Acetylcholinesterase development in extra cells caused by changing the distribution of myoplasm in ascidian embryos

By J. R. WHITTAKER

From the Wistar Institute of Anatomy and Biology and the Marine Biological Laboratory, Woods Hole

SUMMARY

This research shows that myoplasmic crescent material of the ascidian egg has both functional autonomy and functional specificity in establishing the differentiation pathway of muscle lineage cells. The cytoplasmic segregation pattern in eggs of Styela plicata was altered by compression of the embryos during third cleavage. This caused a meridional division instead of the normal equatorial third cleavage; first and second cleavages are meridional. Since eggs of S. plicata have a pronounced yellow myoplasmic crescent, one observes directly that third cleavage under compression resulted in a flat 8-cell stage with four cells containing yellow myoplasm instead of the two myoplasm-containing cells that would be formed by normal equatorial division at third cleavage. If such altered 8-cell-stage embryos were released from compression and kept from undergoing further divisions by continuous treatment with cytochalasin B, some embryos eventually developed histospecific acetylcholinesterase in three and four cells instead of in just the two muscle lineage cells found in cleavage-arrested normal 8-cell stages. The wider myoplasmic distribution effected by altering the division plane at third cleavage apparently caused a change in developmental fate of the extra cells receiving myoplasm. This meridional third cleavage also resulted in a changed nuclear lineage pattern. Two nuclei that would ordinarily be in ectodermal lineage cells after third cleavage were now associated with yellow myoplasm. Acetylcholinesterase development in these cells demonstrates that nuclear lineages are not responsible for muscle acetylcholinesterase development in the ascidian embryo.

INTRODUCTION

Many papers in the embryological literature indicate that the eggs of some animal groups have cytoplasmic determinants localized in particular regions of the egg and that these agents are segregated by a determinate cleavage pattern into certain cell lineages where they appear to play a role in programming the differentiation pathways of the cells (Wilson, 1925; Davidson, 1976). Perhaps the best-known example of a cell lineage originating from a distinct cytoplasmic region of the egg involves the yellow crescent region of myoplasm in the ascidian egg (subphylum Urochordata, class Asciidiacea). This crescent material of the

1 Author's address: The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, Pennsylvania 19104, U.S.A.
Styela partita egg is eventually found within the muscle lineage cells of the developing larva (Conklin, 1905). The autonomous development of blastomeres in cleavage-arrested ascidian embryos and the developmental autonomy of surgically isolated early blastomeres have suggested that a cytoplasmic determinant for muscle acetylcholinesterase is localized in the egg myoplasm and segregated into the muscle lineage cells (Whittaker, 1973, 1979; Whittaker, Ortolani & Farinella-Ferruzza, 1977).

The most thoroughly investigated example of a cytoplasmic determinant is the germinal plasm at the posterior pole of the insect egg and the vegetal pole of the anuran amphibian egg (reviewed by Beams & Kessel, 1974; Eddy, 1975; Smith & Williams, 1975). This plasm becomes segregated into the germ cell lineage where it has a determinative role in differentiation of primordial germ cells. Microinjection of the appropriate plasm into eggs has cured the sterility resulting from ultraviolet irradiation of the germ plasm region (Smith, 1966; Okada, Kleinman & Schneiderman, 1974; Warn, 1975). Wakahara (1978) induced extra primordial germ cells by microinjecting a cytoplasmic fraction from vegetal pole cytoplasm of Xenopus eggs into a region near the vegetal pole of unfertilized eggs.

An important advance in this problem of cytoplasmic determinants was achieved by the microinjection experiments of Ilmensee & Mahowald (1974, 1976) on embryos of Drosophila melanogaster. They have shown functional autonomy of the germ plasm determinant by altering its position in the egg and obtaining primordial germ cells in a new location. These cells were then transferred to the gonadal region of other larvae where they gave rise to functionally specific germ cells which produced offspring.

The aim of the present study is to demonstrate a similar functional autonomy and functional specificity of the determinant for tail muscle acetylcholinesterase in the ascidian embryo. In these experiments microcompression of the embryo has been used to distribute myoplasm into additional cells at third cleavage thereby causing these extra cells to become programmed for the development of acetylcholinesterase.

MATERIALS AND METHODS

Embryos

Styela plicata (Lesueur) of the Florida Gulf was obtained during two breeding seasons (November through April) from the Gulf Specimen Company, Panacea, Florida. The animals were held in an Instant Ocean aquarium (15 °C) under constant light. Fresh animals continued to produce fertile gametes for about 10 days under these conditions. Some observations were made on two other species, Styela partita (Stimpson) and Styela clava (Herdman), obtained during summer months in the vicinity of Woods Hole, Massachusetts and maintained in tanks with running sea water.

Eggs and sperm were obtained by dissection of the gonads and gonoducts. Eggs of S. plicata still retain their germinal vesicles when they are liberated in
Ascidian egg compression

This way and at least 40 min (18 °C) are required for germinal vesicle breakdown and first meiotic division to occur, after which the eggs become fertilized. Few of the eggs obtained in this way are fertile (1 % or less); best results were obtained if eggs were not decanted from the sperm suspension until 30 min after first mixing of the eggs and sperm. Embryos reaching the 2-cell stage (95–105 min after mixing eggs and sperm) were isolated manually and maintained at 18 ± 0.1 °C in a constant temperature water bath.

Microcompression experiments

Eggs of S. plicata are approximately 150 µm in diameter exclusive of the chorionic membrane. Microcompression chambers 50 µm in depth were prepared using wax ribbons cut on a microtome and mounted on microscope slides (Bell, 1962). The chambers had a circular well 8 mm in diameter. A hemacytometer coverglass flattened by capillary action against the wax surface will compress eggs placed in the circular well to 50 µm thickness. The time between second and third cleavage is approximately 25 min. Four-cell-stage embryos which were compressed to 50 µm thickness for the last 15–20 min of this period and during the event of third cleavage itself divided meridionally rather than equatorially. Compressed embryos were released by floating the coverglass off in a dish of sea water; the flattened 8-cell stages were immediately transferred to 2 µg/ml cytochalasin B (Sigma Chemical Company) where they were maintained 16–18 h at 18 °C. Normal 8-cell stage control embryos were also placed in cytochalasin B and kept for the same length of time at 18 °C.

Since compressed eggs in a group do not all divide simultaneously there is some variation in their age in relation to the cell cycle when they are released from compression. Consequently, some embryos always underwent an additional complete or partial cleavage in cytochalasin B; these were discarded. The numbers reported in Table 1 refer only to experimental and control embryos in which none of the cells divided further in cytochalasin B and which retained the typical sizes and shapes of 8-cell-stage blastomeres.

Histochemistry

Acetylcholinesterase was localized histochemically by the method of Karnovsky & Roots (1964) after fixation of embryos 3 min in cold (5 °C) 80 % ethanol (Durante, 1956). Incubation was for 20 h at 18 °C. After the reaction embryos were dehydrated in ethanol, cleared in xylene, and mounted in damar resin.

Acetylcholinesterase properties

Acetyltiocholine iodide at 1.7 mM was the reaction substrate. The S. plicata enzyme reaction was inhibited by the Burroughs–Wellcome acetylcholinesterase inhibitor BW 284 C51 dibromide (1.7 mM), but not by 3.4 mM tetraisopropylpyrophosphoramide (iso-OMPA), a pseudocholinesterase inhibitor (Silver, 1974).
S. plicata acetylcholinesterase was unusual in giving a moderate reaction with 1.7 mM butyrylthiocholine iodide. This reaction with butyrylthiocholine was inhibited by BW 284 C51 but not by iso-OMPA. Broad substrate specificity seems to be characteristic of larval acetylcholinesterase in the genus Styela since larvae of S. plicata, S. partita, and S. clava all gave histochemical localizations with butyrylthiocholine iodide, propionylthiocholine iodide, and benzoylthiocholine iodide, each at 1.7 mM. Benzoylthiocholine iodide was purchased from ICN Pharmaceuticals, Inc. All other histochemical reagents were obtained from the Sigma Chemical Company.

RESULTS

Microcompression during third cleavage

This project was suggested by Morgan’s (1910) finding that grossly abnormal embryos result from altering the planes of cleavage by compression during early cell divisions in the ascidian egg (Ciona intestinalis). A number of compression studies by earlier investigators with embryos of other groups had established that compressing eggs during cleavage would cause the division spindles to reorient perpendicular to the direction of the compressing force and parallel to the longest axis of the cell. The relationship is sometimes called Hertwig’s Rule (Hertwig, 1893). Compressed cells then divide in a different plane of cleavage, thereby altering their normal pattern of cytoplasmic distribution.

Morgan’s experiment has been improved upon in the present work by using a species with a visible marker of the region of myoplasmic cytoplasm in the fertilized egg. S. plicata has a very strongly colored yellow crescent of myoplasm (Hirai, 1958; West & Lambert, 1976). Conklin (1905) first noted that a similar yellow crescent of S. partita became segregated entirely into the tail muscle lineages of the developing larva. In the present study, the yellow crescent of S. plicata was also found to be segregated into the muscle lineages.

The work reported here is concerned entirely with the results of changing the plane of third cleavage in the S. plicata egg. Ascidian eggs have an animal–vegetal polar axis which is established soon after fertilization. The peripheral position of the female pronucleus in the mature unfertilized egg marks the central animal pole of subsequent development through which this axis of symmetry passes. In species with a visible myoplasmic crescent, the crescent occurs in the immediate subcortical cytoplasm just below the equator of the egg (Fig. 1A). This crescent marks an axis of bilateral symmetry since first cleavage is meridional along the animal–vegetal axis and bisects the crescent into equal halves (Fig. 1B).

Second cleavage is also meridional: it occurs at right angles to the first and isolates the myoplasmic crescent into two of the four cells (Figs. 1C, 2). Third cleavage is equatorial and perpendicular to the plane of the first two cleavages: it divides the embryo across the equator into animal and vegetal quartets of cells. Crescent material becomes localized in only two of the eight cells (Figs. 1D, 3) and these are the muscle lineage cells. Oviparous ascidian eggs all apparently
Ascidian egg compression

Fig. 1. Location of the myoplasmic yellow crescent in the first cleavage stages of the *Styela plicata* embryo, and its distribution after compression during third cleavage. Fertilized egg (A), 2-cell stage (B), 4-cell stage (C), and 8-cell stage (D), showing the animal–vegetal axis and the position of the two polar bodies. Orientation of the compression of 4-cell-stage embryos (E) and the resulting flat plate of eight cells formed by third cleavage taking place while the embryo is compressed to one third of its thickness (F).

have this same determinate pattern of cleavage in relation to predetermined axes and localized cytoplasmic materials. This cleavage pattern results in isolating certain cytoplasmic areas of the egg into particular cell and tissue lineages of the embryo.

When the 4-cell-stage embryo of *S. plicata* was compressed it invariably flattened in the direction of the animal–vegetal axis (Fig. 1E). During third cleavage, cell division in these flattened embryos became meridional rather than equatorial. As a consequence, the embryo was cut into eight almost wedge-shaped segments in a single plane (Figs. 1F, 4), instead of the two upper and lower quartets of cells that would result from a normal equatorial cleavage. This induced pattern of division resulted in four cells containing myoplasmic crescent instead of the normal two. The advantage of using *S. plicata* is that one observes directly the change in myoplasmic distribution (Fig. 4).

Embryos which have been compressed during third cleavage did not produce normal or even recognizable larvae when they were released from compression and permitted to go on with development. Although cell division continued, the
Ascidian egg compression

349

embryos did not gastrulate and no blastopore could be seen at the appropriate times (at seventh cleavage and later). Unfortunately, the disorganized multicellular embryos that result from a changed third cleavage do not lend themselves to a simple analysis of what possible changes in cell fate might result from the altered cytoplasmic distribution.

Acetylcholinesterase development in cleavage-arrested embryos

A feature of muscle differentiation in *S. plicata* and many other ascidian species is the development of an acetylcholinesterase beginning at about neurulation and continuing throughout later embryogenesis (e.g. Fig. 5). This enzyme does not develop in the larval brain of *S. plicata* and occurs in the adult brain only in older larvae and after larval metamorphosis. Embryos of *Ciona intestinalis* which were cleavage-arrested during early stages of development eventually gave rise to acetylcholinesterase in the muscle lineage blastomeres of the arrested embryos (Whittaker, 1973). This was true also for *S. plicata*. Normal 8-cell stages which were prevented from undergoing further cleavage by placing them in 2 μg/ml cytochalasin B eventually developed acetylcholinesterase in the two muscle lineage blastomeres of many embryos (Fig. 6).

This technique offers a simple method of evaluating possible cellular transformation that results from changing the myoplasmic distribution at third cleavage. Compressed embryos in which yellow myoplasm was segregated into four rather than two of the eight cells at third cleavage were placed in cytochalasin B immediately after they were released from compression. A significant number of such embryos (19 %) eventually developed acetylcholinesterase in three or four of the myoplasm-containing cells. This number is similar to the number (20 %) of control embryos developing enzyme in both of the muscle

---

**Figures 2-7**

Fig. 2. Four-cell-stage embryo of *Styela plicata*. The myoplasmic yellow crescent (granular dark area) is visible in the two bottom blastomeres.

Fig. 3. Normal 8-cell stage after third cleavage. Myoplasm occurs in the two lower blastomeres which are the muscle lineage cells.

Fig. 4. Embryo compressed to 50 μm thickness at the 4-cell stage and photographed after third cleavage. Granular myoplasm occurs in the four lower blastomeres.

Fig. 5. Localization of acetylcholinesterase activity in tail muscle of the normal *Styela plicata* embryo. Reacted histochemically at the late-tailbud stage.

Fig. 6. Normal 8-cell-stage embryo cleavage-arrested in cytochalasin B for 16 h then reacted for acetylcholinesterase activity. Reaction occurs in the two muscle lineage blastomeres.

Fig. 7. Compressed 8-cell stage embryo, as in Fig. 4, cleavage-arrested in cytochalasin B for 16 h and then reacted for acetylcholinesterase activity. Reaction occurs in the four myoplasm-containing cells. Figs. 2-4 were photographed in Normarski interference phase-contrast (Leitz) and Figs. 5-7 with bright-field illumination. All figures are of the same magnification, bar is 50 μm.
Table 1. Acetylcholinesterase development in embryos compressed during third cleavage and cleavage-arrested in the 8-cell stage for 16–18 h by treatment with cytochalasin B

(Based on 27 separate experimental series (450 embryos) and 19 parallel control series (681 embryos).)

<table>
<thead>
<tr>
<th>Number of blastomeres containing acetylcholinesterase</th>
<th>Experimental embryos</th>
<th>Control embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>(%)</td>
</tr>
<tr>
<td>0</td>
<td>207</td>
<td>54</td>
</tr>
<tr>
<td>1 or more</td>
<td>243</td>
<td>36</td>
</tr>
<tr>
<td>2 or more</td>
<td>165</td>
<td>19</td>
</tr>
<tr>
<td>3 or more</td>
<td>84</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

lineage cells (Table 1). Twenty-eight out of a total of 450 experimental embryos produced enzyme in all four myoplasm-containing cells (Fig. 7).

There is no simple explanation of why so few embryos of either the experimental or control groups produced the maximal possible numbers of enzyme-containing cells. The results in the two groups are comparable, but the experimental embryos have a greater tendency to develop enzyme (Table 1), perhaps as a result of their augmented numbers of myoplasm-containing cells. The relationship between numbers of myoplasm-containing cells and numbers of embryos producing acetylcholinesterase is not, however, strictly proportional.

Enzyme development in the experimental embryos did not appear to be an all-or-nothing response. Sometimes, as in Fig. 7, all three or four of the reactive cells had the same apparent enzyme activity, as judged by the relative histochemical staining intensity, but frequently the concentration of activity varied widely. Ordinarily two or three of the responding cells had much less activity than one or two more strongly staining cells. Crescent material seems to be distributed almost equally to the four crescent-containing blastomeres of the compression-treated embryos; Fig. 4 is typical of the result obtained. The response obviously varies for reasons other than a quantitative effect of myoplasm concentration.

In normal 8-cell stages that are placed in cytochalasin B the animal quartet blastomeres often fuse together. They do not seem, however, to form a syncytium but are nonetheless quite inseparable. The four vegetal quartet blastomeres, which include the two muscle lineage cells, remain quite separate and distinct. The cells of experimental embryos all tend to stay discrete and unfused during their exposure to cytochalasin. This observation considered along with the failure of gastrulation suggests that the abnormal third cleavage must also redistribute the cell surface in such a way that precludes cells and their progeny from associating and interacting normally.
Fig. 8. Normal and compressed ascidian eggs showing a hypothetical qualitative nuclear lineage at third cleavage. Division is depicted in relation to the segregation of yellow crescent myoplasm. At cell division, the daughter nuclei are presumed to differ, as indicated, in some intrinsically programmed and sequentially oriented way. (A, B) Normal third cleavage in which division cuts across the animal-vegetal axis resulting in animal and vegetal quartets of blastomeres. A daughter nucleus from each of the two myoplasm-containing cells at the 4-cell stage is now in a presumptive ectoderm cell of the animal quartet. Side view parallel to the axis. (C, D). Third cleavage of an egg under compression. Cleavage occurs along the animal-vegetal axis. Side view. (E, F) Third cleavage of the compressed egg in C and D viewed downwards from the animal pole in the direction of compression. All four nuclei originating from two blastomeres at the 4-cell stage are in cells containing myoplasm.

Altered nuclear–cytoplasmic relationships

Another consequence of the abnormal third cleavage is a change in the nuclear–cytoplasmic relationships of 8-cell-stage blastomeres. There is no evidence that an intrinsic lineage progression of qualitative nuclear change directs the choice of differentiation pathways in so-called mosaic embryos. The idea of nuclear lineage originated with the germ-plasm theory of Weismann (1892) and has persisted since that time. If there were such evidence, and Fig. 8 depicts a hypothetical nuclear lineage at third cleavage, then two of the nuclei that would ordinarily be found in the presumptive ectodermal cells of the animal quartet after a normal third cleavage (Fig. 8A, B) are now associated with myoplasm instead (Fig. 8C–F). The results of cell lineage and microsurgical studies indicate clearly that the major pathways of differentiation are already...
established at the 8-cell stage (Reverberi, 1961, 1971). Since extra myoplasm-containing blastomeres respond to the new relationship by producing acetylcholinesterase, this finding strongly implicates the role of cytoplasm in selecting the differentiation pathway.

**DISCUSSION**

The first microcompression experiments on ascidian embryos were done by Morgan (1910) to investigate the mosaic nature of the embryos. His results indicated that ascidian embryos could not regulate, as did sea-urchin embryos, to compensate for changes in the early cleavage pattern; the resulting larvae were abnormal. Unfortunately, the disorganized complexity of the larvae precluded any investigation of cell fates that might have been changed by altering the cytoplasmic segregation pattern. A more definitive microcompression experiment was done by Wilson (1896) on embryos of another mosaic species, the annelid *Nereis*. Embryos which were compressed during the first three cleavages formed additional macromeres in the trochophore. There was, however, no test of functional specificity for these extra macromeres since macromeres remain undifferentiated in the trochophore larva.

Ambiguities in the interpretation of the previous microcompression experiments were avoided by the nature and design of the present experiment. Some experimental embryos have three and four large acetylcholinesterase-producing cells rather than only two as in controls. This unquestionably demonstrates a change in the number of muscle lineage cells caused by altering the distribution of myoplasm. Myoplasm does, therefore, have a functional autonomy in relation to muscle differentiation that is evident by its displacement to other cellular locations. Functional specificity of the change is demonstrated by the development of acetylcholinesterase. Ordinarily, acetylcholinesterase develops only in the muscle lineage cells of ascidian embryos.

Results of experiments by Tung et al. (1977) show that selection of differentiation pathways in ascidian embryos is not mediated primarily by a stable intrinsic nuclear lineage. Anucleate *Ciona* egg fragments into which they microinjected the nucleus of older differentiated cells then cleaved to produce abnormal or partial embryos containing different tissues. The tissue types formed were determined mainly by regional cytoplasmic differences in the egg fragments. In the present experiments myoplasm initiated developmental changes that would ordinarily not occur in cells containing that particular nuclear lineage, as indicated by the diagrams in Fig. 8. Both lines of evidence strongly favor cytoplasmic determinants as playing a major role in the cellular differentiation of ascidian embryos. They do not support the view that nuclear lineages are a necessary basis of the mosaic development in ascidian embryos.

The myoplasmic crescent of ascidian embryos contains large numbers of mitochondria. An ascidian species producing anural larvae in which no mitochondrial localization and segregation were associated with the myoplasm
nevertheless developed some of the tail muscle acetylcholinesterase in their embryos (Whittaker, 1979). Cytoplasmic determinants of muscle differentiation are not, therefore, associated with the mitochondria but apparently occur elsewhere in the myoplasm. The results of experiments with inhibitors of RNA synthesis suggest that a stage-specific genetic transcription is required for acetylcholinesterase development in muscle tissues (Whittaker, 1973, 1977, 1979). It seems likely that the cytoplasmic determinant of acetylcholinesterase development is a factor initiating genetic expression in the muscle lineage cells.

Muscle acetylcholinesterase development of ascidian embryos offers a very advantageous model for embryological research. Localization and segregation of a cytoplasmic determinant can be related directly to synthesis of a histospecific protein, rather than solely to complex morphological and biochemical features. The findings reported here indicate both functional autonomy and functional specificity of the acetylcholinesterase determinant. They suggest the possibility that materials extracted from unfertilized eggs and microinjected into non-muscle lineage blastomeres of the early embryo might induce some of these cells to express acetylcholinesterase synthesis. If so, microinjection into blastomeres could become a suitable test system for monitoring the isolation and purification of a possible gene activator substance.

This work was supported by Grant HD 09201, awarded by the National Institute of Child Health and Human Development, DHEW.

REFERENCES


(Received 14 August 1979, revised 25 September 1979)