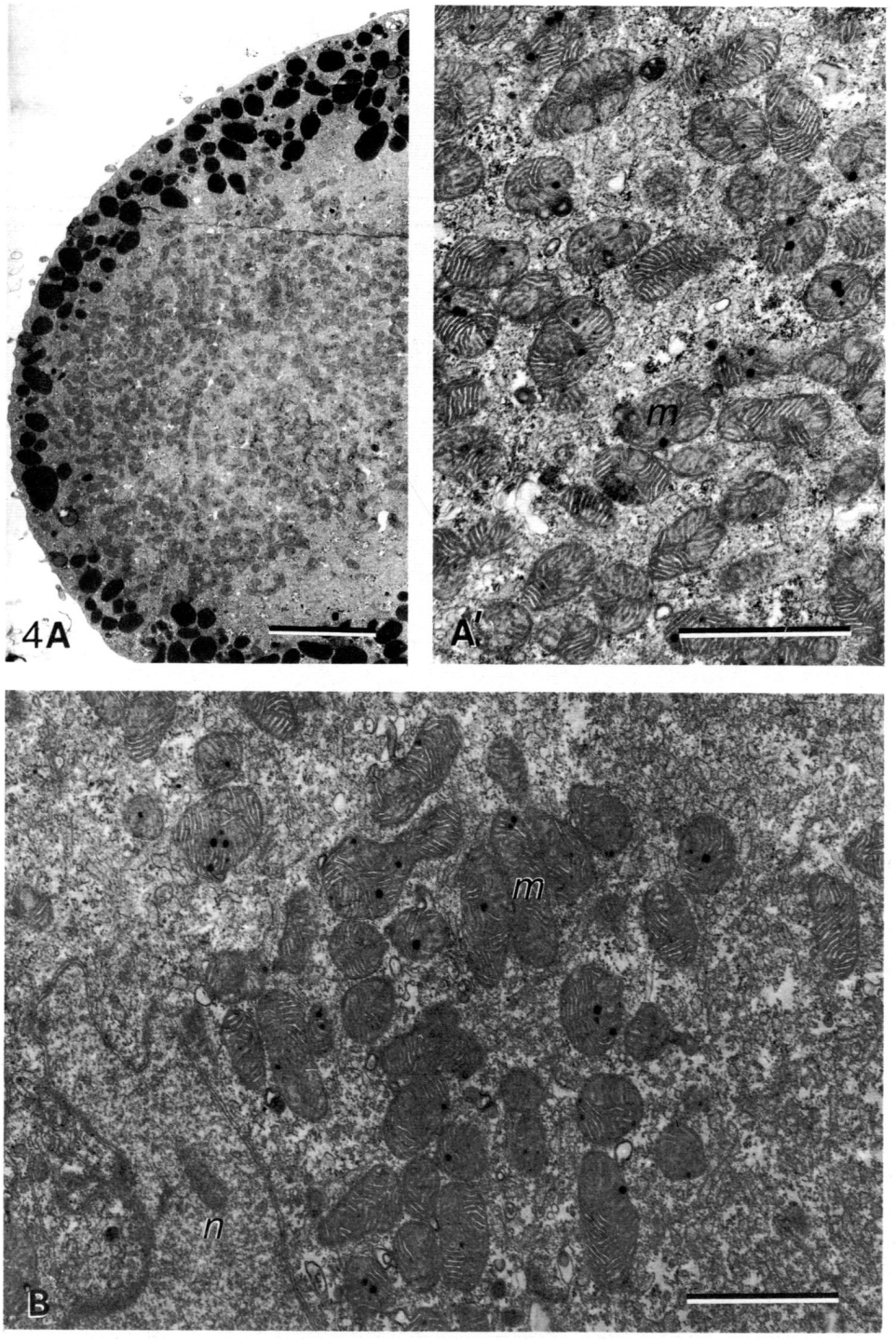

Fig. 4

Table 2. *Results of acetylcholinesterase determination in progeny cells of isolated B5.1 blastomeres (presumptive muscle cells) of* Ciona intestinalis

Cleavage after isolation*	No. of experiments in which some of B5.1 progeny cells showed the enzyme activity	
	Series A	Series B
2	0/42 (0%)	0/14 (0%)
3	12/46 (26%)	4/15 (27%)

* Isolated B5.1 cells were allowed to divide either twice or three times thereafter then further divisions of the cells were permanently inhibited by a simultaneous treatment with aphidicolin and cytochalasin B.

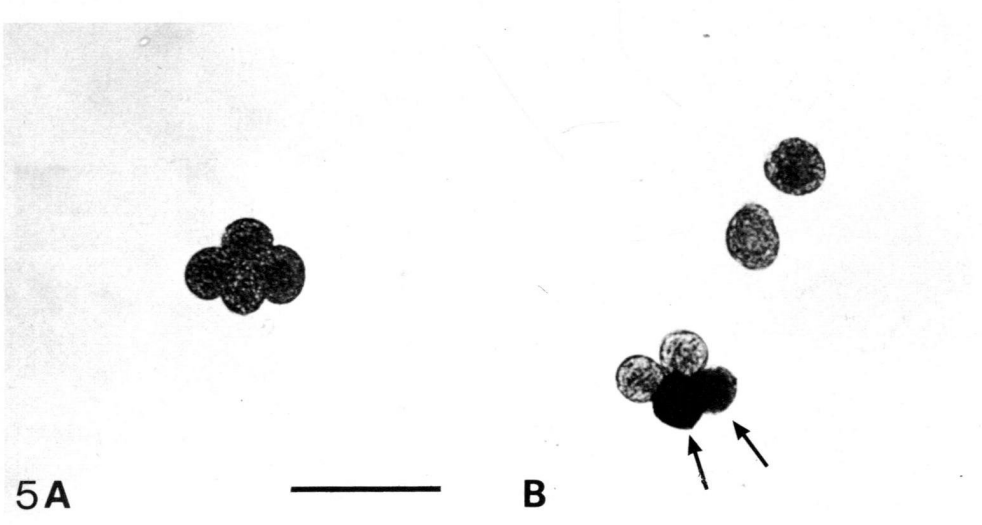

Fig. 5. Histochemical detection of muscle-specific acetylcholinesterase in the progeny cells of isolated B5.1 blastomeres. (A) Four progeny cells resulting from two divisions of an isolated B5.1 cell have been continuously blocked with aphidicolin and cytochalasin B. No histochemically detectable acetylcholinesterase has developed. (B) Six progeny cells resulting from three divisions of an isolated B5.1 cell are permanently arrested with the inhibitors. Clear acetylcholinesterase activity is seen in only two blastomeres, because only these two cells are muscle lineage cells. Scale bar, 10 μm.

Fig. 4. Electron micrographs of B5.1 blastomeres in an intact embryo (A) and an isolated cell (B). (A, A′) The muscle lineage cells in ascidian embryos are characterized by the presence in the cytoplasm of dense aggregates of mitochondria (*m*). (B) The existence of a large number of mitochondria (*m*) near the nucleus (*n*) suggests that the isolated and selected blastomere is a muscle lineage cell. Scale bar in (A), 5 μm and in (A′, B), 1 μm.

12 out of 46 B5.1 blastomeres (26 %) in series A that had divided three times after isolation before they were continuously arrested with aphidicolin and cytochalasin B, developed distinct enzyme activity (Table 2; Fig. 5B). In addition, 4 out of 15 B5.1 blastomeres (27 %) in series B also showed the enzyme activity (Table 2). The enzyme activity was usually observed in both of two muscle lineage cells (B8.7 and B8.8) among the six progeny cells of B5.1 blastomere (Fig. 5B).

DISCUSSION

A hypothesis examined in this study is that muscle cell differentiation in ascidian embryos will occur if two necessary conditions are satisfied; (i) the cell is provided with a segregated egg cytoplasmic determinant responsible for muscle cell differentiation and (ii) the cell completes a definite number of DNA replications. According to this hypothesis, no cell–cell interactions are required for muscle development in ascidian embryos, and neither cytokinesis nor nuclear division is necessary for this differentiation. As originally shown by Whittaker (1979) and confirmed in the previous study (Satoh, 1979) as well as in the present experiment, cytochalasin-arrested 16-cell-stage embryos are able to synthesize acetylcholinesterase in the B5.1 and B5.2 blastomeres. In addition, as shown in this study, if the B5.1 blastomeres are isolated from the 16-cell-stage embryos and either allowed to develop in isolation or their division is arrested with cytochalasin B, the isolated B5.1 cells could differentiate the histospecific enzyme activity. Furthermore, development of myofibrils in the progeny cells of isolated B5.2 blastomeres has recently been verified by Crowther & Whittaker (1983). All these results clearly indicate that at least the B5.1 and B5.2 blastomeres have already been provided with the cytoplasmic determinant which destines the cells to be muscle cells.

When a B5.1 blastomere was allowed to divide two times after it was isolated then DNA synthesis of the progeny cells was blocked with aphidicolin, the four progeny cells did not develop acetylcholinesterase at all. However, when a B5.1 blastomere was allowed to divide three times after isolation then DNA synthesis was inhibited, two of the six progeny cells eventually expressed the enzyme activity. These results indicate that a definite number of DNA replications is necessary for acetylcholinesterase development in isolated presumptive muscle cells in ascidian embryos. Therefore, the hypothesis is plausible. These results also support our previous proposal that the egg may time initiation of differentiation by the cycles of DNA replication and that during a certain DNA replication cycle an interaction between the cytoplasmic determinant and the genome might occur, which would bring about activation of the genes responsible for the enzyme synthesis (Satoh & Ikegami, 1981a, b; Satoh, 1982, 1984).

At present, it is difficult to obtain the necessary number of isolated muscle lineage cells to analyse the intracellular changes occurring during different DNA replications at the biochemical level. At present, we are devising a technique to do this.

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