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# p58, a Cytoskeletal Protein, Is Associated With Muscle Cell Determinants in Ascidian Eggs

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**Abstract.** The theory that p58, a cytoskeletal protein, has an important role in ascidian muscle cell development was tested by altering normal distributions of orange-pigmented myoplasm in *Boltenia villosa* embryos and determining if muscle development is correlated with the presence of p58. Removal of the animal region of fertilized *Boltenia* eggs resulted in the redistribution of myoplasm into the anterior endoderm cells of the embryo. Despite alterations in the normal distribution of myoplasm, these embryos developed into larvae. However, when four-celled embryos that exhibited altered distributions of pigmented myoplasm were stained with NN18, an antibody that stains p58, a maximum of two blastomeres were stained, as in control embryos. Compression of *Boltenia* embryos at the four-celled stage caused the myoplasm to be partitioned into four blastomeres of an eight-celled embryo, instead of into two blastomeres. Compressed and cleavage-arrested eight-celled embryos developed myosin and muscle actin RNA in a maximum of four blastomeres, compared to a maximum of two blastomeres in control embryos. When compressed eight-celled embryos were stained with NN18, p58 was present in a maximum of four blastomeres. These results support the idea that the cytoskeletal protein p58 is associated with muscle cell determinants in ascidian eggs.

## Introduction

Egg cytoskeletons are known to have important roles in the determination of embryonic cells (Jeffery, 1982; Jeffery, 1989; Elinson, 1990; Hill *et al.*, 1990). Experiments performed by Jeffery and Meier (1983), Jeffery and Swalla (1990a), Swalla *et al.* (1991), and Marikawa (1995) support the idea that cytoplasmic factors required for the development of larval muscle cells are associated with myoplasmic cytoskeletal domain (MCD) of ascidian eggs. The MCD consists of two distinct, yet integrated, cytoskeletal systems: a plasma membrane lamina (PML) and a deep filamentous lattice (DFL). The PML is an actin-containing skeleton, sensitive to DNase I treatment, that contracts towards the vegetal pole during ooplasmic segregation (Jeffery and Meier, 1983). The DFL (Jeffery and Meier, 1983; Swalla *et al.*, 1991) is situated beneath the PML; contains pigment granules, mitochondria, and other components; and co-segregates with the PML during ooplasmic segregation. Swalla *et al.* (1991) have shown that a protein termed p58, which binds to a vertebrate intermediate filament antibody, is a component of the DFL. In ascidian species that produce tadpoles, the DFL was present in the cortical cytoplasm of the unfertilized egg and was partitioned exclusively into embryonic muscle progenitor cells. Eggs produced by direct-developing ascidians, which do not produce larvae with muscular tails, lack p58 (Swalla *et al.*, 1991; Bates, 1995).

Normal distributions of myoplasm were altered in ascidian embryos by using a microcompression technique (Whittaker, 1980). Whittaker positioned four-celled *Styela* embryos between glass plates during the third cell cycle and applied pressure that resulted in the reorientation of mitotic spindles according to Hertwig's Rule. In compressed eight-celled embryos, all of the blastomeres were in the same plane, instead of four animal blastomeres being positioned over four vegetal blastomeres. Furthermore, in compressed embryos, myoplasm was present in four blastomeres, instead of in two blastomeres. Compressed and non-compressed eight-celled embryos were then treated with cytochalasin B to pre-

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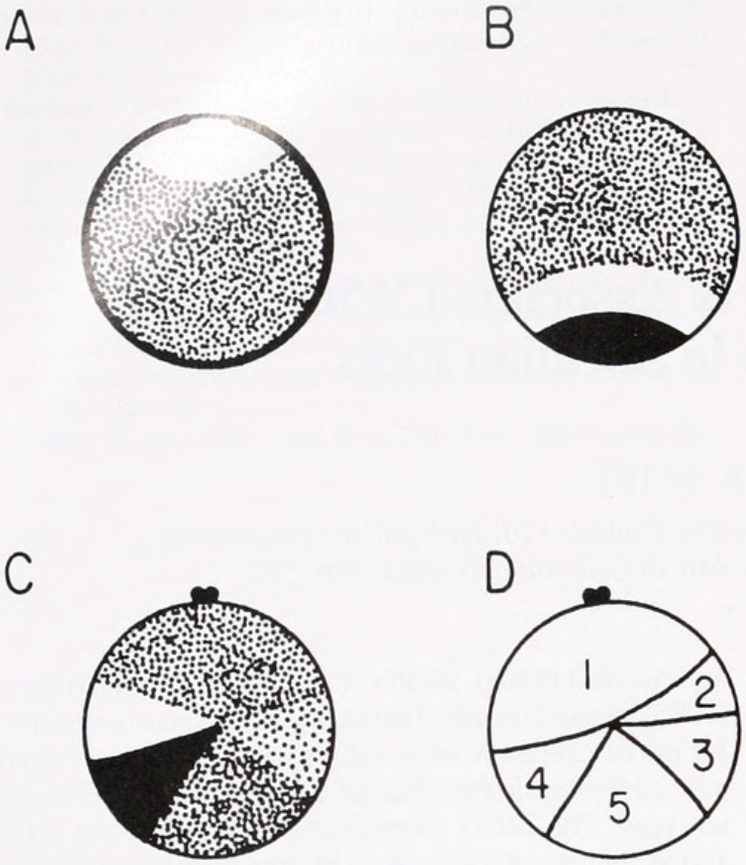


**Figure 1.** A schematic drawing showing the distribution of cytoplasmic determinants in unfertilized and fertilized ascidian eggs. (A) In the unfertilized egg, the myoplasm (bold line) is situated in the egg periphery. The ectoplasm (white region) is located inside the germinal vesicle, and the endoplasm (stipled region) fills the inner egg region. (B) After an egg is fertilized, the myoplasm moves into the vegetal pole region. The ectoplasm forms a band of cytoplasm above the myoplasm, and the endoplasm resides above the ectoplasm. (C) During the second phase of ooplasmic segregation, the myoplasm and ectoplasm shift into the equatorial region. At the end of the second phase of ooplasmic segregation, the egg cytoplasm is divided into five regions: (1) ectoplasm; (2) neuroplasm; (3) notochord plasm; (4) myoplasm; and (5) endoplasm (based on Conklin, 1905).

vent subsequent cell divisions. Treatment with cytochalasin facilitated the identification of myoplasm-containing blastomeres in eight-celled embryos after they were cultured for the time required for control embryos to develop into hatched larvae. When these embryos were examined for the activity of acetylcholinesterase (AchE), a muscle-specific enzyme, a maximum of four blastomeres comprising a compressed embryo expressed AchE activity, whereas in non-compressed eight-celled embryos a maximum of two blastomeres exhibited AchE activity. These elegant experiments performed by Whittaker provide rigorous support for Conklin's idea, published in 1905, that ascidian eggs contain muscle cell determinants.

Bissection of fertilized eggs has also been used to alter the normal distributions of myoplasm in ascidian embryos. When the animal hemispheres of fertilized eggs were removed, myoplasm was partitioned into more than the normal number of blastomeres (Bates, 1988). During the first phase of ooplasmic segregation, fertilized eggs were bissected into vegetal fragments that contained all of the egg myoplasm, termed myoplasm-enriched (ME) fragments, and into animal fragments that lacked myoplasm. ME fragments composed of 40%–50% of the total egg volume usually cleaved normally, completed gastrulation, and in some cases developed into larvae. At each developmental stage, it was shown that more than the normal number of blastomeres contained myoplasm. In larvae derived from ME fragments, most of the myoplasm was contained in tail muscle cells, although significant quantities of myoplasm were present in some of the head endoderm cells.

When the spatial expressions of tissue-specific markers were examined in ME larvae, each marker was normally expressed. For example, AchE activity and myosin were expressed in the cytoplasm of tail muscle cells, and alkaline phosphatase (AP) activity was expressed in the cytoplasm of endoderm cells situated in the head region. In another set of experiments, smaller ME fragments composed of 10%–30% of the total egg volume were produced from the vegetal pole region of fertilized eggs at the first stage of segregation, and these fragments could sometimes undergo several rounds of cleavage before cell division stopped. When cleavage-arrested four-celled ME embryos composed of 10%–30% of the total egg volume were tested for AchE activity after the controls developed into larvae, in some cases AchE activity was detected in all four blastomeres. In cleavage-arrested control four-celled embryos and ME four-celled embryos composed of 40%–50% of the total egg volume, AchE

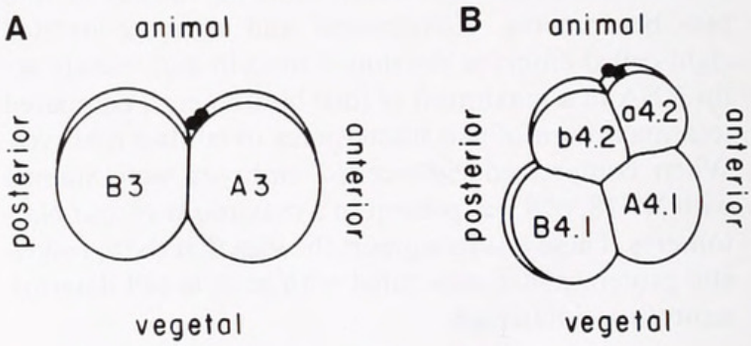


**Figure 2.** Schematic drawings showing the designation of blastomeres composing four-celled and eight-celled ascidian embryos. (A) The A3 blastomeres of a four-celled embryo lack myoplasm, whereas the B3 blastomeres contain myoplasm and develop muscle cell features when isolated from the rest of the embryo. (B) B4.1 blastomeres contain myoplasm and produce most of the tail muscle cells, mesenchyme cells, and some of the endoderm cells and notochord cells. A4.1 blastomeres produce spinal cord cells, endoderm, notochord, and distal tail muscle cells. a4.2 blastomeres produce epidermal and brain cells. b4.2 blastomeres produce epidermis, spinal cord cells, and distal tail muscle cells (Conklin, 1905; Nishida and Satoh, 1983).

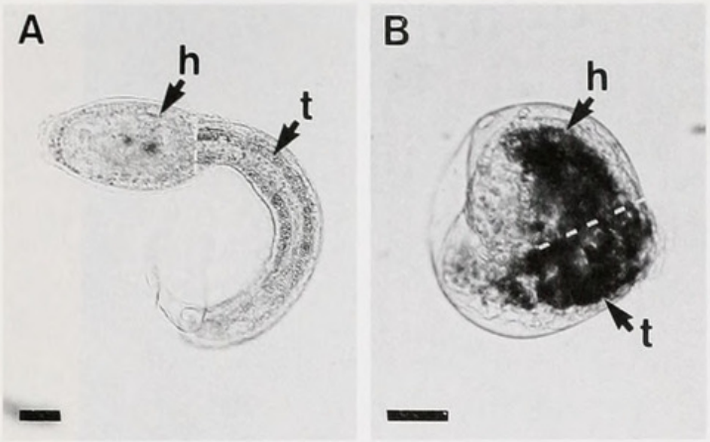


**Figure 3.** Light photomicrographs of detergent-extracted larvae. (A) A control larva showing the pigmented myoplasm within tail muscle cells (t). Endoderm cells situated within the head region (h) do not contain myoplasm. (B) A myoplasm-enriched (ME) larva showing the pigmented myoplasm within tail muscle cells (t) and the pigment granules evident within some of the endoderm cells situated in the head region (h). The dotted white lines shown in (A) and (B) designate the boundary between the larval head and and tail regions. Scale bars equal 50 μm.

activity was detected in a maximum of two blastomeres that corresponded to the posterior B3 muscle progenitor cells.

The contrasting results obtained from these two kinds of myoplasm redistribution experiments present an intriguing paradox. Why were muscle cell fates altered in compressed embryos and in small ME embryos produced from the vegetal pole region, whereas muscle cell fates were normal in ME embryos composed of 40%–50% of the total egg volume? The present study resolves this paradox by examining the distribution of MCD components in ME and compressed *Boltenia villosa* embryos and provides experimental support for the idea that p58 anchors ascidian muscle cell determinants.

## Materials and Methods

### *Adult ascidians and embryo cultures*

*Boltenia villosa* (Stimpson 1864) adults were purchased from Westwind Sealab Supplies, Victoria, British Columbia, Canada. Adults were maintained under conditions of constant light to prevent spawning (West and Lambert, 1975). Eggs and sperm were dissected from the gonads of two or more individuals. The eggs were cross-fertilized, washed several times with large volumes of seawater, and cultured at 11°C until the desired developmental stages were obtained.

### *Surgical methods*

The spatial patterns of five kinds of cytoplasmic factors present in unfertilized and fertilized ascidian eggs are shown in Figure 1. These factors determine epidermal, muscle, endoderm, notochord, and nerve cell fates. The present study is focused on muscle cell factors localized in the myoplasm of ascidian eggs. Prior to fertilization, the myoplasm is present in the cortical cytoplasm that surrounds the egg. Within minutes of fertilization, the myoplasm is dramatically segregated into the vegetal hemisphere by a series of precisely controlled cytoplasmic movements, termed ooplasmic segregation. A fixed cleavage pattern subsequently partitions the myoplasm into specific blastomeres of the embryo (Fig. 2). In four-celled embryos, the posterior B3 blastomeres contain myoplasm, whereas the anterior A3 blastomeres lack it. At the eight-cell stage, the B4.1 blastomeres contain myoplasm, and these cells produce most of the tail muscle cells of the larva (Conklin, 1905; Nishida and Satoh, 1983).

The normal distribution of myoplasm within embryonic progenitor cells was altered using a surgical method previously described by Bates and Jeffery (1987). Eggs were fertilized and allowed to undergo the first phase of ooplasmic segregation, in which the cortical myoplasm is moved into the vegetal hemisphere and surrounds the vegetal pole. At a position corresponding to the animal pole, a tear was made in the follicular envelope (FE) that surrounds the egg. In some cases, 50%–60% of the total egg was extruded through the torn FE, leaving the my-

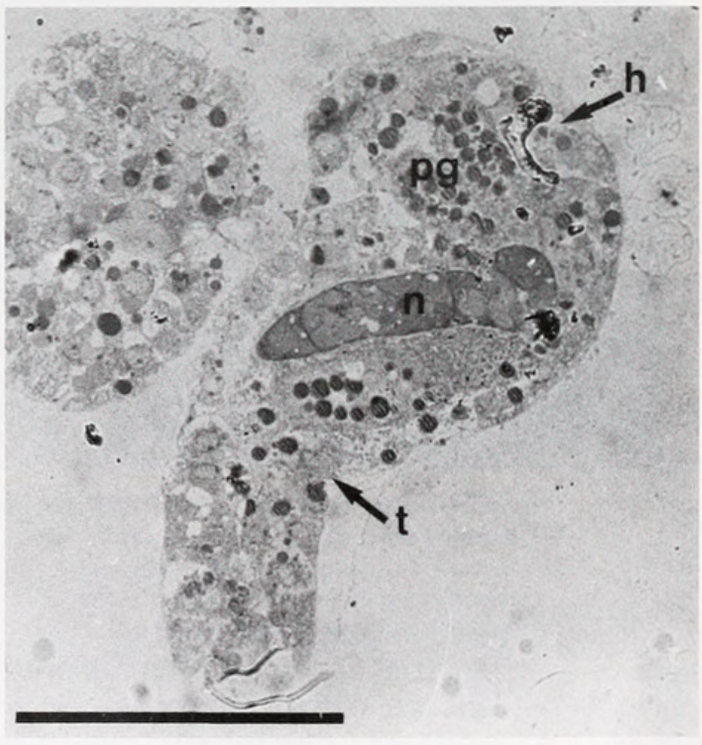


**Figure 4.** Light photomicrograph of a thick section through a myoplasm-enriched (ME) larva showing pigment granules (pg) in tail muscle cells and in the cytoplasm of endoderm cells situated in the head region (h). The notochord (n) is displaced into a more anterior position than normal. Scale bar equals 100 μm.

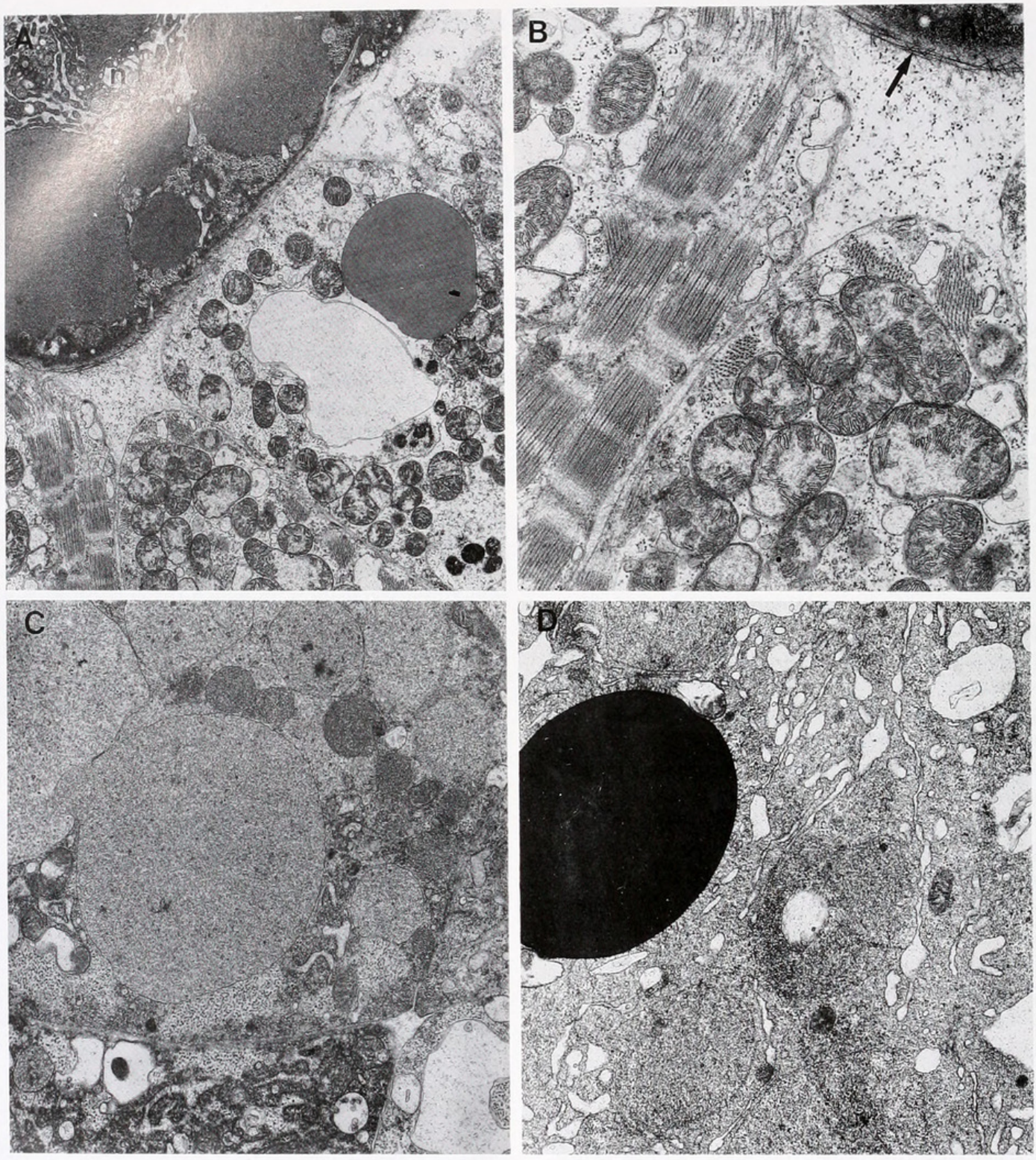


**Figure 5.** Transmission electron micrographs showing the ultrastructural features of muscle and endoderm cells of a myoplasm-enriched (ME) larva. (A) Sacromeres are evident in tail muscle cells near the notochord (n). Magnification equals 6,300×. (B) A higher magnification of (A) showing well-developed sacromere structure. Magnification equals 13,500×. Arrow points to the notochord sheath that surrounds the notochord (n). (C) Fine structure of yolky endodermal cells situated in the head region. Magnification equals 10,500×. (D) Features of endoderm and epidermal cells in the head region. Magnification equals 10,500×.

oplasm-containing vegetal hemisphere within the FE. The cytoplasmic bridge connecting the extruded egg region with the myoplasmic region was cut using a fine needle. In other cases, 70%–90% of the total egg volume was extruded through the tear, and the cytoplasmic bridge was cut leaving the vegetal pole ME fragment

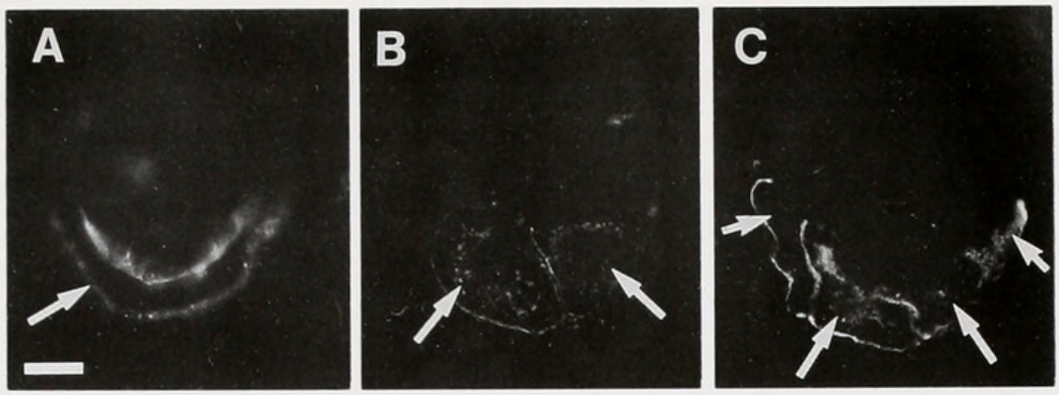


**Figure 6.** Photomicrographs showing myosin heavy chain expression in a control larva, a cleavage-arrested eight-celled embryo, and a compressed and cleavage-arrested eight-celled embryo. (A) Section of a control larva showing the expression of myosin heavy chain protein in tail muscle cells (arrow) situated along the notochord. (B) Section of a cleavage-arrested eight-celled embryo showing myosin in the peripheral cytoplasm of B4.1 blastomeres (long arrows). (C) Section of a compressed and cleavage-arrested eight-celled embryo showing myosin in the peripheral cytoplasm of B4.1 blastomeres (long arrows) and two additional blastomeres (short arrows). Scale bar in (A) equals 50 μm. Same magnifications in (B and C) as in (A).

within the FE. ME fragments of both size classes were either fixed for electron microscopy or immunocytochemistry, or they were transferred to tissue culture wells containing filtered seawater and cultured until the desired developmental stages were obtained.

*Microcompression of embryos*

Whittaker (1980) modified T. H. Morgan's microcompression technique (1910) to reorient mitotic spindles of eight-celled *Styela* embryos. Compression resulted in the partitioning of the myoplasm of eight-celled *Styela* embryos into four blastomeres instead of two blastomeres. A compression method similar to Whittaker's was used to alter the normal distributions of myoplasm in *B. villosa* embryos. Microcompression chambers were prepared by positioning strips of lens paper about 20 mm apart on a glass microscope slide. A drop of seawater containing four-celled embryos was positioned between the paper strips, and a coverslip was placed over the embryos. Seawater was gradually withdrawn from beneath the coverslip by capillary action to exert a gentle pressure on the embryos. The extent of compression was monitored under a microscope. Pressure was applied to embryos for about 15 to 20 min. Next, the coverslip was floated away from the compressed embryos and the embryos were transferred to a well containing 2 μg/ml cytochalasin B (CB; Sigma Chemical Co., St. Louis, MO) dissolved in seawater. CB treatment inhibits cell division, as previously described by Whittaker (1980). Control and compressed eight-celled CB embryos were cultured until the control eggs developed into hatched larvae. CB embryos were subsequently processed for *in situ* hybridization or immunocytochemistry.

*Identification of myoplasm*

Orange pigment granules embedded in the cortex of *B. villosa* eggs were used to produce myoplasm-enriched (ME) egg fragments. Some specimens were treated with 0.5% Triton X-100 detergent to make the myoplasm-containing cells more visible in photographs (Jeffery and Meier, 1983; Bates, 1988).

*Transmission electron microscopy*

Larvae were fixed with 2% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 30 min at room temperature, as previously described by Jeffery and Meier (1983). Specimens were rinsed with phosphate buffer and post-fixed in 1% osmium tetroxide dissolved in 0.1 *M* phosphate buffer, pH 7.4, for 1 h at room temperature. After the samples were rinsed with the phosphate buffer, they were dehydrated through a graded series of ethanol. Following gradual infiltration with Spurr's resin, specimens were embedded. Thick sections (0.5 μm) and thin sections were cut. The thin sections were stained with 2% aqueous uranyl acetate and lead citrate and viewed using a Phillips 420 electron microscope at 80 kV.

*Scanning electron microscopy*

Specimens were prepared for scanning electron microscopy (SEM) using a modification of a method previously described by Jeffery and Meier (1983). Specimens were extracted with 0.5% Triton X-100 detergent dissolved in seawater for 45 to 60 min at room temperature and then washed in seawater. Triton X-100 detergent removes cell membranes to expose the underlying MCD for SEM (Jeffery and Meier, 1983). Specimens

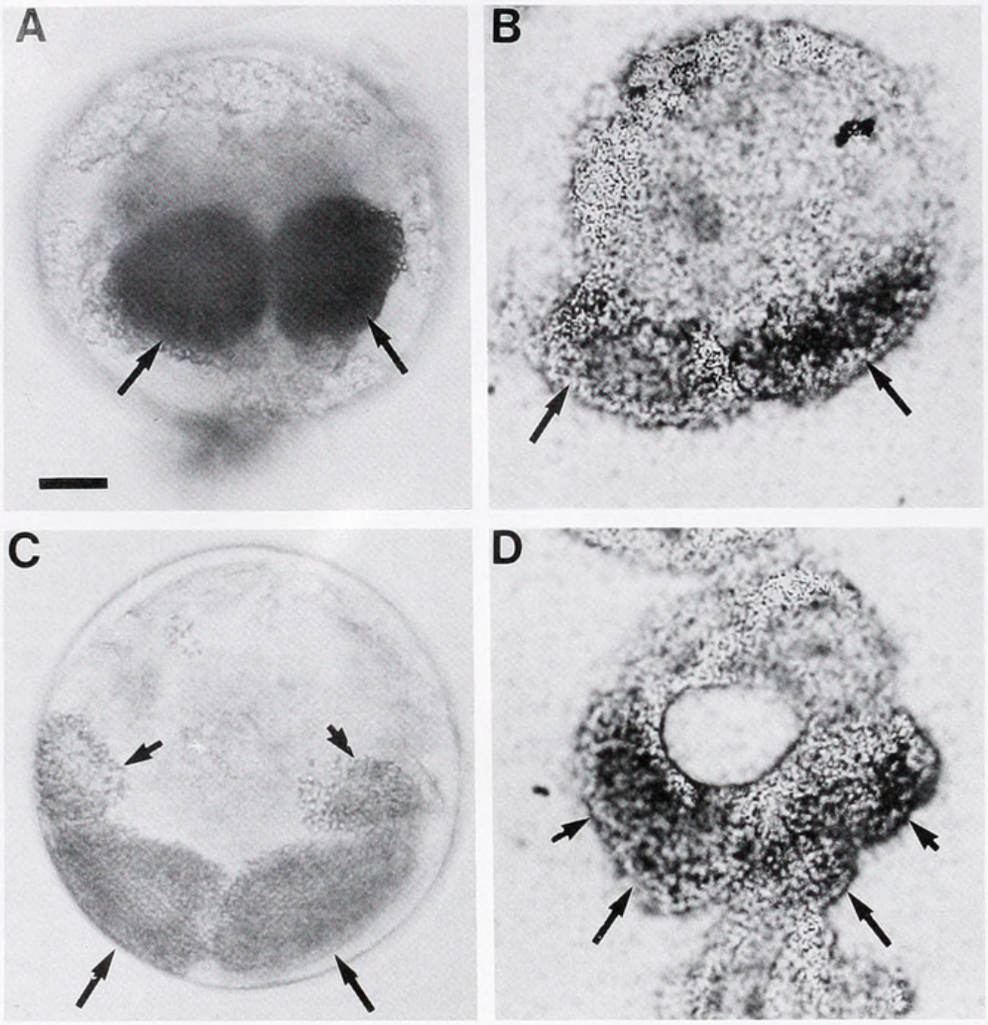


**Figure 7.** Light photomicrographs showing the spatial distribution of myoplasm in control and compressed eight-celled embryos and *in situ* hybridizations of sectioned control and compressed cleavage-arrested eight-celled embryos using SpMA3C anti-sense RNA. (A) A detergent-extracted control embryo showing myoplasm contained in the B4.1 blastomeres (long arrows). (B) Bright-field image of a cleavage-arrested embryo showing the hybridization of SpMA3C probe to the cytoplasm of B4.1 blastomeres (long arrows). (C) A detergent-extracted, compressed embryo showing myoplasm in the B4.1 blastomeres (long arrows) and in two additional blastomeres (short arrows). (D) Bright-field image of a compressed and cleavage-arrested eight-celled embryo showing the hybridization of SpMA3C probe to the cytoplasm in the B4.1 blastomeres (long arrows) and in two additional blastomeres (short arrows). Scale bar in (A) equals 50 $\mu$m. Same magnification for (B–D) as (A).

were rinsed with PBS and then fixed in 2% glutaraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.4, for 30 min at room temperature. Samples were washed three times in 0.1 *M* sodium phosphate buffer. Fixed specimens were immersed in 1% osmium in the same buffer for 1 h at room temperature, followed by dehydration through a graded series of ethanol (10%, 30%, 50%, 70%, 95%, 100%) for 10 min at each step.

Specimens were transferred into a specimen holder that was inserted inside a Tousimis Autosamdri-814 critical point drying chamber. Dried specimens were adhered to double-sided tape on an aluminum stub and placed inside a Sputtering System, Hummer VII coating machine. A 20-nm gold/palladium metal alloy coating was applied to the surface of each specimen, and the specimens were viewed using a JSM-6400 scanning electron microscope.

### In situ *hybridization*

The *in situ* hybridization method previously described by Tomlinson *et al.* (1987) was used in the present study. Normal and compressed eight-celled embryos, cultured in seawater containing cytochalasin B until the controls developed into larvae, were fixed for 20 min in 3:1 ethanol-glacial acetic acid at −20°C. After the fixed embryos were dehydrated in a graded series of ethanol, they were gradually infiltrated with Paraplast and embedded in BEEM capsules. Specimens were sectioned at 7 $\mu$m and dried on gelatin-coated slides. SpMA3C DNA cloned

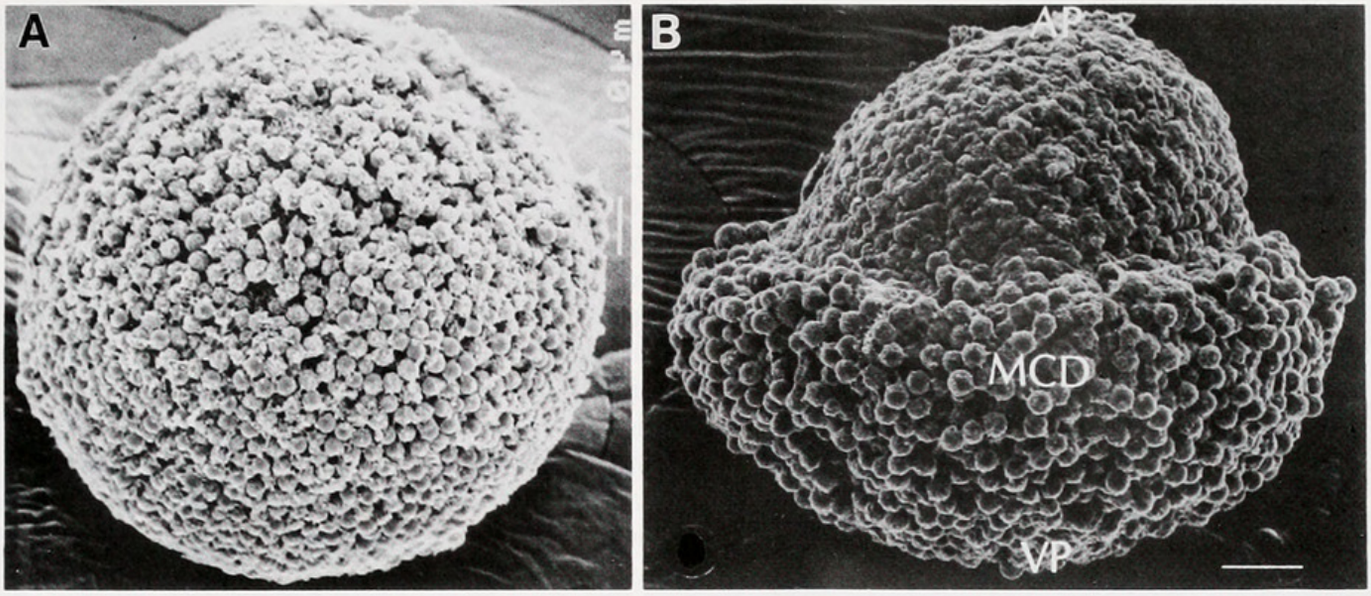


**Figure 8.** Scanning electron micrographs of detergent-extracted unfertilized and fertilized eggs. (A) An unfertilized egg showing the myoplasmic cytoskeletal domain (MCD) in the peripheral egg cytoplasm. (B) A fertilized egg at the first stage of ooplasmic segregation showing the segregation of the MCD in the cytoplasm of the vegetal hemisphere. AP—animal pole; VP—vegetal pole. Same magnification for (A) and (B); Scale bar in (A) equals 10 μm.

into Bluescribe vector was linearized using EcoR1 or Hind III to serve as templates for anti-sense and sense RNA probes respectively. Linearization was checked by running some of the cut and uncut DNA on 1% agarose gels followed by ethidium bromide staining. SpMA3C DNA cut with EcoRI was transcribed in the presence of 50 μCi of $^{3}$H-UTP (Amersham) or $^{35}$S-UTP (Amersham) and cold ATP, CTP, and GTP (400 μM of each nucleotide) using T3 polymerase. SpMA3C DNA was cut with Hind III and transcribed with T7 polymerase to produce sense probes.

The hybridization buffer contained 50% formamide, 10% w/v dextran, 0.01 *M* Tris, 0.3 *M* NaCl, 0.001 *M* EDTA, 500 μg/ml tRNA, Denhardt's solution (1:10 dilution of 50× stock), and 500 μg/ml polyadenylic acid. Slides were probed at low stringency (washed in 1× SSC for 30 min at room temperature) or at high stringency (washed in 1× SSC for 30 min at 45°C), air dried, dipped in Kodak NTB-2 nuclear track emulsion, and exposed for up to 6 weeks.

*Immunocytochemistry*

Specimens were fixed and embedded in polyester (Steedman's) wax, as previously described by Mita-Miyazawa *et al.* (1987). Specimens were fixed for 20 min in absolute methanol and then immersed in cold absolute ethanol for 20 min. Specimens were infiltrated in 50% polyester wax in absolute ethanol for 1 h at 42°C, then infiltrated in 100% polyester wax for 1 h at 42°C. Specimens were embedded in BEEM capsules, sectioned at 7 μm, and mounted on gelatin-coated stripes of coverslips. Sections were de-waxed in 100% ethanol, rehydrated, and washed in PBS prior to treatment with the primary antibody.

Myosin was detected using Mu-2 antibody (Mita-Miyazawa *et al.*, 1987) diluted 1:300 with PBS. Sections were immersed in the primary antibody for 1 h at room temperature, washed in PBS at room temperature, and immersed in FITC-conjugated anti-mouse IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:60 with PBS. After a 30-min incubation at room temperature, sections were washed in PBS for 30 min, mounted in 80% glycerol in PBS, and viewed with an Olympus fluorescence microscope. Stained sections were photographed using Tri X film, ASA 400.

Monoclonal anti-neurofilament 160 antibody (clone NN18; Sigma Chemical Company, St. Louis, MO) was the primary antibody used to detect p58 in embryos (Swalla *et al.*, 1991). Sections were immersed in NN18 diluted 1:25 with PBS for 1 h at room temperature. Sections were washed with PBS and treated for 1 h at room temperature with anti-mouse IgG-POD (Sigma Chemical Company, St. Louis, MO) diluted 1:60 with PBS. Peroxidase activity was detected using Sigma FAST DAB peroxidase substrate tablets. Sections were incubated in the substrate solution for 10 min, washed in PBS, and viewed with a Zeiss Axioplan microscope.

## Results

The myoplasm of an ascidian egg is normally partitioned into the embryonic progenitor cells that produce

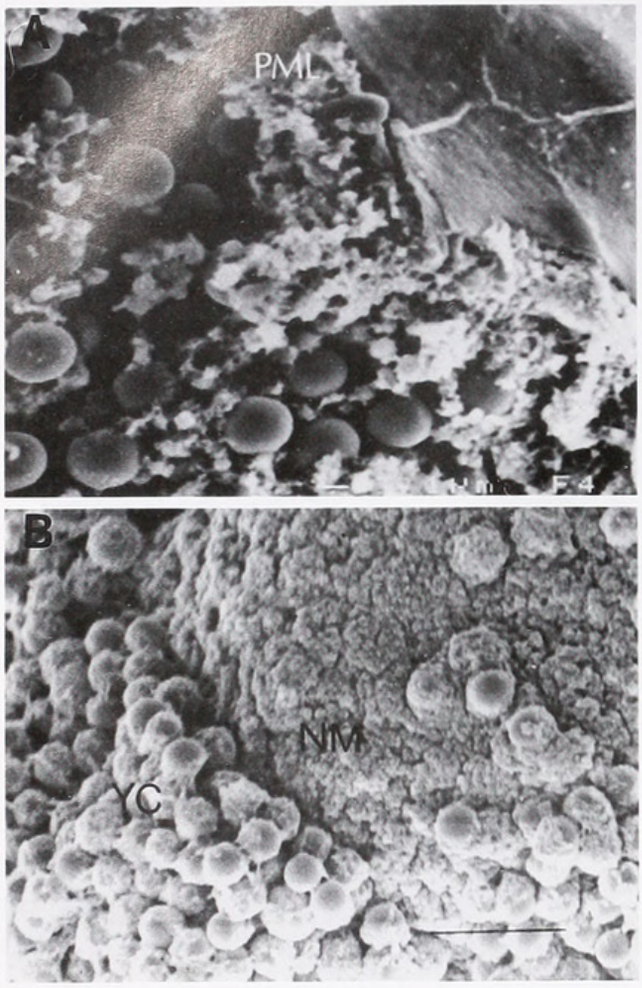


**Figure 9.** Higher magnification scanning electron micrographs of myoplasmic and non-myoplasmic egg regions. (A) The plasma membrane lamina (PML) of the myoplasmic cytoskeletal domain (MCD) is evident beneath a small patch of plasma membrane that was not dissolved by Triton X-100 treatment. Numerous pigment granules are evident. Scale bar equals 1 $\mu$m. (B) A distinct boundary is evident between the MCD and non-MCD regions of a fertilized egg. Scale bar equals 10 $\mu$m.

muscle cells situated in the larval tail region (Fig. 3A). When the animal hemisphere region was surgically deleted from fertilized eggs at the first stage of ooplasmic segregation, the nucleated vegetal merogons that contained segregated myoplasm developed into myoplasm-enriched (ME) larvae (Fig. 3B). In striking contrast to normal larvae, in which the pigmented myoplasm was present only in the tail muscle cells, in ME larvae myoplasmic pigment granules were present in tail muscle cells and many of the endoderm cells situated in the larval head region (Fig. 3B). To determine more precisely which cell types of ME larvae contained pigmented myoplasm, thick sections of ME larvae were examined, as shown in Fig. 4. Pigmented myoplasm was evident in the cytoplasm of many endoderm cells situated in the head region of sectioned ME larvae, whereas myoplasmic pigment granules were not evident in the cytoplasm of epidermal and notochord cells. Thick sections of ME larvae also revealed that their notochords were displaced into more anterior positions, as compared to normal larvae.

Can endoderm cells of ME embryos that contain pigmented myoplasm develop muscle cell features? This important question was addressed by using transmission electron microscopy to examine the cytoplasm of head-region endoderm cells of ME larvae for myofilaments. Twelve ME larvae were sectioned and thick sections were produced to identify head and tail regions prior to cutting thin sections. The ultrastructural features of ME tail muscle cells and head endoderm cells are shown in Figure 5. Sacromeres were evident in the peripheral cytoplasm of tail muscle cells (Fig. 5A, B); however, there was no evidence for the development of myofilaments in the head region of ME larvae in the more than 300 sections that were examined (Fig. 5C, D).

Next, the expressions of two muscle-specific markers, myosin and muscle actin RNA, were examined in cleavage-arrested control (non-compressed) and compressed eight-celled embryos. Figure 6 shows the expression patterns of myosin heavy chain protein in a control larva (Fig. 6A), in a cleavage-arrested eight-celled embryo (Fig. 6B), and in a compressed and cleavage-arrested eight-celled embryo (Fig. 6C). Myosin development was restricted to the tail muscle cytoplasm in normal larvae (in all 40 larvae tested). In cleavage-arrested eight-celled embryos, a maximum of two blastomeres expressed myosin (40 larvae tested). In contrast, compressed and cleavage-arrested eight-celled embryos developed myosin in a maximum of four blastomeres (40 embryos tested).

In another set of experiments, the development of muscle-specific actin RNA was studied in control and compressed cleavage-arrested eight-celled embryos (Fig. 7). A maximum of two blastomeres in control embryos showed hybridization signals (50 embryos examined; Fig. 7B), whereas a maximum of four blastomeres exhibited hybridization signals in the compressed embryos (40 embryos examined; Fig. 7D). The redistribution of pigmented myoplasm into more than the normal number of blastomeres by compression (compare A and C in Fig. 7) promoted the ectopic development of these muscle cell markers.

Why were muscle cell fates altered in compressed *Boltenia* embryos, whereas cell fates were normal in ME *Boltenia* embryos? This question was examined by studying the distribution of MCD components in normal embryos compared to ME embryos and compressed embryos.

### *Spatial distributions of the myoplasmic cytoskeletal domain and p58*

The structure of the pigmented MCD of an unfertilized *Boltenia* egg treated with Triton X-100, as revealed

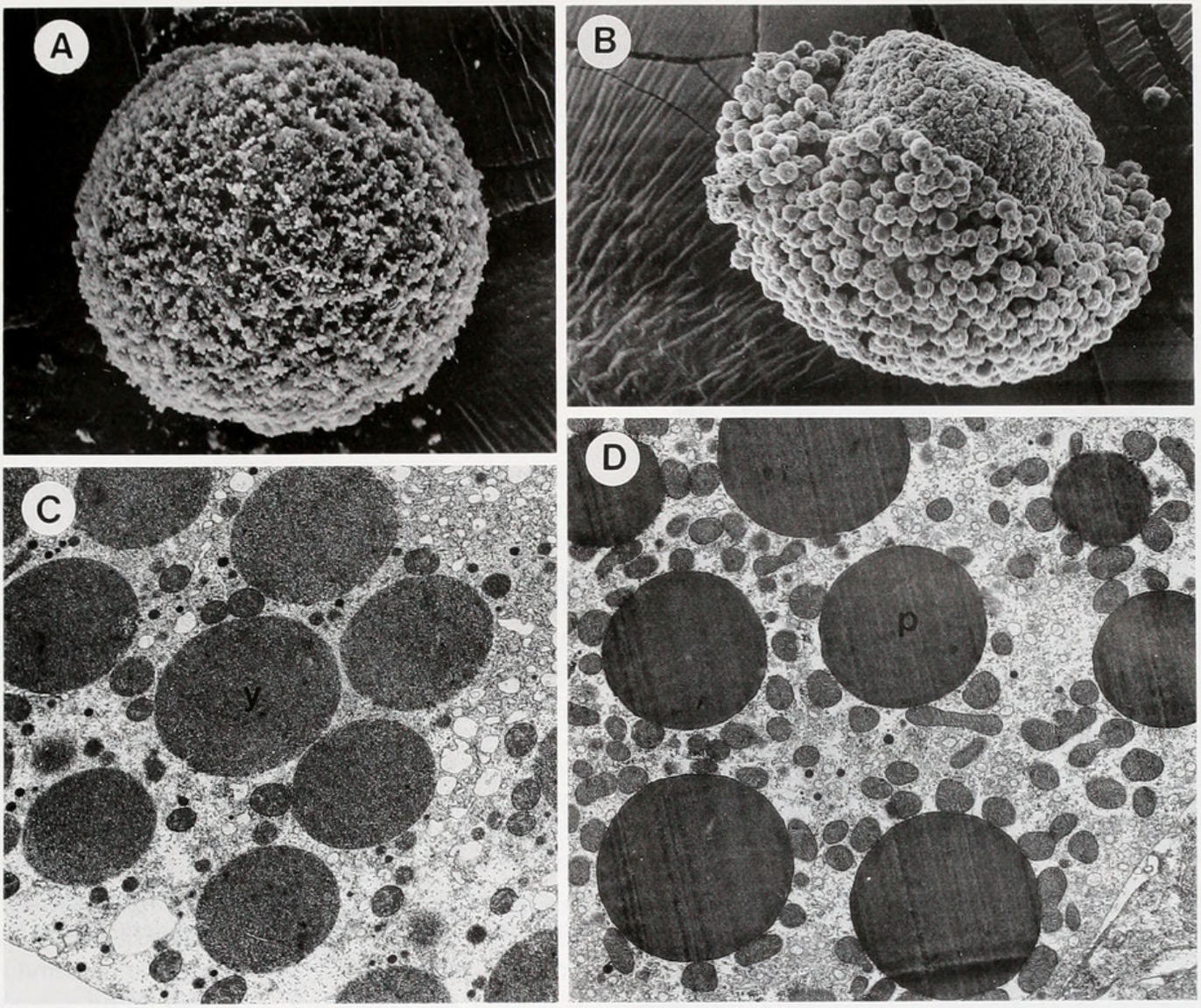


**Figure 10.** Scanning electron micrographs (SEM) and transmission electron micrographs (TEM) of myoplasm-deficient and myoplasm-enriched egg fragments. (A) SEM of a detergent-extracted myoplasm-deficient egg fragment. The pigmented myoplasmic cytoskeletal domain (MCD) is absent. Scale bar equals 10 $\mu$m. (B) SEM of a detergent-extracted myoplasm-enriched egg fragment. The pigmented MCD covers about 60%–70% of the surface. Scale bar equals 10 $\mu$m. (C) TEM of cytoplasm of a myoplasm-deficient egg fragment. Yolk granules and a few scattered mitochondria are evident. Magnification equals 6,300×. (D) TEM of cytoplasm of a myoplasm-enriched egg fragment. Pigment granules surrounded by many mitochondria. Magnification equals 6,300×.

by scanning electron microscopy, is shown in Figure 8A. After fertilization, the MCD segregated into the vegetal hemisphere and covered about 40%–50% of the total egg perimeter (Fig. 8B). The animal hemisphere region lacked the MCD and contained only a few scattered pigment granules. Higher magnification SEM images of the animal and vegetal hemisphere regions of a fertilized egg are shown in Figure 9. A small patch of plasma membrane that was not dissolved by Triton X-100 is evident in Fig. 9A. The distinct edge of the MCD in a fertilized egg is shown in Fig. 9B.

Animal fragments produced from fertilized eggs during the first phase of ooplasmic segregation lacked the MCD (Fig. 10A) and contained fewer mitochondria than found in the ME fragments (compare C and D in Fig. 10). The MCD surrounded about 60%–70% of the perimeter of ME fragments (Fig. 10B), and the MCD contained more mitochondria than did the egg fragments produced from the animal hemisphere (Fig. 10D). Removal of the animal hemisphere resulted in a 10%–20% increase in the surface area of an ME fragment composed of MCD (compare Figs. 8B and 10B). This increase in the surface area that contained the pigmented MCD resulted in the development of orange-headed larvae (Fig. 3B).

Seasonal variations in the distribution of myoplasmic

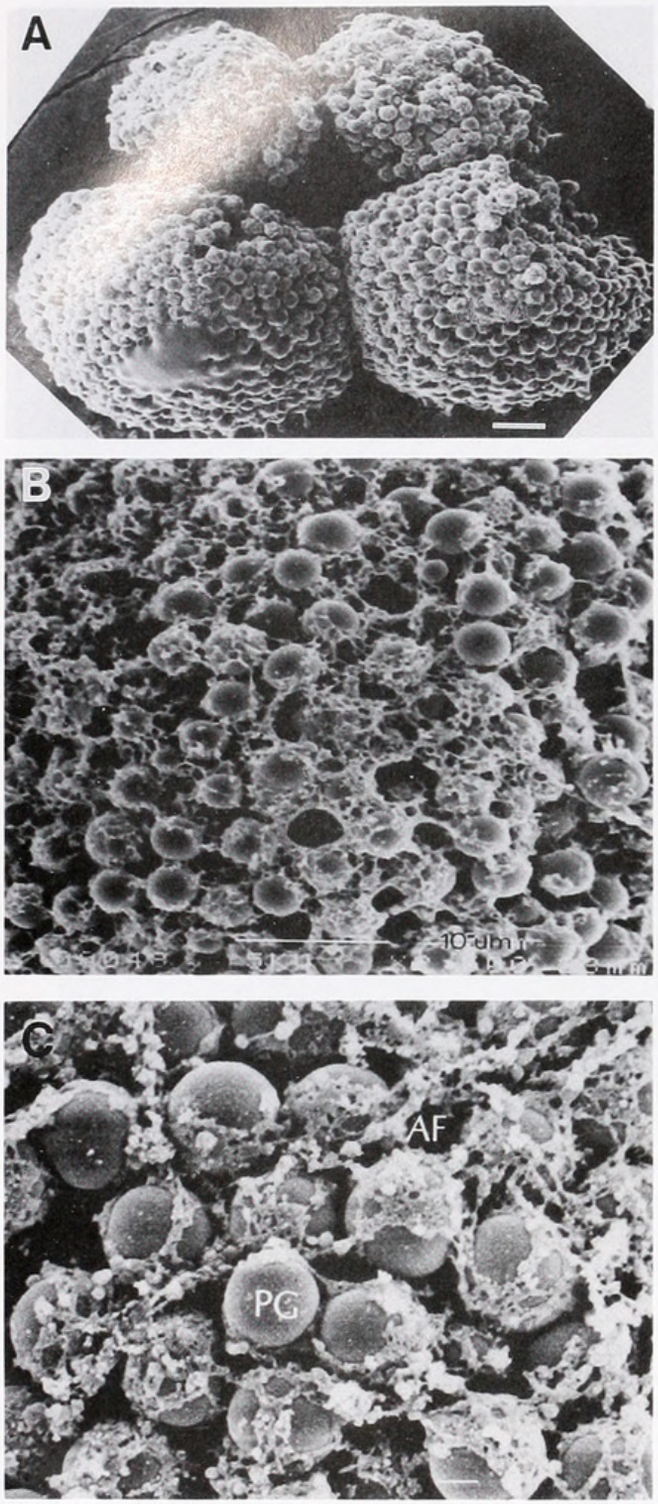


**Figure 11.** Scanning electron micrographs of a four-celled embryo showing the pigmented myoplasmic cytoskeletal domain (MCD) present in all four blastomeres. (A) The MCD is present in the cytoplasm of posterior B3 blastomeres and in the cytoplasm of anterior A3 blastomeres. Scale bar equals 10 μm. (B) Higher magnification showing the structure of the MCD in B3 cytoplasm. The MCD consists of a network of filaments with underlying pigment granules. Scale bar equals 10 μm. (C) Higher magnification of the MCD in the posterior region of an A3 blastomere. Actin filaments (AF) composing the plasma membrane component of the MCD cover the underlying pigment granules (PG) that are embedded in the deep filamentous lattice (PG). Scale bar equals 1 μm.

**Table I**

*Distribution of the myoplasmic cytoskeletal domain (MCD) in the blastomeres of four-celled embryos produced in the autumn that showed pigment granules in all blastomeres*

| Number of blastomeres containing MCD | Number of embryos |
|---|---|
| 0 | 0 |
| 1 | 0 |
| 2 | 0 |
| 3 | 1 |
| 4 | 29 |

pigment granules in *Boltenia villosa* embryos were observed. In late autumn, about one-quarter of the *B. villosa* adults produced clutches of eggs in which orange pigment granules were present in the cortical cytoplasm of all four blastomeres in four-celled embryos, as compared to clutches of eggs produced at other times of the year in which pigment granules were restricted to the cortical cytoplasm of B3 blastomeres of four-celled embryos. Most of the embryos that exhibited altered pigmentation patterns developed into normal larvae. The spatial distributions of the MCD were mapped in four-celled embryos; SEM was used to determine if the MCD was restricted to the B3 blastomeres or was also found in A3 cytoplasm. These results are shown in Figure 11 and Table I. Whereas most of the pigmented MCD was con-

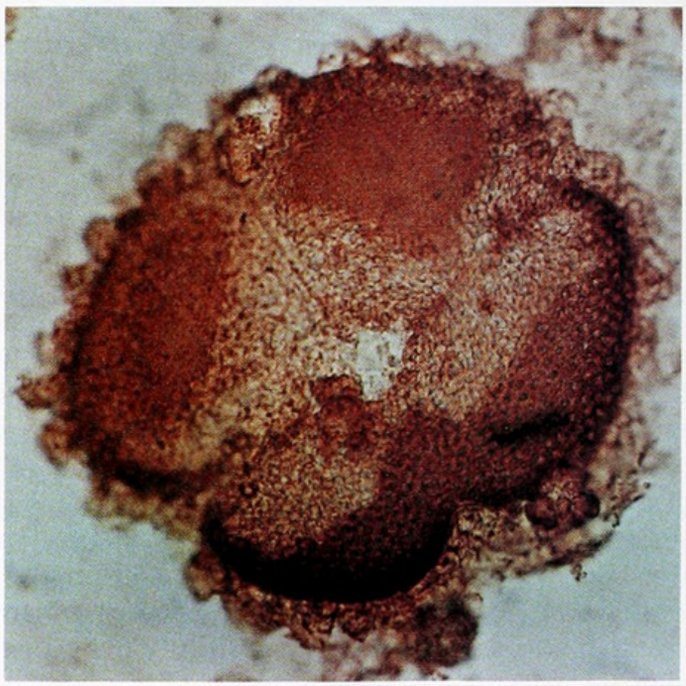

**Figure 12.** Light photomicrograph of a four-celled embryo stained with NN18 antibody in which the myoplasmic cytoskeletal domain is present in all four blastomeres. Two blastomeres are stained with NN18 antibody. Scale bar equals 100 μm.

**Table II**

*Distribution of p58 in unoperated and myoplasm-enriched (ME) four-celled embryos*

| Specimens | Maximum number of blastomeres stained with NN18 antibody | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| 'Autumn' embryos | 8 | 7 | 25 | 0 | 0 |
| ME embryos composed of 40%–50% of total egg volume | 2 | 4 | 15 | 0 | 0 |
| ME embryos composed of 20%–30% of total egg volume | 0 | 0 | 1 | 8* | 10 |

* Three blastomere per embryo were in the plane of section.

tained in the B3 blastomeres, A3 blastomeres contained significant quantities of the pigmented MCD.

The spatial distributions of p58, a protein associated with the DFL component of the MCD, were examined using NN18 antibody in four-celled embryos that contained pigmented MCD in all blastomeres. A maximum of two blastomeres per embryo were stained with NN18 antibody (Fig. 12 and Table II). Therefore, although A3 blastomeres contained the anteriormost region of the MCD, this region of the MCD lacked p58.

In another set of experiments, four-celled ME embryos composed of 40%–50% and 20%–30% of the total egg volume were stained with NN18 antibody. In embryos made up of 40%–50% of the total egg volume, a maximum of two blastomeres were stained with NN18 as in control four-celled embryos (Fig. 13A and Table II). In contrast, when four-celled embryos derived from smaller ME fragments produced from the vegetal pole region were stained with NN18, in most cases (95%; $n$ = 19) more than two blastomeres reacted with the antibody (Fig. 13B and Table II). When compressed eight-celled embryos were stained with NN18, a maximum of four blastomeres reacted with this antibody (Fig. 13C and Table III). In control eight-celled embryos, a maximum of two blastomeres were stained with NN18.

## Discussion

The results of the present study demonstrate that (1) compression of four-celled *Boltenia villosa* embryos increased the number of blastomeres of eight-celled embryos that could develop muscle cell features; (2) the development of muscle cell features in compressed embryos was correlated with the presence of the cytoskeletal protein p58; (3) p58 was restricted to the cytoplasm of posterior B3 blastomeres of four-celled ME embryos composed of 40%–50% of the total egg volume; (4) p58 was present in the cytoplasm of anterior A3 and posterior B3 blastomeres of four-celled embryos that were derived from small ME fragments produced from the vegetal pole region of fertilized eggs at the first stage of ooplasmic segregation; and (5) p58 was concentrated in the vegetal pole region of the MCD at the first stage of ooplasmic segregation.

The present results provide rigorous experimental support for the idea, first suggested by Swalla *et al.* (1991), that the cytoskeletal protein p58 is associated with muscle cell determinants in ascidian eggs. Another cytoskeletal protein, myoplasmin-C1, is localized in ascidian myoplasm (Nishikata *et al.*, 1987), and it has been suggested that myoplasmin-C1 may interact with p58 through $\alpha$-helical rod regions formed by hydrophobic heptad repeats present in both of these proteins (Nishikata and Wada, 1996; B. J. Swalla, pers. comm.). Therefore, $\alpha$-helix coiled-coil complexes composed of p58 and my-

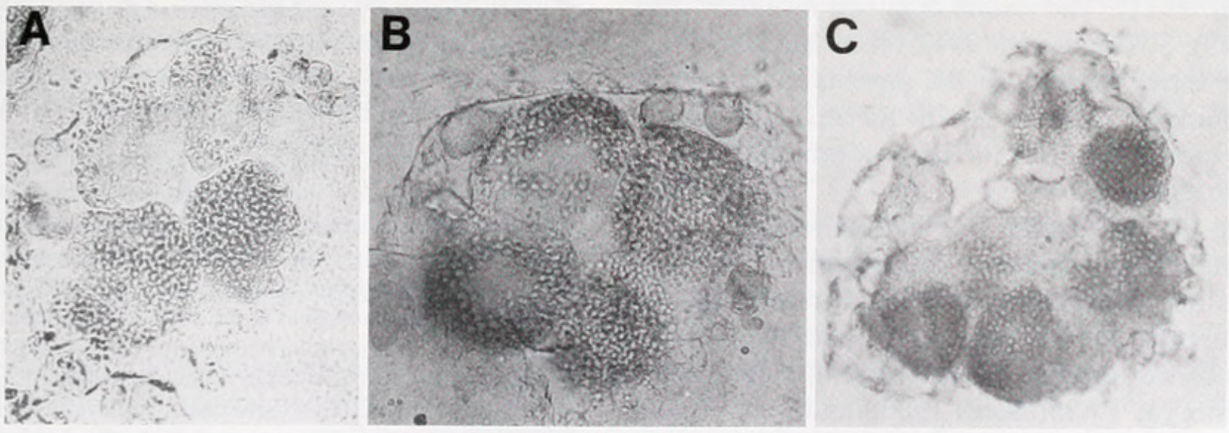


**Figure 13.** Light photomicrographs of NN18 staining patterns of myoplasm-enriched (ME) and compressed embryos. (A) A section of a four-celled ME embryo composed of 40% of the total egg volume that shows two blastomeres stained with NN18. (B) A section of a four-celled ME embryo composed of 30% of the total egg volume in which four blastomeres reacted with NN18. (C) A section of a compressed embryo showing four blastomeres stained with NN18. Scale bars equal 50 $\mu$m.

Table III

*Distribution of p58 in normal and compressed eight-celled embryos*

| Specimens | Maximum number of blastomeres per embryo stained with NN18 antibody | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Normal embryos | 2 | 2 | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| Compressed embryos | 3 | 1 | 1 | 0 | 22* | 0 | 0 | 0 | 0 |

* In five embryos, five or six blastomeres were in the plane of section.

oplasmin-C1 may be the cytoskeletal scaffold that anchors muscle cell determinants.

The expression of a muscle actin promoter-*lacZ* reporter gene construct, MocuMA1/*lacZ*, was recently examined in the tailless larvae of *Molgula occulta*. In these larvae, which lack p58 and do not develop functional muscle cells, low levels of $\beta$-galactosidase activity were detected in a few posterior cells (Kusakabe *et al.*, 1996). This result and the presence of insertions, deletions, and codon substitutions in the coding regions of orthologous larval muscle actin genes isolated from the urodele species *Molgula oculata* suggest that mutations in muscle genes rather than changes in trans-acting regulatory factors are responsible for the regression of muscle cells. These investigators also reported that MocuMA1/*lacZ* constructs are expressed in mesenchyme and other non-muscle cell types in urodele larvae and that the *M. occulta* cells expressing $\beta$-galactosidase activity did not correspond to the cells expressing low levels of vestigial AchE activity. Therefore, these results suggest that the activity of the MocuMA1 promoter is somewhat "leaky" in some cell types, as is the transcription of AchE genes in vestigial muscle cells of several anural ascidian species (Whittaker, 1979; Jeffery and Swalla, 1990b; and Bates and Mallett, 1991). The results obtained by Jeffery and Meier (1983), Swalla *et al.* (1991), Nishikata and Wada (1996) and those reported in the present study suggest that determinants associated with the myoplasmic cytoskeleton are required for the normal promoter activities of muscle genes.

Mapping the distributions of p58 in ME and compressed embryos has resolved the paradox of why cell fates were normal in embryos derived from ME fragments composed of 40%–50% of the total egg volume (Bates, 1988), but muscle cell fates were altered in compressed embryos (Whittaker, 1980; and the present study) and small ME embryos produced from the vegetal pole region of fertilized eggs (Bates, 1988). Removal of the animal hemisphere of a fertilized egg during the first stage of ooplasmic segregation resulted in a 10%–20% increase in the cortical cytoplasm of an ME fragment that contained the MCD. Increasing the surface area of MCD would produce larger than normal myoplasmic crescents after the second phase of ooplasmic segregation. These enlarged crescents would position the anteriormost MCD into the endodermal determinant domain of the egg (Conklin, 1905; Nishida, 1994). The normal cleavage pattern of an ME embryo (Bates, 1988) would then partition the anterior MCD into endoderm cells, resulting in the development of larvae in which some of the head endoderm cells contain pigmented cytoplasm.

When the regional expressions of three muscle-specific markers, myosin heavy chain (Bates, 1988), AchE activity (Bates, 1988), and myofilaments (present study) were examined in ME larvae, only tail muscle cells were able to develop these markers. The endoderm cells present in

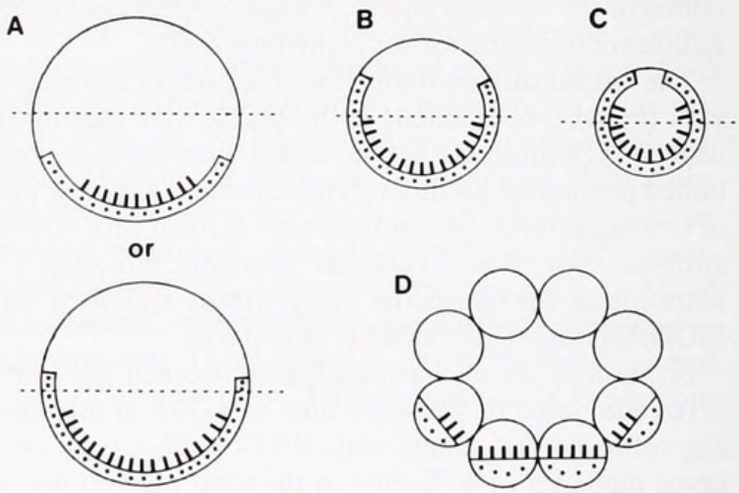


**Figure 14.** A summary of the present results. (A; top) The myoplasmic cytoskeletal domain (MCD) resides in the more posterior cytoplasm of a fertilized egg at the second stage of ooplasmic segregation (anterior—top; MCD is outlined). The MCD contains pigment granules (dots), proteins, RNA, and mitochondria. The posterior region of the MCD contains p58 (short lines). (A; bottom) In late autumn, some adults produce eggs in which some of the MCD was extended into the anterior cytoplasm, yet p58 was restricted to the more posterior MCD. (B) In myoplasm-enriched (ME) fragments composed of 40%–50% of the total egg volume, there was an increase in cortical cytoplasm that contained the MCD. As in autumn eggs that exhibited altered pigmentation patterns (Fig. 14A; bottom), some of the pigmented MCD was extended into the more anterior cytoplasm. This pigmented MCD was partitioned into some of the head endoderm cells of the larva; however, endoderm cell fates remained normal because p58 was localized in the more posterior region of the MCD. (C) In smaller ME fragments produced from fertilized eggs, MCD that contained p58 was present in the anterior cytoplasm as well as in the posterior cytoplasm. After two cell divisions, the resulting four blastomeres contained p58, and all four blastomeres of cleavage-arrested smaller ME embryos expressed AchE activity (Bates, 1988). (D) Compression of four-celled embryos resulted in the partitioning of MCD that contained p58 into four blastomeres of eight-celled embryos, in contrast to normal eight-celled embryos that have p58 in the two B4.1 blastomeres. The presence of p58 in four blastomeres of a compressed and cleavage-arrested eight-celled embryo promoted the development of myosin and muscle actin RNA in four blastomeres.

**Table IV**

*Summary of results*

| Type of embryo[1] | Maximum number of cells/embryo expressing a muscle cell marker | Distribution of p58 |
|---|---|---|
| Cleavage-arrested four-celled control embryos | two cells expressed AchE activity [2] | two cells |
| Cleavage-arrested four-celled ME embryos 40%–50% TEV | two cells expressed AchE activity[2] | two cells |
| Cleavage-arrested four-celled ME embryos 10%–30% TEV | four cells expressed AchE activity[2] | four cells |
| Cleavage-arrested eight-celled embryos | two cells expressed myosin and muscle actin RNA | two cells |
| Cleavage-arrested and compressed eight-celled embryos | four cells expressed myosin and muscle actin RNA | four cells |

[1] ME = myoplasm-enriched; TEV = total embryo volume.
[2] From Bates (1988).

the head region of ME larvae developed an endoderm-specific marker, alkaline phosphatase activity, as did the control larvae (Bates, 1988). The detection of p58 in the cytoplasm of B3 blastomeres of four-celled ME embryos but not in A3 blastomeres explains why muscle cell fates in these embryos were normal.

Two observations described in the present study indicate the probable distribution of p58 in the MCD in fertilized eggs just prior to first cleavage. During most of the year, *Boltenia* adults produce clutches of eggs that develop into four-celled embryos in which the MCD resides exclusively in the cytoplasm of the B3 blastomeres (Jeffery and Meier, 1983). However, in late autumn some animals produce four-celled embryos in which the MCD is present in A3 blastomeres and in B3 blastomeres. When these embryos were stained with NN18, only two blastomeres contained p58. These results suggest that p58 may be more concentrated in the posterior region of the MCD prior to first cleavage than in the more anterior region of the MCD. This idea is further supported by the detection of p58 in the cytoplasm of all four blastomeres of four-celled ME embryos produced from the vegetal pole region of fertilized eggs (see Fig. 14).

The present results indicate that the distribution of the cytoskeletal protein p58 is correlated with the ectopic development of muscle cell features in compressed ascidian embryos. Whittaker (1980) observed that the maximum number of blastomeres that can develop AchE activity is four in compressed and cleavage-arrested eight-celled *Styela* embryos, but only two in non-compressed and cleavage-arrested embryos. The present results have confirmed and extended Whittaker's findings by showing that compression can alter muscle cell fates in *Boltenia villosa* embryos and that p58 is present in four, instead of two, blastomeres of compressed eight-celled embryos.

The results of the present study are summarized in Figure 14 and in Table IV. During the first phase of ooplasmic segregation following fertilization, the MCD is moved towards the vegetal pole. The MCD is then secondarily shifted into the equatorial-vegetal region of the egg prior to first cleavage (Fig. 14A, top), and this region designates the future posterior region of the larva (Conklin, 1905). Embedded within the MCD are pigment granules, mitochondria, RNA, and proteins. The cytoskeletal protein p58 is concentrated in the deep filamentous lattice of the MCD (Swalla *et al.*, 1991). In some clutches of eggs produced in late autumn, the surface area of an egg containing pigmented MCD was greater than in eggs produced at other times of the year (Fig. 14A, bottom). This increase resulted in the development of four-celled embryos in which the MCD was present in the cytoplasm of all four blastomeres. However, staining with NN18 showed that only the primary muscle progenitor B3 cells contained p58. These observations suggest that p58 is associated with the posterior MCD contained within the cytoplasm of B3 blastomeres, but absent from the anterior region of the MCD contained in A3 blastomeres.

In ME fragments composed of 40%–50% of the total egg volume, there was an increase in the surface area that contained the pigmented MCD (Fig. 14B). This increase resulted in the redistribution of some of the pigmented MCD into the anterior blastomeres. When four-celled ME embryos derived from this size class of egg fragments were immersed in NN18, a maximum of two blastomeres were stained. These results demonstrate that p58 distributions were not altered in the ME embryos and explains why the fates of embryonic ME cells were normal. In contrast, nearly the entire cortical cytoplasm of smaller ME fragments produced from the vegetal pole region of fertilized eggs contained the MCD (Fig. 14C). When four-celled ME embryos derived from these fragments were stained with NN18, p58 was detected in all four blastomeres. This observation explains why AchE activity was sometimes detected in all four blastomeres of small cleavage-arrested ME embryos, as compared to a maximum of two blastomeres in the control cleavage-arrested four-celled embryos (Bates, 1988). Compression of four-celled embryos caused p58 to be partitioned into four, rather than two, blastomeres of an eight-celled embryo (compare Figs. 2B and 14D). The presence of p58 in four blastomeres was correlated with the development of myosin and muscle actin RNA in four blastomeres of compressed and cleavage-arrested eight-celled embryos.

In conclusion, mapping the distribution of p58 has resolved the paradox of why cell fates were normal in ME embryos composed of 40%–50% of the total egg volume, but muscle cell fates were altered in small ME embryos and in compressed embryos. Immunoprecipitation experiments, now in progress, will coprecipitate muscle cell determinants with p58 for microinjection into non-muscle lineage blastomeres of urodele ascidian embryos and injection into anural ascidian eggs.

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