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**DETERMINATION AND DIFFERENTIATION OF MUSCLE
CELLS IN THE TADPOLE LARVA OF THE ASCIDIAN
*BOLTENIA VILLOSA***

by

Michael Andrew Swallow

**A thesis submitted to the faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Master of Science**

Department of Biology

**Joint Institute
Carleton University
University of Ottawa
Ottawa, Canada
September 9, 1992**

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**Determination and Differentiation of Muscle
Cells in the Tadpole Larva of the Ascidian
*Boltenia villosa***

**submitted by Michael Andrew Swallow, B.Sc. (Hons.)
in partial fulfilment of the requirements for the
degree of Master of Science**

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ABSTRACT

The establishment of cell fates in ascidian embryos are determined primarily by factors localized in the cytoplasm. The role of localized myoplasmic factors in determining the muscle lineage of *Boltenia villosa* was investigated by redistributing this cytoplasmic domain into a non-muscle lineage. Compressed 8-cell embryos expressed muscle actin mRNA, myosin, actin and myofibrils in four blastomeres due to the presence of myoplasm in two additional cells. These results suggest that factor(s) localized in the myoplasm of ascidian eggs play a role in the establishment of the muscle lineage.

The temporal aspects of muscle differentiation in ascidian larvae were investigated by determining the time of appearance of actin and myosin and their assembly into a contractile apparatus. Actin is expressed prior to myosin and is first to be assembled into filaments. Myosin filaments are simultaneously assembled and incorporated into myofibrils. The rapid development of the ascidian larva reflects the rapid differentiation of the muscle lineage.

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List of Abbreviations

Abbreviation	Description
A	A Band of Sarcomere
Af	Actin Filament
Ch	Chorion
h	Head Region of Tadpole
H	H Band of Sarcomere
I	I Band of Sarcomere
m	Muscle Cell of Tadpole
Mf	Myofilament
Mt	Mitochondrion
n	Notochord of Tadpole
P	Pigment Granule of Myoplasm
S	Sarcolemma of Muscle Cell
Sf	Sheath fibers of notochord
t	Tail Region of Tadpole
Z	Z Line of Sarcomere

1. INTRODUCTION

1.1 Ascidians as a Model System for Studies in Development

Ascidians are sessile marine Urochordates which normally develop from an egg to a free swimming tadpole larva prior to settlement and metamorphosis into an adult organism. The general anatomy of the larva is relatively simple in that the tadpole is bilaterally symmetrical and can be divided into a head and tail region. The head region is comprised primarily of large endoderm cells surrounded by ectoderm. The head region also contains a palp, or adhesive organ that temporarily anchors the larva, and usually two sensory organs, the otolith and ocellus, which detect gravity and light respectively (Conklin, 1905a). Ascidian larvae are especially interesting because of the transient expression of chordate features at this stage. The tail region of the tadpole consists of a central notochord which is lost when the tail is resorbed during metamorphosis. The notochord is flanked dorsally by spinal cord cells, ventrally by endodermal strand cells and bilaterally by muscle cells with an additional four to six muscle cells, depending on the species, at the most caudal end of the tail.

Ascidian embryos are ideally suited to the study of cell fate determination because of their well established cell lineage and determinate cleavage pattern as well as an extremely rapid development with a swimming tadpole larva that develops in less than twenty-four hours after insemination. Ascidians are also known to exhibit a highly mosaic form of development suggesting that localized cytoplasmic factors play an important role in the early embryo. The eggs of some species, including

Boltenia villosa , contain cytoplasm with pigmented inclusions that provide a natural marker to follow the segregation of localized cytoplasmic domains into specific cell lineages.

1.2 Cytoplasmic Determinants and the Establishment of Cell Fate

1.2.1 What is a Cytoplasmic Determinant?

The determination of cell fates in ascidian embryos are thought to be regulated primarily by the unequal partitioning of localized cytoplasmic factors into specific cell lineages during early cleavages. Because these factors are thought to play a role in establishing cell fate, they have been termed cytoplasmic determinants. The molecular nature of these determinants has yet to be positively identified, but it has been hypothesized that these factors could play a role in specifying cell fate by regulating the activity of lineage-specific genetic programs (Davidson, 1986). The difficulty in identifying the exact nature of a cytoplasmic determinant arises from a lack of suitable methods for isolating sufficient quantities of localized cytoplasmic domains to perform any molecular analysis. It has been suggested that these determinants may be messenger RNAs which are synthesized during oogenesis and are stored in the egg until fertilization (Jeffery, 1985, Jeffery et al, 1986). This is supported by studies in which treatment of embryos with transcriptional inhibitors has failed to prevent the expression of tissue-specific markers thought to be regulated by cytoplasmic determinants (Whittaker, 1973,1977). However, the expression

of other tissue-specific markers which exhibit developmental autonomy is prevented by treatment with transcriptional inhibitors (Whittaker, 1973, Crowther and Whittaker, 1984). This suggests that some determinants may be regulatory factors, ie. proteins, which promote differential gene expression. Thus the exact nature of localized determinants remains unclear.

The cytoplasmic nature of these determinants has been shown by nuclear transplantation studies in which the nucleus of one species was transferred into an enucleate fertilized egg of another. The resulting embryo exhibited characteristics of the egg and not the nucleus indicating that factors localized in the cytoplasm were directing the expression of nuclear genes (Crowther et al, 1988). This is supported by studies in which the nucleus of one differentiated cell type was redistributed into the cytoplasm of another cell type (Morgan, 1927). The resulting embryo developed normally in every respect indicating that the genetic program of the differentiated nucleus was respecified by factors localized in the cytoplasm of the recipient blastomere.

A well documented example of localized cytoplasmic factors directing cell fate in ascidian embryos is the determination of the muscle cells via the segregation of the myoplasm into this lineage. The myoplasm is a collection of mitochondria, pigment granules and cytoskeletal elements which is distributed throughout the cortex of the unfertilized egg (Jeffery and Meier, 1983). During ooplasmic segregation, a complex series of cytoplasmic movements initiated at insemination first observed by Chabry (1887), the myoplasm is localized to the vegetal pole of the fertilized egg

and is eventually concentrated into a crescent at the posterior vegetal region of the uncleaved zygote. This pigmented crescent is then divided equally between the two blastomeres resulting from first cleavage but during subsequent cleavages it is unequally segregated into only two cells of the 4- and 8-cell embryo, four cells of the 16-cell embryo, six cells of the 32-cell embryo and eight cells of the 64-cell embryo (Conklin, 1905a). These eight cells are the progenitors of most of the muscle cells in the tail of the ascidian larva (Nishida, 1987). Other cytoplasmic domains are localized in a similar manner during ooplasmic segregation including the material released from the ruptured germinal vesicle. This clear cytoplasm is localized immediately anterior to the myoplasmic crescent and is termed the ectoplasm because it is segregated into ectodermal lineages such as the epidermis (Uzman and Jeffery, 1986, Nishida, 1987).

The localization of the determinants found in specific cytoplasmic domains may be mediated by their interaction with cytoskeletal elements consisting of a plasma membrane lamina, integral membrane proteins and cytoskeletal actin filaments (Jeffery and Meier, 1983, Jeffery, 1984). Cytoskeletally bound determinants were hypothesized by Conklin (1931) after he subjected ascidian eggs to centrifugation. Despite the stratification of visible cytoplasmic components, the centrifuged eggs developed normally suggesting that any determinants present were somehow anchored to an unidentified structure of the egg. The exact nature of the cytoskeletal elements may be specific to the cytoplasmic domain with which they are associated.

1.2.2 Role of Cytoplasmic Determinants in Ascidian Development

The establishment of different cell types during embryonic development in most organisms is thought to be mediated by a combination of localized cytoplasmic factors and inductive interactions between various cell and tissue types in the embryo. Although the relative importance of these two mechanisms appears to vary from organism to organism, experimental evidence suggests that cell fate specification for the majority of the larval cell types in ascidians is regulated via specific cytoplasmic determinants.

General evidence suggesting the importance of determinants in ascidian development includes the following. Egg fragments created prior to fertilization will develop into normal larvae if inseminated while removal of certain regions after fertilization of the egg will prevent further development (Ortolani, 1958, Reverberi, 1971, Bates and Jeffery, 1987a, Bates, 1991). This suggests that cytoplasmic determinants required for normal development are localized to specific domains within the egg after fertilization. Prior to insemination, the determinants are distributed uniformly throughout the egg cytoplasm. Blastomere ablation and isolation studies suggest that as cleavage proceeds, cytoplasmic determinants are segregated into specific cell lineages. If a two-cell embryo has one of the blastomeres removed or destroyed, a normal ascidian tadpole will develop. However if the same procedure is carried out on a later stage embryo, the isolated blastomeres only develop tissues specified by that isolated lineage or whole embryos which have a single blastomere destroyed will be deficient in the tissue(s) specified by the ablated blastomere (Chabry, 1887,

Conklin, 1905b, Deno et al, 1985). Furthermore, if an ascidian embryo is cleavage-arrested using cytochalasin, a number of tissue-specific markers will develop in the correct spatial and temporal arrangement (Whittaker, 1973, Crowther and Whittaker, 1983,1984, Deno et al, 1984, Nishikata et al, 1988) suggesting developmental autonomy of a number of tissue-specific characteristics. Redistribution of specific regions of cytoplasm into ectopic locations in the embryo have been shown to alter the fate of the lineage which receives the cytoplasm (Whittaker, 1980,1982).

A number of specific aspects of early ascidian development which appear to require the presence of cytoplasmic determinants for their expression have been identified. These include the endodermal lineage histospecific marker alkaline phosphatase (AP) (Whittaker, 1977, Bates and Jeffery, 1987a, Bates, 1991), and embryonic axis formation, both dorsal/ventral and anterior/posterior (Bates and Jeffery, 1987b). The expression of a variety of muscle-specific markers also appears to involve cytoplasmic determinants including: myosin heavy chain (Nishikata et al, 1987b), myofibrils (Crowther and Whittaker, 1983,1986, Crowther et al 1990), muscle actin mRNA (Jeffery et al, 1983, Jeffery, 1989, Tomlinson et al, 1987) and acetylcholinesterase (AChE) (Whittaker, 1973, Whittaker et al, 1977) which is expressed only in the tail muscle cells of the tadpole and not in neural tissues . It should be noted that a few muscle cells at the most caudal tip of the tail do not arise from the same cell lineage as the majority of the tail muscle cells. Most of the larval muscle cells are derived from the B4.1 cell lineage of the 8-cell embryo (see Fig III), the sole blastomeres to receive the pigmented myoplasm thought to contain muscle-specific

determinants. The most caudal tail muscle cells are derived from the A4.1 and b4.2 lineages (see Fig III) which do not contain myoplasm. It is thought that the fate of these muscle cells is not determined by the presence of localized cytoplasmic factors but rather by induction via interaction with other cell lineages later in embryogenesis (Deno et al, 1984,1985, Tomlinson et al, 1987, Nishida, 1987,1990, Jeffery, 1989).

The molecular nature of the factors responsible for the development of the above features remains a matter of some contention as the above studies have utilized various transcriptional and translational inhibitors which suggest that some of these determinants may be maternally stored mRNA while others may be proteins.

1.2.3 Role of Cytoplasmic Determinants in the Development of Other Organisms

The important role played by cytoplasmic determinants in the determination of cell fate is by no means restricted to ascidians. Although embryonic development in mammals is regulated by the interaction between various cell and tissue types, a number of other organisms which exhibit determinate cleavage patterns also rely on the localization of cytoplasmic factors to establish the fate of specific cell lineages.

Molluscan embryos appear to contain localized cytoplasmic factors exemplified by the snail *Ilyanassa obsoleta* that appears to utilize determinants segregated into the polar lobe, a transient cytoplasmic protrusion observed during early cleavages, and are responsible for the

development of the velum, shell and foot of the veliger larva as well as the establishment of the dorsal/ventral axis (Render, 1989). Similarly, the vegetal pole of uncleaved zygotes of the nemertean worm *Ceratomyxus lacteus* contain localized determinants which appear to specify larval apical tuft structures (Freeman, 1978). Eggs of dipterans such as *Drosophila* contain an ultra violet (UV) sensitive cytoplasmic factor localized in the vegetal region of the embryo which specifies the germ cell lineage (Okada et al, 1974). Even simple protozoans such as *Volvox* contain a UV labile cytoplasmic component which appears to specify the gonidia, or reproductive cells, of this green algae (Kochert and Yates, 1970). Invertebrates are not the only organisms that utilize cytoplasmic determinants to specify cell fate. *Xenopus* embryos appear to contain cytoplasmic information that specifies both the germ cell lineage (Wakahara, 1978) and the dorsal/ventral axis (Moen and Namenworth, 1977). After insemination of eggs of the mexican axolotl *Ambystoma mexicanum*, a specific subset of the total egg protein becomes segregated to specific regions of the uncleaved zygote (Jackle and Eagleson, 1980) suggesting that cytoplasmic information is being localized in the egg such that it will be placed into specific cell lineages during early cleavage. Thus a wide range of phylogenetically diverse organisms appear to utilize localized cytoplasmic determinants in their early development.

1.2.4 Interaction Between Different Cytoplasmic Determinants

Although there is an abundance of experimental evidence that suggests a role for localized cytoplasmic factors in the establishment of cell fate in the embryos of a variety of organisms, little is known about how the various determinants localized within the same zygote interact with one another. There is some evidence to suggest that certain combinations of egg cytoplasm, and so localized determinants, may act in an antagonistic fashion with respect to the establishment of cell fate. Cytoplasmic deletion studies indicate that cytoplasmic material localized in the polar lobe of the polychaete worm *Sabellarium cementarium* may inhibit the formation of apical tuft structures in specific cell lineages in which the ability to form this structure is inherent (Render, 1983). By centrifuging uncleaved zygotes of the ctenophore *Mneimiopsis leidyi*, egg fragments containing specific cytoplasmic domains can be produced (Freeman, 1976). If a specific region of cytoplasm, the endoplasm, was excluded from these merogons, ectopic development of comb plate structures was observed. If the merogons contained even small amounts of endoplasm, no ectopic structures developed. These experiments seem to suggest that the presence of specific regions of egg cytoplasm, which presumably contain localized cytoplasmic factors, may inhibit the activity of other localized determinants responsible for the establishment of a specific cell lineage. These results are interesting in light of recent results obtained by Bates and Swallow (unpublished) in which two experimental methods were utilized to redistribute the myoplasm of ascidian embryos into ectopic cell lineages.

Microsurgically created myoplasm enriched (ME) larvae were observed to contain myoplasm in the head region of the tadpole but muscle-specific features were restricted to the tail region. In contrast, compressed embryos were observed to contain myoplasm in non-muscle lineage cells which exhibited a variety of muscle features. These results will be discussed in the context of a possible antagonistic interaction between the various cytoplasmic domains present as a result of each experimental situation.

1.3 Differentiation of Muscle Cells in the Ascidian Tadpole Larvae

Previous studies on the differentiation of muscle in the tail of the ascidian tadpole larvae have dealt with the ultrastructural characteristics of these cells (Cavey and Cloney, 1972, Terakado, 1972, 1975). The tail musculature of ascidian tadpoles appears morphologically similar to vertebrate cardiac muscle in that ascidian muscle cells remain as uninucleated cells which do not fuse to form syncytia but are functionally similar to vertebrate striated muscle (Cavey and Cloney, 1972). The tail contains two muscle bands consisting of two longitudinal rows of muscle cells in parallel to the long axis of the tail. Swimming movements occur as a result of alternating contraction of these two lateral bands. (Cavey and Cloney, 1972). Because the development of the ascidian larvae is rapid, less than 17.5 hrs from insemination to a hatched tadpole for *Boltenia villosa*, the expression and subsequent assembly of muscle-specific proteins must be tightly regulated. It has been suggested that the thin actin and thick myosin filaments which make up a myofibril, the contractile unit of

muscle, are assembled simultaneously in some ascidian species (Terakado, 1972). Proteins which must be synthesized and assembled into supramolecular structures in a rapid fashion may have to be organized in a tandem manner in the genome such that their expression may be regulated coordinately. This possibility was investigated by studying the temporal pattern of expression of the muscle-specific proteins sarcomeric actin and myosin heavy chain in developing ascidian tadpole larvae and the subsequent assembly of these proteins into myofibrils and intact sarcomeres.

The goal of this study was to determine the role of the myoplasm in the determination of the muscle cell lineage in embryos of the ascidian *Boltenia villosa* by microsurgically redistributing this cytoplasmic domain into a non-muscle lineage. Using the techniques of *in situ* hybridization, immunocytochemistry and transmission electron microscopy, the effects of the presence of ectopic myoplasm were determined by assaying the resulting embryos for expression of muscle actin mRNA, the muscle-specific proteins actin and myosin heavy chain and the presence of muscle filaments in inappropriate cells.

A second aim of this thesis was to investigate the temporal aspects of muscle differentiation in the ascidian larva by studying the timing of expression and assembly of muscle-specific proteins using the techniques of immunocytochemistry and transmission electron microscopy.

2. MATERIALS AND METHODS

2.1 Organisms, Gametes, and Embryos

Gravid *Boltenia villosa* were purchased from Westwind Sea Labs Inc. (Victoria, BC). The animals were maintained at 15 °C under constant light conditions in aquaria containing Instant Ocean artificial seawater. *B. villosa* gametes were obtained by dissection of excised adult gonads (Jeffery and Capco, 1983) *B. villosa* is a self-sterile hermaphrodite; therefore, fertilization was carried out *in vitro* by mixing the sperm and eggs of at least two individuals in Syracuse dishes. Gametes were incubated together for 15 min at room temperature and then washed repeatedly with Millipore-filtered artificial seawater (MFASW). Embryos were subsequently cultured at 15 °C in MFASW.

2.2 Microcompression

Microcompression chambers were prepared using strips of lens paper placed on a microscope slide (modified from Whittaker, 1980). Embryos were compressed by being placed between the paper strips and then covered with a coverslip. MFASW was withdrawn by capillary action until embryos were compressed to the point at which the entire chorion was observed to be filled by the flattened blastomeres. At 15 °C, the time between second and third cleavage was 20 min. Four-cell stage embryos were compressed for the final 10-15 min of this period and for 10 min during the event of cleavage itself to ensure a meridional rather than equatorial cleavage (Whittaker, 1980) This ensured the redistribution of myoplasm into ectopic blastomeres. Compressed embryos

were released by forcing MFASW under the coverslip, the flattened and control 8-cell embryos were cultured separately at 15 °C in 2 µg/ml cytochalasin B (Sigma Chemical Co., St. Louis, MO.) to arrest further cleavage. Experimental and unoperated control embryos were cultured in MFASW at 15 °C for 17.5 hrs at which time the controls had hatched. These embryos were subsequently processed for *in situ* hybridization. Control embryos were also allowed to develop for 12 hrs and were collected at 30 min intervals until development had proceeded for a total of 18 hrs. At each interval, the embryos were fixed and processed for electron microscopy or immunocytochemistry.

2.3 Confirmation of Myoplasmic Redistribution

Three methods were utilized to confirm that myoplasm had been redistributed into ectopic, non B4.1 blastomeres:

A) Milligan Trichrome for Myoplasm

Myoplasmic pigment granules were identified by their staining characteristics in Milligan trichrome stained sections (Milligan, 1946) which stains the myoplasm of primary muscle cells dark red, while other cell types are stained light pink or blue-green (Jeffery, 1989) Milligan trichrome staining (Milligan, 1946, Jeffery, 1989) was carried out on paraplast embedded sections (see below) as follows.

Paraplast was removed from the sections by immersion in 100% toluene followed by successive washes in 100% and 95% ethanol. Sections were allowed

to mordant in potassium dichromate-HCl for 2 min, stained with acid fuchsin for 1 min and then fixed in phosphomolybdic acid for 3 min. Slides were then counterstained with orange G for 5 min, with fast green for 11 min and then washed in 1 % acetic acid for 3 min. The stained sections were then dehydrated in two washes each of 95% and 100% ethanols, cleared in 100% toluene and mounted in permount.

B) Triton Extraction

Control and compressed embryos were extracted in 0.5% Triton X-100 in MFASW to enhance the contrast of the myoplasm. The detergent extracts the majority of the cytoplasm from the blastomeres leaving the detergent insoluble pigment granules of the myoplasm which are readily visible using bright field microscopy (Jeffery and Meier, 1983).

C) Transmission Electron Microscopy (TEM)

Control and compressed embryos were prepared for TEM (see below) in order to visualize the presence of myoplasm in the B4.1 and ectopic blastomeres. Myoplasm is characterized by an abundance of pigment granules and mitochondria (Conklin, 1905b).

2.4 Preparation of RNA Probes

Complementary (experimental) and homologous (control) RNA probes were synthesized from the Bluescribe SpMA3C subclone (supplied by W.R. Jeffery)

using T3 and T7 RNA polymerase respectively. The probe was developed using poly(A) RNA isolated from the the mantle muscle lining the body wall of the adult ascidian *Styela plicata* . This RNA fraction was then used to constuct a cDNA library that was then screened with a muscle actin gene of *Drosophila* . A subclone was selected which was specific for 3' region of the gene including 580 base pairs of the coding region and 85 base pairs of the non-coding region. The presence of the non-coding region was required to ensure the specificity of the probe for muscle actin as opposed to cytoskeletal actin. Probes were transcribed *in vitro* using [³⁵S] UTP (1000-1200 Ci mmole⁻¹) or [³H] UTP (30-50 Ci mmole⁻¹); (NEN Du Pont, Mississauga, Ont.) and 400 µM of each unlabelled nucleotide. Refer to Figure I for a flow chart of the procedures used.

2.5 *In Situ* Hybridization

The *in situ* hybridization procedure was modified from that outlined by Tomlinson et al (1987a). Control embryos, compressed embryos and tadpoles were fixed for 20 min in ice-cold 95% ethanol:acetic acid (3:1) at intervals as outlined above under microcompression . The fixed embryos were dehydrated in successive washes with ice-cold 70%, 80%, 90%, 95% ethanols followed by staining with eosin saturated 95% ethanol for 15 min each. Dehydration was completed with two washes in ice-cold absolute ethanol followed by treatment with ice-cold 100% ethanol:100% toluene (3:1, 1:1, 1:3, 15 min each) and clearing in two washes of 100% toluene (the first ice-cold, the second at room

I Synthesis of Radiolabelled Muscle Actin mRNA Probe From SpMA3C

Flow chart indicating the synthesis of the muscle actin mRNA probe SpMA3C and its use in the technique of *in situ* hybridization

temperature), 15 min each. Specimens were then infiltrated gradually with 100% toluene:100% paraplast (3:1, 1:1, 1:3, 30 min each) at 60 °C and embedded in 100% paraplast, 30 min at 60 °C followed by transfer to fresh

100% paraplast in beem capsules for 24 hrs at 60 °C. The specimens were sectioned to 8 µm and placed on gelatin subbed glass slides. Deparaffinized, rehydrated slides were immersed in 0.1 M Tris Base/0.05 M EDTA (pH 8.0), 5 min at room temperature (RT), and treated with 1 µg ml⁻¹ proteinase K in Tris/EDTA for 30 min at 37 °C, washed in Tris/EDTA, 5 min, and immersed briefly in 0.1 M sodium phosphate buffer (pH 7.2) at RT. Slides were postfixed in 4% paraformaldehyde, 30 min at RT, washed in Tris/EDTA, 5 min at RT and treated with freshly prepared 0.25% acetic anhydride/0.1 M triethanolamine, 10 min at RT. A final wash in Tris/EDTA, 5 min, RT, was followed by dehydration in an ethanol series and slides were air dried. Approximately 1X10⁶ cpm of ³⁵S or ³H labelled RNA probe was applied to each slide in 50 µl of hybridization buffer (50% formamide, 10% dextran sulphate, 0.01M Tris Base, 0.3 M NaCl, 0.001 M EDTA, 500 µg ml⁻¹ tRNA, 1X Denhardt's solution, 500 µg ml⁻¹ poly A) and covered with an acid washed coverslip. Hybridization was carried out for approximately 15 hrs at 45 °C in a humid chamber. High stringency washing conditions were required to eliminate hybridization with ubiquitously expressed cytoplasmic actins and were carried out as follows. Coverslips were removed in 250 mls 4X SSC and slides were washed twice in 250 mls of 4X SSC, 5 min each at RT, treated with 20 µg ml⁻¹ pancreatic RNase A in 0.01 M Tris/0.5 M NaCl, 30 min at 30 °C and washed in 3 l of 2X SSC, 30 min at RT. High stringency required a further wash in 3 l of 1X SSC, 30 min at 45 °C. If very high stringency washes were applied to the sections (0.1X SSC, 30 min at 45 °C) no signal was detected presumeably due to complete removal of

the probe. The washed slides were dehydrated in an ethanol series to 95%, air dried and prepared for autoradiography using Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, N.Y.). Slides hybridized with ^{35}S probes were exposed for 7 days at $-20\text{ }^{\circ}\text{C}$, those with ^3H exposed for 56 days at $-20\text{ }^{\circ}\text{C}$, developed using Kodak D-19 developer and subsequently stained with Milligan trichrome.

2.6 Transmission Electron Microscopy

Control and compressed CA embryos and control tadpoles were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at RT, washed three times in 0.1 M phosphate buffer and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer for 1 hr at RT. Specimens were dehydrated in a graded ethanol series and gradually infiltrated and finally embedded in Spurr resin (Jeffery and Meier, 1983). Thin sections (85 nm) were cut on a Reichert-Jung ultracut E ultramicrotome with a glass knife, placed on copper grids, double stained with 2% aqueous uranyl acetate and lead citrate and examined with a Phillips 420 transmission electron microscope at 80 kV.

2.7 Immunocytochemistry

Control and compressed CA embryos and control tadpoles were prepared for indirect immunofluorescence in sections. All specimens were stained separately

with both Sr-1 ascites (anti-sarcomeric actin; Sigma Immunochemical, St. Louis, MO) diluted 1:350 with phosphate buffered saline (PBS) and Mu-2 ascites (anti-myosin heavy chain, supplied by Dr. N. Satoh) diluted 1:300 with PBS. Both antibodies were subsequently detected using fluorescein-isothiocyanate conjugated sheep anti-mouse Ig serum (Boehringer Mannheim, Laval, P.Q.) diluted 1:30 with PBS. Immunocytochemistry was carried out on polyester wax embedded, sectioned material according to Mita-Miyazawa et al, 1987. Specimens were fixed for 20 min in absolute methanol at -20 °C, followed by 20 min in absolute ethanol at -20 °C, and gradually infiltrated with Steedman polyester wax (BDH, Toronto, Ont.) at 40 °C. Sections were cut onto gelatin subbed coverslips, polyester wax was removed in absolute ethanol and sections were rehydrated in a graded ethanol series ending in two washes of PBS. Each coverslip was then incubated with 70 µl of the appropriate primary antibody (see above) in a humid chamber for 1 hr at RT. Coverslips were then washed for 30 min in PBS at RT followed by incubation with 70 µl of secondary antibody for 30 min at RT and a final PBS wash of 30 min at RT. Coverslips were then mounted in a drop of 80% glycerol in PBS and observed with an Olympus CH-2 compound microscope equipped with a mercury burner. Photomicrographs were taken on Kodak Tri-X Pan film.

2.8 Miscellaneous Procedures

Whole mount preparations for bright field microscopy were prepared by fixing the specimens with 5% formalin in MFASW. Bright field and dark field microscopy was performed on a Zeiss Axioplan compound microscope. Thick sections of Spurr embedded material were prepared to indicate the orientation of TEM photomicrographs were mounted on glass slides and stained with 1% azure B:1% methylene blue, 1:1. Quantitative determination of ectopic myoplasm was estimated on 8-cell compressed embryos extracted with 0.5% Triton X-100 and fixed in 5% formalin using a calibrated ocular micrometer.

3. RESULTS

3.1 The Role of Localized Myoplasmic Determinants in Muscle Cell Determination of the Ascidian Larva

3.1.1 Microcompression of 8-Cell Embryos

Normal cell division takes place at right angles to the plane of the mitotic spindles and through their equator (Morgan, 1927). Early studies on the density of various regions of the egg cytoplasm indicate that a semi-rigid gel forms immediately around the spindle of a cleaving blastomere (Morgan, 1927). This dense matrix could cause the spindle apparatus to behave as a single structure when subject to an external force that would alter its orientation within the cell. Under compression, ascidian eggs are observed to cleave at right angles to the plane of compression (Whittaker, 1980). This can be explained by a reorientation of the mitotic spindles which shift to the most stable position in the compressed cell, that being perpendicular to the plane of compression and parallel to the long axis of the cell. This reorientation of the spindles is defined by Hertwig's Rule (Hertwig, 1893) which is shown diagrammatically in Figure II. Because ascidian eggs exhibit a localization of specific cytoplasmic regions, any modifications in the plane of cleavage will alter the normal distribution of these regions into ectopic positions in the resulting blastomeres. This redistribution of localized cytoplasmic regions is especially evident with respect to the pigmented myoplasm of *Boltenia villosa* eggs.

II The Effect of Compression on the Plane of Cleavage as Determined by Hertwig's Rule

A diagram indicating the effects of compression on the orientation of the mitotic spindle and subsequently the plane of cleavage of a dividing blastomere. By Hertwig's rule (1892), compression during the third cleavage will orient the mitotic spindle such that cell division is meridional rather than equatorial with respect to the animal-vegetal axis.

Legend

F - compression force

- aster

←→ - plane of spindle

The normal distribution of myoplasm in the 8-cell embryo of this species is restricted to the B4.1 blastomere pair (Figure III). While under compression, the myoplasm is redistributed ectopically into the b4.2 pair also (Figure IV). This redistribution of myoplasm was used to test the hypothesis that factors localized in this cytoplasmic domain are involved in the determination of the primary muscle cell lineage in the ascidian larva. It should be noted that after being subject to compression, the resulting embryos did not gastrulate or form any recognizable body axis. Normal tadpoles never developed but rather a disorganized mass of cells was observed. In order to identify the normal muscle cell lineage and those containing ectopic myoplasm, it was necessary to cleavage-arrest both compressed and control 8-cell embryos by culturing them in cytochalasin B until unoperated controls had hatched.

Diagram indicating the distribution of cytoplasm in control 8-cell embryos. The third cleavage is normally equatorial across the animal-vegetal axis giving rise to an embryo in which myoplasm is restricted to the B4.1 blastomere pair. The ectoplasm is found in both the B4.2 and A4.1 blastomere pairs. Nomenclature of blastomeres after Conklin (1905).

IV Distribution of Localized Cytoplasmic Regions in Compressed 8-Cell Embryos

Diagram indicating the distribution of cytoplasm in compressed 8-cell embryos. Under compression, the third cleavage is meridional giving rise to an 8-cell embryo in which four blastomeres contain myoplasm, the B4.1 pair and the ectopic B4.2 pair. Ectoplasm is found in the same four blastomeres as controls. Nomenclature after Conklin (1905).

3.1.2 Characteristics of the Muscle Lineage in Control Tadpoles

At 15 °C, *Boltenia villosa* tadpoles were found to hatch at approximately 17.5 hrs post-fertilization. At this time the larvae are motile and continue to swim using muscle cells in the tail until a suitable substrate is found. At that time, settlement and subsequent metamorphosis into the juvenile life form takes place. The musculature of the ascidian larva is found only in the tail, flanking either side of the presumptive notochord. Figure V (A) shows that only this region contains the pigmented myoplasm (stained bright red with Milligan trichrome) which is known to be partitioned into the muscle lineage in early ascidian embryos (Chabry, 1887). The tail muscle cells also express muscle-specific actin transcripts (Fig V, C and D) as well as the muscle-specific proteins myosin heavy chain and sarcomeric actin (Fig V, E and F respectively) which are not found in any other cell type in the larva. These muscle cells exhibit ultrastructural characteristics similar to those of vertebrate striated muscle (Fig V, B) including myofibrils consisting of thin actin and thick myosin filaments as well as Z lines and H, I and A bands.

3.1.3 Redistribution of Myoplasm in Compressed 8-Cell Embryos

Normal 8-cell embryos contain myoplasm in only the B4.1 blastomeres which are located in the posterior vegetal region of the embryo (Fig III) and give rise to most of the tail musculature in the tadpole (Conklin, 1905a). In compressed embryos, the myoplasm is redistributed into two additional blastomeres, the b4.2 pair, resulting in an 8-cell embryo with

Fig.V. Micrographs showing the characteristics of muscle cells in the control tadpole larva. The tail muscle cells (m) contain myoplasm as seen by the characteristic red staining of these cells with Milligan trichrome (A). Only the muscle cells of the tail (t) contain myoplasm and not cells in the notochord (n) or the head (h) . The muscle cells of the tail exhibit typical muscle ultrastructure as seen by the presence of an intact sarcomere (B). Identifiable structures include the Z line (Z), H band (H), A band (A), I band (I) and myofibrils (Mf) consisting of actin and myosin. The sarcolemma (S) and mitochondria (Mt) are also visible. Muscle actin mRNA as detected by *in situ* hybridization with SpMA3C, is also localized in the muscle cells of the tail and is not found in the notochord or head region of the tadpole. Both bright field (C) and dark field (D) microscopy were used. Immunocytochemistry was used to detect the presence of muscle-specific proteins in the larva. Both myosin heavy chain (E) and sarcomeric actin (F) are strictly localized to the muscle cells of the tail and are not found in the notochord or head region. Magnification; (A) X90, (B) X30,000, (C-F) X70.

myoplasm in four of the eight blastomeres. The b4.2 blastomeres normally give rise to epidermal tissues as well as the secondary muscle lineage at the most caudal region of the larval tail (Conklin, 1905a, Nishida, 1987). Figure VI indicates the distribution of myoplasm in both control and compressed 8-cell embryos. The presence of the myoplasm can be visualized by treating living embryos with Triton X-100 (Figure VI, A and C) which extracts most of the cytoplasm leaving only the detergent insoluble pigment granules of the myoplasm (Jeffery and Meier, 1983). The distribution of myoplasm is also shown using Milligan trichrome staining (Figure VI, B and D). Both techniques clearly indicate that control 8-cell embryos contain myoplasm in only two blastomeres, the B4.1 pair, while compressed 8-cell embryos contain myoplasm in four blastomeres, both the B4.1 and b4.2 pairs. The myoplasm is further characterized ultrastructurally by the presence of abundant mitochondria and pigment granules (Conklin, 1905b). Figure VII shows that compressed 8-cell embryos contain myoplasm in both the B4.1 and b4.2 blastomeres (A and B) but compression does not redistribute myoplasm into other lineages (C). The volume of myoplasm transferred ectopically to the b4.2 pair during compression was estimated to be 30.4 ± 2.8 % of the total cell volume (Table I).

3.1.4 Expression of Muscle-Specific Transcripts in Compressed 8-Cell Embryos

Muscle actin mRNA was detected by *in situ* hybridization using a radiolabelled complementary RNA probe transcribed *in vitro* from

FigVI . Light micrographs indicating the distribution of myoplasm in compressed (A and B) and non-compressed (C and D) 8-cell embryos. (A) and (C) are Triton X-100 extracted embryos in which most of the cytoplasm is extracted leaving the detergent insoluble pigment granules of the cytoplasm. (B) and (D) are Milligan trichrome stained embryos in which the myoplasm is stained bright red while other cell types are stained blue-green. Both embryos can be seen to contain myoplasm in the two B4.1 blastomeres (large arrows) while the compressed embryo also contains myoplasm in two ectopic blastomeres (small arrows). Magnification; X90.

Fig.VII. Electron micrographs showing the ultrastructural differences in various cell lines of an 8-cell compressed embryo. The B4.1 blastomere (A) contains large quantities of myoplasm characterized by an abundance of pigment granules (p) each of which is surrounded by a ring of mitochondria (Mt). In compressed embryos, some of the myoplasm is redistributed to the b4.2 blastomeres (B). The quantity of ectopic myoplasm is less than that in the B4.1 lineage as indicated by the presence of fewer pigment granules and mitochondria. A non-muscle lineage blastomere is seen not to contain any myoplasm (C). Magnification; (A) and (B) X3400, (C) X16,500.

SpMA3C. Stringencies were applied such that only the muscle actin transcripts were detected and not those of the ubiquitously expressed cytoplasmic actins. Although the embryos were cleavage-arrested at the 8-cell stage, "development" was allowed to proceed until the control tadpoles had hatched. Ascidian embryos are known to express tissue-specific characteristics in the expected lineages despite being subject to cleavage-arrest (Whittaker et al, 1977, Whittaker, 1980,1982, Nishikata et al, 1987b). Figure VIII shows the distribution of muscle actin mRNA in compressed and control 8-cell cleavage-arrested embryos (A,B and C,D respectively). It can be seen that only the blastomeres which contain myoplasm express the actin transcript, that being the B4.1 blastomere pair of control embryos and both the B4.1 blastomeres and ectopically in the b4.2 pair of compressed embryos.

TABLE I
Volume of Ectopic Myoplasm Transferred From B4.1 to b4.2 Blastomeres
During Compression as a Percentage of Total Blastomere Volume

Number of 8-Cell Embryos Observed	% of Total Cell Volume Containing Ectopic Myoplasm
50	31 ± 2.8

3.1.5 Expression of Muscle-Specific Proteins in Compressed 8-Cell Embryos

Figure IX shows that the pattern of expression of the muscle-specific

FigVIII Light micrographs showing the distribution of muscle actin mRNA in compressed (A and B) and non-compressed (C and D) 8-cell embryos cleavage-arrested and allowed to develop until controls had hatched. *In situ* hybridization with SpMA3C indicates that both embryos contain muscle actin transcript in the two B4.1 blastomeres (large arrows) which corresponds to the expected number of blastomeres in the muscle lineage at the 8-cell stage. The compressed embryos have the myoplasm redistributed into the b4.2 lineage and this blastomere pair is seen to express the actin transcript ectopically (small arrows). Both bright field (A and C) and dark field (B and D) microscopy were used. Magnification; X150.

proteins myosin heavy chain (A and C) and sarcomeric actin (B and D) were identical to the distribution of actin mRNA in cleavage-arrested 8-cell embryos in that only the blastomeres that contain myoplasm were observed to express these muscle markers. Control embryos expressed both markers in only the B4.1 pair while compressed embryos express the proteins in the B4.1 lineage and ectopically in the b4.2 blastomeres. Again it can be seen that the act of compression itself does not initiate expression of ectopic muscle characteristics as only the myoplasm containing blastomeres, either native or transferred, express myosin and actin.

3.1.6 Ultrastructural Characteristics of Compressed 8-Cell Embryos

Figure X shows the presence of myofibrils in the B4.1 lineage of 8-cell cleavage-arrested embryos at the periphery of the cell. These fibers appear to vary in the extent of their organization from one embryo to another but were never arranged in the extensive parallel arrays characteristic of muscle cells in control tadpoles (see Figure V, B). A similar lack of myofibril organization has also been observed in cleavage-arrested embryos of the ascidian *Ciona intestinalis* (Crowther and Whittaker, 1983). Sarcomere specific structures such as Z lines and H bands were never observed in cleavage-arrested embryos indicating that although muscle development is still following the proper spatial and temporal signals found in control tadpoles, the expression of this tissue-specific is somewhat aberrant perhaps due to a deficiency in the cellular interactions which occur in a normal embryo. Both compressed and control embryos exhibit the presence of

Fig. IX. Immunocytochemistry with Mu-2 and Sr-1 was used to detect myosin heavy chain and sarcomeric actin respectively in compressed (A and B) and non-compressed (C and D) 8-cell cleavage-arrested embryos allowed to develop until controls had hatched. Both of these muscle-specific proteins were localized in the B4.1 blastomere pair of the 8-cell embryos (large arrows). The compressed embryos express both myosin and actin in two additional blastomeres, the b4.2 pair, which contains ectopic myoplasm (small arrows). Magnification; X90.

myofibrils in the B4.1 blastomeres (Fig X, AD and F respectively). In compressed embryos, the b4.2 lineage containing ectopic myoplasm also develops myofibrils that were indistinguishable from those in the B4.1 blastomeres (Fig X, B and E) while the b4.2 lineage of control 8-cell embryos lacked these fibrils (Fig X, G). The cells which do not contain myoplasm in the compressed embryos never developed myofibrils (Fig X, C) indicating that the expression of ectopic muscle ultrastructure in the b4.2 lineage is likely due to the presence of ectopic myoplasm and not the compression event itself. Myofibrils were distinguished from non-muscle filaments on the basis of comparison with myofibrils observed in previous studies with cleavage-arrested embryos (Crowther and Whittaker, 1983). A total of ten compressed embryos were observed and in all cases where fibrils were detected, both the B4.1 and b4.2 lineages contained these structures (Table II). Two compressed embryos were found to lack fibers in all blastomeres despite the presence of myoplasm in both the B4.1 and b4.2 lineages.

TABLE II
Number Of Cleavage-Arrested Embryos Which Exhibit The Presence
Of Myofibrils In Various Cell Lineages

Embryo	Number of embryos	Myofibrils in B4.1	Myofibrils in b4.2	Myofibrils in other cells
8-cell compressed	10	8	8	0
8-cell non-compressed	3	3	0	0

Fig.X. Electron micrographs indicating the ultrastructure of compressed (A-E) and non-compressed (F and G) 8-cell cleavage-arrested embryos allowed to develop until controls had hatched. The B4.1 blastomeres of both compressed (A and D) and non-compressed (F) embryos contain myofibrils (Mf). The b4.2 blastomere of the non-compressed embryo does not contain any myofibrils (G) but this lineage does in the compressed embryo (B and E), which also contains ectopic myoplasm as indicated by the presence of mitochondria (Mt). Other cell lines of the compressed embryo which do not contain myoplasm do not exhibit the presence of myofibrils (C). The chorion (Ch) which surrounds the embryo is visible in (C) and (G). Magnification; (A), (B) X18,500, (C) X51,000, (D), (E) X30,000, (F) X36,000, (G) X25,500.

3.2 Temporal Aspects of Muscle Development in the Ascidian Larva

3.2.1 Muscle Characteristics in Early Tailbud Embryos

Figure XI indicates the expression of muscle characteristics in early tailbud larvae from 12 to 13 hrs of development detected by immunocytochemistry with monoclonal antibodies. It can be seen that sarcomeric actin (A) is synthesized prior to myosin heavy chain (B) at 12 hrs post-fertilization. At this time, actin is faintly visible in the muscle cells of the tail and is absent from the cells of the notochord and head of the tadpole. Myosin is not present at this time as indicated by the absence of a fluorescent signal. Although actin is present, the punctate pattern of its expression indicates that the monomeric form, G-actin, is present and has not yet been assembled into thin filaments. This is supported by the absence of any muscle filaments at the periphery of the muscle cells (C). At 12.5 hrs of development, the levels of actin have increased (D) but the punctate pattern indicates the continued presence of G-actin. Myosin is first synthesized at this time as seen by a similarly punctate staining pattern indicating the presence of the monomeric form of myosin. As is the case for actin, myosin is only expressed in the tail muscle cells and is absent from the notochord and head of the larva (E). The lack of thin or thick filaments at the periphery of the muscle cells (F) also indicates that only the monomeric forms of these proteins are present at this stage of development. The same situation exists at 13 hrs of development (G-I). Note that the expression of actin is clearly localized to the cytoplasm of the muscle cells and is absent from the nucleus.

Fig.XI. Figure indicating the temporal expression of the muscle-specific proteins actin (A,D,G) and myosin (B,E,H) and the corresponding ultrastructure (C,F,I) in the early tailed larva from 12 to 13 hrs. post-fertilization. At 12 hrs of development (A-C), only sarcomeric actin (A) is visible in a punctate pattern as detected using immunocytochemistry and is expressed in only the muscle cells (m) flanking either side of the notochord (n) in the tail (t) and is not found in the head region (h) of the larva. This punctate pattern of expression indicates the presence of the monomeric form of actin prior to its assembly into thin actin filaments. Myosin is not present at this time (B). This is supported by the absence of either of these filament types at this stage of development (C). The mitochondria (Mt) which are abundant in the muscle cells are visible. By 12.5 hrs. post-fertilization (D-F) myosin monomer is first synthesized (E) but neither actin nor myosin have been assembled into filaments (F). The same situation exists at 13 hrs of development (G-I). Magnification; immunofluorescence X60, TEM X18,500.

3.2.2 Muscle Characteristics in Mid Tailbud Embryos

Figure XII shows the development of muscle-specific characteristics in mid tailbud larvae from 13.5 to 14.5 hrs post-fertilization. At 13.5 hrs (A-C) and 14 hrs (D-F) of development, the expression patterns of actin and myosin are identical to those found at 13 hrs of development (Figure XI, G-I) indicating that both proteins are present in their monomeric forms and are restricted to the tail muscle cells. At 14.5 hrs of development, the staining pattern of actin becomes solid and is localized to the periphery of the tail muscle cells (G). This change in the pattern of expression likely indicates the assembly of G-actin into F-actin, the primary component of thin muscle filaments. This is supported by the appearance of thin filaments at the periphery of the muscle cells at this time (I). Myosin is still present in its monomeric form as indicated by the punctate staining pattern (H) and the absence of thick filaments (I).

3.2.3 Muscle Characteristics in Late Tailbud Embryos

Figure XIII indicates the development of muscle-specific characteristics in late tailbud larvae from 15 to 16 hrs post-fertilization. There are no noticeable changes in the patterns of expression of actin and myosin from those observed at 14.5 hrs of development (Figure XII, G-I). The density of the actin filaments appears to be increasing as these thin filaments are organized into parallel arrays at the periphery of the tail muscle cells (C, F and I).

Fig. XII. Figure indicating the temporal expression of the muscle-specific proteins actin (A,D,G) and myosin (B,E,H) and the corresponding ultrastructure (C,F,I) in the mid tailbud larva from 13.5 to 14.5 hrs. post-fertilization. At 13.5 (A-C) and 14 (D-F) hrs of development, the monomeric forms of both actin (A and D) and myosin (B and E) are present in the tail muscle cells (m) but have not yet been assembled into thin and thick filaments respectively (C and F). This is identical to the situation observed at 13 hrs post-fertilization (Fig.XI, G-I). At 14.5 hrs. of development, the expression of sarcomeric actin becomes less punctate and appears to be solid around the periphery of the tail muscle cells (G). This is reflected by the appearance of a network of thin actin filaments (A) immediately adjacent to the notochord (I). Myosin is still present in only the monomeric form as indicated by its punctate pattern of expression (H) and a lack of thick fibers (I). (A) and (H) show the tail in cross-section. Magnification; immunofluorescence X60, (C) and (F) X18,500, (I) X23,000.

Fig. XIII. Figure indicating the temporal expression of the muscle-specific proteins actin (A,D,G) and myosin (B,E,H) and the corresponding ultrastructure (C,F,I) in the late trilobed larva from 15 to 16 hrs. post-fertilization. At 15 hrs. (A-C), actin expression levels have increased as indicated by an increase in the intensity of staining with the actin antibody (A) and an increase in the density of the actin filament network (Af) being laid down in parallel arrays (C). Myosin is still present in only the monomeric form as indicated by its punctate expression pattern (B) and the absence of thick filaments (C). The same situation exists at both 15.5 (D-F) and 16 (G-I) hrs of development. The sheath fibers (Sf) which form a dense mat surrounding the notochord (n) are visible in certain planes of section (I). Magnification; immunofluorescence X60, TEM X18,500.

3.2.4 Muscle Characteristics in Tadpole Larvae

Figure XIV indicates the development of muscle-specific characteristics in tadpole larvae from 16.5 to 17.5 hrs post-fertilization. At 15 °C, *Boltenia villosa* tadpoles will hatch and begin swimming between 17 and 17.5 hrs after fertilization. At 16.5 hrs of development, The staining pattern of myosin is no longer punctate (B) and resembles the linear staining pattern of actin (A). This indicates that myosin has now been assembled into thick filaments which is supported by the appearance of myofibrils consisting of thin actin and thick myosin filaments (C). Coincident with the assembly of the myofibrils is the construction of whole sarcomeres, the supramolecular structure characteristic of differentiated muscle cells. It should be noted that up until this time, the larvae had not exhibited any signs of movement but at 16.5 hrs of development, the tail of the tadpole began to twitch noticeably indicating that the newly formed sarcomeres are functional immediately upon their assembly. The identical situation is present at 17 hrs (D-F) and 17.5 hrs (G-I) of development. The flexation of the tail progressively increased both in intensity and periodicity until the tadpole was able to break free of the chorion with these tail movements and begin swimming.

Fig. XIV. Figure indicating the temporal expression of the muscle-specific proteins actin (A,D,G) and myosin (B,E,H) and the corresponding ultrastructure (C,F,I) in the tadpole larva from 16.5 to 17.5 hrs post-fertilization. At 16.5 hrs of development (A-C), both actin and myosin have been polymerized into thin and thick filaments respectively at the periphery of the tail muscle cells as indicated by the solid pattern of staining with the antibodies. Concurrent with the polymerization of the thick myosin filaments is the assembly of sarcomeres, the contractile unit of a muscle cell (C). The myofilaments (Mf) consist of alternating fibers of actin and myosin as well as the proteins tropomyosin, troponin and alpha-actinin. Other characteristics of a functional sarcomere are also visible including A bands (A), H bands (H), I bands (I) and the Z line (Z). The same situation can be seen at 17 hrs. of development (D-F) except that the sarcolemma (S) is visible in this section (F). By 17.5 hrs. post-fertilization (G-I), the tadpoles have hatched and are motile, using the muscle cells of the tail to swim. Magnification; (A),(B),(D),(F) X60, (G),(H) X70, (C),(F),(I) X18,900.

4. DISCUSSION

The determination of cell fate in early ascidian embryos is thought to be mediated via the action of cytoplasmic determinants that are localized in specific domains of the egg following fertilization. Due to the determinate cleavage pattern of ascidian embryos, these determinants are segregated into specific blastomeres during the early cleavages where they are thought to regulate lineage-specific genetic programs and so establish cell fate. The results of this study suggest that the myoplasm, a pigmented region of cytoplasm segregated into the progenitors of most of the tail musculature in the ascidian tadpole larva, contains information which specifies the muscle lineage. Evidence for the existence of these determinants was obtained by microsurgically redistributing a portion of the myoplasm into a non-muscle lineage by compressing the embryos of *Boltenia villosa* during third cleavage. The resulting embryos were observed to express muscle actin mRNA, the muscle-specific proteins actin and myosin heavy chain, and muscle-specific ultrastructure in the form of actin filaments in inappropriate cells containing ectopic myoplasm.

4.1 Microcompression and Cytoplasmic Redistribution

The first microcompression experiments carried out on ascidian embryos were performed by Morgan (1910) to investigate the apparently mosaic nature of ascidian development. His results indicate that, unlike sea urchin embryos (Driesch, 1892), ascidian embryos cannot regulate after the early cleavage pattern has been altered. The resulting larvae were highly

abnormal, gastrulation did not occur and a body axis did not develop. Unfortunately, the resulting disorganized cell mass did not lend itself to the analysis of any alterations in cell fate resulting from a redistribution of cytoplasmic domains. More recent studies by Whittaker (1980), repeated these experiments on *Styela plicata* and solved the problem of following the cell fate in a non-axial cell mass by cleavage-arresting the embryos after the myoplasm had been redistributed by compression. The resulting 8-cell embryos were allowed to "develop" in cytochalasin until controls had reached a stage at which the muscle-specific marker acetylcholinesterase (AChE) was expressed. Cytochalasin is known to interfere with microfilaments and so prevents cytokinesis but does not inhibit nuclear division (Whittaker, 1973). In some cases, the cleavage-arrested embryos developed AChE in three or four blastomeres rather than only two as in control 8-cell embryos.

The current study utilizes these results but comes closer to investigating the event of cell determination itself. Determination can be defined as the series of molecular events that initiate the establishment of cell fate by permanently "turning off" certain genes while sequentially activating another set of tissue-specific genes. Differentiation is a separate yet dependent event and involves the development of new properties that are specific to a certain cell type. Many molecular events occur between the determination of cell fate and the expression of a tissue-specific marker, i.e. differentiation, such as muscle AChE. Thus assaying for a protein which characterizes the differentiated state of a particular cell type does not necessarily allow one to draw conclusions about the process of determination of that cell type. This study assayed for the presence of a

muscle-specific transcript in inappropriate blastomeres containing ectopic myoplasm. By assaying for a gene product rather than the protein itself, one can study an event further upstream toward the act of cell fate determination rather than observing a characteristic of the differentiated state. Because cytoplasmic determinants are thought to play a role in the establishment of cell fate, any relevant study should look as closely at these molecular events as is experimentally possible.

Ascidian eggs have an animal-vegetal polar axis which is established prior to fertilization (Conklin, 1905a). In some species, the pigmented myoplasm that segregates into a subequatorial crescent at the posterior vegetal region of the egg marks the axis of bilateral symmetry. The first cleavage is meridional along the animal-vegetal axis of the zygote and bisects the myoplasmic crescent into equal halves. The second division is also meridional and occurs perpendicular to the first creating a four cell embryo with the myoplasm distributed unequally into only two of the four blastomeres. The third cleavage is normally equatorial and at right angles to the first two creating an 8-cell embryo with animal and vegetal quartets of blastomeres in which the myoplasm has been segregated into the two posterior-vegetal blastomeres (see Fig. III). These two cells are termed the B4.1 blastomeres (after Conklin, 1905a) and are the progenitors of most of the tail muscle cells in the tadpole larva. This cleavage pattern is highly determinate and results in the reproducible segregation of localized cytoplasmic domains into specific lineages. If ascidian embryos are subject to compression during the third cleavage, a flattening in the plane of the animal-vegetal axis is observed. This causes the mitotic spindles to reorient perpendicular to the direction of the compressing force and parallel to the

long axis of the cell (see Fig. II). This reorientation of the spindles is defined by Hertwig's Rule (Hertwig, 1893) and results in third cleavage also being meridional. The alteration in the plane of cleavage redistributes the localized cytoplasmic domains into new blastomeres but does not alter the nuclear lineage of these cells (Whittaker, 1980) which are now present in a uniplanar rosette rather than an animal and vegetal division of cells as in controls. This is evident from Figure VI which indicates that the myoplasm is now present in four blastomeres of compressed embryos as compared to two in the control embryos. The myoplasm is visible ectopically in the b4.2 blastomeres which normally give rise to epidermal and neural structures (Nishida, 1987). This is also apparent from Figure VII in which an abundance of pigment granules and mitochondria characteristic of the myoplasm (Conklin, 1905b) are present in both the B4.1 and b4.2 blastomeres. Approximately 30% of the total cell volume of the b4.2 blastomeres was observed to consist of ectopic myoplasm (Table I). If a minimum amount of myoplasm is required to initiate the determination of the muscle lineage, 30% of the cell volume appears to be above that minimum threshold value as the non-muscle cells which contain ectopic myoplasm express a variety of muscle-specific characteristics (see below). The physical stress applied to the eggs during compression was not the cause of ectopic muscle expression as only the cells which contain myoplasm were observed to developed muscle-specific features. This is an important point as physical stress has been known to initiate the expression of developmental genetic programs such as the parthenogenic activation of unfertilized eggs by compression or puncture with a needle. Punctured eggs have been observed to initiate cleavage despite the absence of a sperm

nucleus (Morgan, 1927). The cytochalasin treatment used to cleavage-arrest the compressed embryos was not responsible for the ectopic expression of muscle features as non-compressed cleavage-arrested 8-cell embryos never developed muscle markers in more than the two B4.1 blastomeres.

It should be noted that many organisms will develop normally after being subject to compression during early cleavage. For example, sea urchin embryos develop normally after compression (Driesch, 1892) as do nereid worms (Morgan, 1910) and some frogs (Pfluger, 1883). This could be explained by a greater dependence on cell-cell interactions in the establishment of cell fate and a reduced role for localized determinants in these organisms. Thus the redistribution of egg cytoplasm by compression does not disrupt development in these organisms provided that the blastomeres are able to return to their native spatial configuration and in doing so, establish the necessary cell-cell contacts required for induction and subsequent differentiation. This is supported by the fact that organisms which seem to regulate after compression tend to establish cell fate later in embryogenesis and so likely depend on induction to establish cell fate (Morgan, 1927) while in ascidian embryos, many of the cell fate decisions have already been made by the 8-cell stage (Satoh et al, 1990).

4.2 Ectopic Expression of Muscle-Specific Characteristics in Compressed Embryos

In order to determine if a specific region of egg cytoplasm contains material which is capable of determining the fate of a cell, it is necessary to introduce this material into a specific cell lineage and observe if that cell

takes on the fate which is specified by the donor cytoplasm. Fortunately, in the case of the myoplasm there are a number of muscle-specific markers which can be utilized to determine if the fate of a non-muscle cell has been respecified by the introduction of ectopic myoplasm. The histochemical marker acetylcholinesterase (AChE) has been used in previous studies to identify the muscle cell lineage in ascidian embryos and larvae (Whittaker, 1980, 1982, Crowther and Whittaker, 1984, 1986, Whittaker and Meedel, 1989). However, this marker is not applicable to studies with *Boltenia villosa* as the reaction product for the AChE assay is indistinguishable from the normal pigmentation of the myoplasm of this species under normal optical conditions. Thus a positive AChE reaction in inappropriate cells would be masked by the presence of the ectopic myoplasm itself.

Fortunately a number of other muscle-specific markers are available including a probe for muscle actin mRNA as well as monoclonal antibodies specific for sarcomeric actin and myosin heavy chain. Muscle cells can also be identified ultrastructurally by the presence of thin actin and thick myosin filaments which make up a myofibril, the contractile unit of a muscle cell. All of these characteristics were examined to ensure that the presence of ectopic myoplasm had indeed contributed to the respecification of cell fate in a non-muscle lineage.

4.2.1 Muscle Actin mRNA

A probe specific for muscle actin mRNA was developed using poly(A) RNA isolated from the mantle muscle lining the body wall of the adult ascidian *Styela plicata*. This RNA fraction was then used to construct a

cDNA library which was in turn screened with a muscle actin gene isolated from *Drosophila*. The final isolated sequence (SpMA3C) was shown to correspond to muscle actin mRNA due to its restricted expression in the muscle cell lineage of *S. plicata* larvae and adults (Tomlinson et al, 1987b). The specificity of the probe for muscle actin, as opposed to the ubiquitously expressed cytoskeletal actins, is due to the presence of the 3' noncoding sequence of the muscle actin gene in the probe. (Tomlinson et al, 1987b) The present study used the cDNA sequence to transcribe a radiolabelled sequence complementary to the muscle actin transcript. The probe was then used to detect the presence of muscle specific mRNA in blastomeres which contain ectopic myoplasm to determine if the presence of this cytoplasmic domain is sufficient to promote the expression of muscle features. The technique of *in situ* hybridization was used so that the location and not just the presence of the transcript could be detected. Before experimental embryos could be probed, it was necessary to determine if the probe would cross react with the muscle actin of *B. villosa*. Figure V (C and D) indicates that the probe does recognize the actin of this species and is specific for the muscle isoform as the signal is restricted to the tail muscle cells of the tadpole. The fact that this sequence is common to not only two species of urochordates but also to the dipteran genus *Drosophila*, indicates that the gene coding for muscle actin must be highly conserved and so is likely subject to a great deal of evolutionary constraint.

The antisense probe was also useful in detecting the muscle actin transcript in 8-cell cleavage-arrested embryos (Fig. VIII). It can be seen that the transcript is only present in the two B4.1 blastomeres of control

embryos which correspond to the known muscle lineage progenitors in ascidian embryos (Nishida, 1987). However, in compressed embryos the actin transcript was detected in four of the eight blastomeres which correspond to the cells which contain myoplasm. This result suggests that the presence of myoplasm is sufficient to initiate the expression of a muscle-specific gene in a non-muscle lineage. It is unlikely that this mRNA is the determinant itself as embryos which were compressed and immediately prepared for *in situ* hybridization rather than being allowed to "develop" in cytochalasin did not express the transcript in any of the eight blastomeres (data not shown). This is in agreement with previous work by Jeffery (1989) in which he determined that at least four rounds of cell division are required for readily detectable levels of muscle actin mRNA expression. A "clock" mechanism based on the requirement for a minimum number of rounds of DNA replication prior to the expression of some tissue-specific markers has been suggested as a possible mechanism regulating the temporal aspects of tissue differentiation (Satoh and Ikegami, 1981). The "clock" regulating the muscle actin genes may require four rounds of nuclear division prior to their expression. Because cytochalasin prevents cellular but not nuclear division, this intrinsic clock based on rounds of DNA synthesis is allowed to operate and so muscle actin is expressed under conditions of cleavage-arrest. Transcription of genes under the control of such a mechanism should not occur prior to the minimum number of nuclear divisions required. This appears to be the case for muscle actin mRNA as indicated by the absence of the transcript in compressed embryos which were prevented from carrying out a fourth round of nuclear division.

4.2.2 Expression of Actin and Myosin Heavy Chain

An antibody specific to myosin heavy chain was developed using a larval homogenate of the ascidian *Halocynthia roretzi* as the antigen (Mita-Miyazawa et al, 1987). The resulting monoclonal antibody (Mu-2) recognizes an antigen that is restricted to the tail muscle cells of ascidian tadpoles and has been identified as myosin heavy chain. A monoclonal antibody which is specific for the sarcomeric actin of chicken muscle cells (Sr-1) is commercially available. These antibodies were used in this study to determine if the presence of myoplasm is sufficient to promote the expression of the muscle-specific proteins actin and myosin in non-muscle cells.

Figure IX indicates that both of these antibodies recognize their respective antigens in *B. villosa*. The expression of both actin and myosin is restricted to the two B4.1 muscle progenitors in control 8-cell embryos while in embryos subject to compression, these proteins are expressed in four of the eight blastomeres. This suggests that despite being directed to enter an epidermal lineage in normal embryos (Nishida, 1987), the fate of the b4.2 blastomeres can be respecified by the presence of ectopic myoplasm. The role of myoplasm in establishing the majority of the muscle lineage in ascidian embryos is supported by studies in which the B4.1 blastomeres are removed from 8-cell embryos and cultured in isolation. The resulting cell mass will express myosin (Nishikata et al, 1987b) which suggests that the myoplasm contains determinants which specify the muscle lineage autonomously and that no inductive interactions are required. It is

unlikely that the myoplasmic determinant is the myosin transcript itself as isolated B4.1 blastomeres exposed to transcriptional inhibitors are deficient in their expression of myosin (Nishikata et al, 1987b) which suggests that muscle cells must transcribe myosin mRNA after determination has already occurred. *De novo* transcription of myosin mRNA and not maternal storage of this transcript is further supported by the fact that the treatment of embryos with inhibitors of DNA replication up to the 32-cell stage will prevent the expression of myosin (Nishikata et al, 1987b). This suggests that a clock mechanism based on rounds of DNA synthesis may regulate the timing of myosin expression in a similar manner as that already discussed for actin. The observation that myosin expression is not initiated until at least five rounds of DNA synthesis have occurred (32-cell stage) and the future muscle lineage has already been established by the 8-cell stage (Nishida, 1987) indicates that myosin is not involved in muscle determination but rather in differentiation.

The fact that Mu-2 cross reacts with *H. roretzi* (Nishikata et al, 1988), *B. villosa* (this study) and *Ciona intestinalis* (Nishikata, unpublished) suggests that at least some epitope of myosin is highly conserved across a wide range of urochordate species. Similarly, the fact that Sr-1 recognizes muscle actin from both chickens and ascidians further substantiates the degree of conservation of the gene coding for this protein.

4.2.3 Muscle-Specific Ultrastructure

Previous studies have shown that cleavage-arrested ascidian embryos have the ability to express muscle-specific ultrastructure in the form of

myofilaments (Crowther and Whittaker, 1983,1984) which are present only in cells of the presumptive muscle lineage. The myofilaments in cleavage-arrested embryos are as well developed as those of controls in that the accessory protein tropomyosin is incorporated into these filaments as in normal embryos (Crowther et al, 1990). Partial ascidian embryos consisting of muscle progenitor blastomeres, the B4.1 pair, isolated from 8-cell embryos will also develop these myofilaments if cultured in isolation (Crowther and Whittaker, 1983, 1984). The fact that muscle-specific features can develop without the normal cellular interactions which occur in control situations indicates the developmental autonomy of the muscle lineage and so suggests that the determination of this tissue may be regulated by cytoplasmic determinants. The results of the current study are in agreement with these findings as 8-cell cleavage-arrested embryos were observed to develop fine myofilaments (see Fig. X). In control 8-cell embryos, the myofilaments are restricted to the two B4.1 muscle progenitor blastomeres while compressed embryos develop these filaments in four of the eight blastomeres. Because these four blastomeres contain myoplasm, the expression of myofilaments suggests that myoplasm contains a factor(s) responsible for the determination of muscle cell fate.

Myofilaments were observed in all blastomeres containing myoplasm in each of the embryos examined except in two compressed embryos (Table II). These two embryos did not develop myofilaments in either the ectopic blastomeres or the normal B4.1 blastomeres. It is likely that these embryos were aberrant due to a defect in the egg or sperm. Each fertilization results in a number of embryos which reach varying stages of development before arresting completely or developing abnormally. The two embryos which

did not develop myofilaments are likely examples of these abnormal embryos. Although the presence of the myofilaments appears to be normal both spatially and temporally despite cleavage-arrest of the embryo, the organization of the fibers never equals that observed in control tadpoles as intact sarcomeres are never observed (Crowther and Whittaker, 1983,1984, and this study). These results indicate that although the establishment of cell fate has occurred due to the presence of myoplasmic determinants, some component of the differentiation process is being altered by cleavage-arrest. Perhaps the latter stages of muscle differentiation require cellular inductions which are eliminated by cleavage-arresting the embryo. Another possibility is that the interaction between various cytoplasmic domains in the 8-cell embryo are preventing the normal expression of the muscle-specific genetic program. If normal cell division is allowed to occur, these cytoplasmic domains are segregated into specific cell lineages and so are effectively isolated from one another. Cleavage-arrest prevents "isolation by cleavage" of these domains and so a number of different determinants may be present in a single blastomere which could disrupt the subsequent differentiation of that cell. This hypothesis will be discussed below in the context of recent results by Bates and Swallow (unpublished).

An important point that must be addressed in this study is that ectopic muscle features were observed in the b4.2 blastomeres presumably as a result of the introduction of myoplasm into these cells, and so the determinants localized within this cytoplasmic domain. However, cell lineage analysis has shown that some of the cells derived from the b4.2 blastomeres are responsible for the formation of the muscle cells at the

caudal tip of the tail, the so called secondary muscle lineage (Nishida, 1987). Thus the question may be asked, is the expression of muscle features in the b4.2 blastomeres a result of the presence of ectopic myoplasm or merely the development of a cell fate inherent in this lineage? The former situation seems to be the case as a number of studies have shown that development of muscle from the b4.2 lineage is not regulated by cytoplasmic determinants but rather requires inductive interactions from neighbouring cells in the developing larva. Muscle actin mRNA has been detected in the B4.1 blastomeres, the primary muscle lineage progenitors, of cleavage-arrested 8-cell embryos but not in the b4.2 blastomeres at this stage (Jeffery, 1989, this study). Similarly, the expression of this transcript is restricted to the descendants of the B4.1 blastomeres of 64-cell cleavage-arrested embryos (Jeffery, 1989) and is present in this lineage as early as gastrulation in control embryos while the secondary muscle lineage does not accumulate the transcript in the b4.2 lineage until a few hours later in development at the mid tailbud stage (Tomlinson et al, 1987b). Finally, b4.2 blastomeres removed from 8-cell embryos and cultured in isolation will not develop muscle-specific AchE while isolated B4.1 blastomeres will (Nishida, 1990). These results suggest that the b4.2 blastomeres do not contain localized information which directs the autonomous development of the secondary muscle lineage but rather the determination of this lineage requires an inductive interaction with the multicellular environment which is established as normal development proceeds. Thus it can be stated that the expression of muscle features in the b4.2 blastomeres of compressed 8-cell embryos is due to the presence of ectopic myoplasm.

The results of this and other studies suggest that the presence of

myoplasm is sufficient to promote the expression of muscle-specific features. Unfortunately, the molecular nature of the factor(s) localized in the myoplasm responsible for the establishment of cell fate remains a mystery, ie. the actual myoplasmic determinant has yet to be identified. The definitive experiment to prove the existence of a cytoplasmic determinant would be the respecification of cell fate in a non-muscle lineage after the introduction of a molecule isolated from the myoplasm. The problem is unlikely to be that simple in that the determinant may not be a single entity but rather a class of molecules which must act in a coordinate manner to establish cell fate. Furthermore, it is difficult to isolate quantities of a localized cytoplasmic domain sufficient to perform a biochemical analysis of its molecular constituents. The techniques for isolating these domains must take into account the specific macromolecule(s) being sought as procedures for biochemical separation often isolate one molecule at the expense of many others. Because the molecular identity of the determinants are not known, the techniques used for the isolation cannot be tailored to suit their molecular nature. Jeffery (1985) devised a technique for the mass isolation of myoplasmic crescents which were subsequently assayed for their protein and mRNA content. A subset of total egg proteins was found to be restricted to the myoplasmic crescent but as of yet they have not been isolated and identified. There were no differences in the mRNA species found in the crescent relative to the total egg mRNA but it is possible that less prevalent mRNA were below detectable levels or were lost during the isolation. These experiments have not been followed up as a more promising avenue for the study of cytoplasmic determinants has been undertaken by Jeffery and others.

A recent trend in the study of cytoplasmic determinants involves the comparative analysis of the development of ascidians which undertake different life strategies. Most ascidians exhibit urodele development in that they form a transient larval tadpole stage prior to metamorphosis into the adult. A few species have anural development in which the tadpole stage has been modified or eliminated. In the anural species, the larva often does not develop a tail and so there is never expression of larval muscle. It is possible that an evolutionary modification involving the elimination of myoplasmic determinants has occurred in anural ascidians (Jeffery and Swalla, 1990). A comparative analysis of the molecular contents of the eggs of closely related anural and urodele ascidians may lead to the identification of a myoplasmic determinant. One such study has found that a cytoskeletal protein, p58, is present in the myoplasm of the urodele ascidian *Molgula oculata* and is absent in the con-specific anural species *M. occulta* (Swalla et al, 1991). Continued analysis of this system may lead to the molecular identification of a cytoplasmic determinant.

4.3 Interaction Between Localized Cytoplasmic Determinants

As previously discussed, a number of experimental studies such as blastomere ablation (Chabry, 1887), cleavage-arrest (Whittaker, 1973), cytoplasmic deletion (Bates and Jeffery, 1987a) and cytoplasmic redistribution (Whittaker, 1980) suggest that localized cytoplasmic determinants may be involved in specifying various cell fates in ascidian embryos, yet little is known about how these localized determinants may interact with one another. Some experimental evidence suggests that certain

combinations of cytoplasmic determinants may act in an antagonistic fashion with respect to the establishment of cell fate.

The polychaete worm *Sabellaria cementarium* develops polar lobes, transient cytoplasmic protrusions thought to contribute to the segregation of cytoplasmic information, during early cleavage of the embryo (Render, 1983). If blastomeres of the 4-cell embryos of this organism are isolated and cultured, only the "C" blastomere will develop apical tuft structures while the "D" blastomere, the major recipient of polar lobe cytoplasm, will not. If the lobe is removed prior to shunting its contents into the D blastomere, this cell will develop apical tuft structures indicating that the D blastomere contains the developmental potential to form apical tuft and that this potential is inhibited by the presence of polar lobe cytoplasm. Furthermore, if the lobe material is equally distributed to both the C and D blastomeres, both will develop apical tuft structures (Render, 1983) suggesting that a threshold level of material in the lobe cytoplasm is required to inhibit the formation of apical tuft. Similar conclusions can be drawn from studies in which egg fragments of the ctenophore *Mnemiopsis leidyi* were created by centrifugation (Freeman, 1976). If normal 4-cell embryos are cleavage-arrested and allowed to develop, only one blastomere will express comb plate structures. If the basophilic merogon created by centrifugation is cleavage-arrested at the same stage, all four blastomeres will produce a comb plate. If a small amount of cytoplasm from the other egg fragment is present in the basophilic fragment, only one of the four blastomeres will develop a comb plate as in the controls. This suggests that the cytoplasm in the less dense egg fragment contains material which can restrict the developmental potential of cell lineages derived from the

cytoplasm in other regions of the egg.

Recent studies by Bates and Swallow (unpublished) suggest that antagonism between factors localized in specific cytoplasmic domains may interact in an antagonistic manner in ascidians. Two methods were utilized to redistribute localized regions of cytoplasm into ectopic lineages of *B. villosa* embryos. Microsurgery of fertilized eggs was used to create egg fragments that were enriched in myoplasm (Bates, 1988) and microcompression was applied to 8-cell embryos to redistribute the myoplasm into additional blastomeres (this study). Larvae that developed from myoplasm enriched (ME) egg fragments were observed to contain myoplasm in the tail and head regions as opposed to restriction of this cytoplasmic domain to the tail of control tadpoles. The compressed embryos were found to contain myoplasm in four blastomeres rather than the normal two blastomeres of control 8-cell embryos. The ME larvae expressed muscle features, including actin mRNA, myosin and myofibrils, in only the tail region despite the presence of myoplasm throughout the larvae while compressed 8-cell embryos express these features in ectopic lineages as already discussed. Although both experimental situations were found to cause a redistribution of myoplasm, they differed in how relative amounts of other localized cytoplasmic regions, specifically the ectoplasm, were distributed in the resulting embryo. The ectoplasm is released from the ruptured germinal vesicle after fertilization and may contain ectoderm-specific determinants as this domain is segregated into blastomeres destined to enter ectodermal lineages such as the epidermis (Nishida, 1987). The difference in relative amounts of ectoplasm between ME and compressed embryos could explain the absence of ectopic muscle features in ME larvae

if antagonism between determinants in the myoplasm and ectoplasm is considered. The ME larvae develop from egg fragments consisting of 40-50% of the total egg volume that are produced such that the cytoplasm of these merogons is almost entirely made up of myoplasm and ectoplasm. This effectively doubles the relative amounts of these two cytoplasmic domains as the total egg volume is reduced by half. The higher than normal proportion of ectoplasm in ME embryos may prevent the expression of ectopic muscle features. In compressed embryos, the distribution and not the relative proportions of these cytoplasmic regions are altered and so a quantitatively dependant antagonism between the myoplasm and the ectoplasm does not exist. ME fragments can be produced such that they consist solely of myoplasm and so lack or at least have greatly reduced levels of ectoplasm. These smaller merogons represent 30-40% of the total egg volume and lack the necessary information to develop into larvae (Bates, 1988). However, if these merogons are cleavage-arrested at the 8-cell stage and allowed to "develop", muscle AchE will be expressed in more than the expected two cells due to the presence of myoplasm in most of the eight blastomeres present (Bates, 1988). Ectopic expression of muscle characteristics due to the redistribution of myoplasm in embryos with levels of ectoplasm which are reduced (small ME) or normal (compressed embryos) and a lack of ectopic expression in embryos with increased levels of ectoplasm (large ME) despite the presence of myoplasm in non-muscle cells suggests that material in the ectoplasm and myoplasm are interacting in an antagonistic manner. A threshold level of ectoplasmic factors appears to be required for the suppression of the activity of myoplasmic determinants similar to the situation discussed for the polar

lobe of *S. cementarium*.. This threshold was obtained in the creation of large ME fragments and so the resulting larvae did not express muscle features in inappropriate lineages despite the presence of ectopic myoplasm. Small ME and compressed embryos contain ectoplasm at levels below this threshold and so ectopic myoplasmic factors are free to promote the expression of muscle features.

Although the temporal and spatial expression of muscle features appears to be normal in the normal and ectopic muscle lineages of compressed embryos, the development of myofibrils never reaches the same degree of organization observed in control tadpoles (discussed above). Although determination of the muscle lineage appears to occur in cells containing myoplasm, perhaps the presence of ectoplasm or some other cytoplasmic domain is inhibiting the normal stages of muscle differentiation in these cells.

Antagonistic interactions between the material localized in various cytoplasmic domains could explain the theory of exclusivity of tissue differentiation proposed by Nishikata et al (1988). This theory suggests that the expression of one genetic program somehow excludes the expression of another. Cleavage-arrested early stage embryos of the ascidian *Halocynthia roretzi* were assayed for the expression of muscle and epidermal features in the same blastomeres. The expression of these features was found to be exclusive in that both tissue types were never expressed in the same blastomere despite the potential to give rise to every type of cell at early stages of development (Nishikata et al, 1988). Similar results were obtained with embryos of the nematode *C. elegans* in which the development of intestine, muscle and hypodermis were found to be exclusive (Cowan and

McIntosh, 1985). The molecular mechanisms of this exclusion are not presently known but could be explained by a suppressive force exerted by one cytoplasmic determinant on another. Ascidians of the genus *Ciona* appear to be an exception to this exclusivity hypothesis as *C. savignyi* (Marikawa and Satoh, 1991) and *C. intestinalis* (Crowther and Whittaker, 1984, Whittaker and Meedel, 1989) both exhibit the expression of multiple tissue-specific markers in egg fragments and cleavage-arrested single-cell zygotes respectively. Nishikata et al (1988) suggests that this could be due to species differences but does not expand on this idea. Explained in the context of antagonism between localized cytoplasmic factors, threshold levels of determinants required for this exclusion may be species specific and the levels present in the eggs of *Ciona* may be below the exclusion threshold for this genus.

4.4 Temporal Aspects of Muscle Differentiation

The structure of the larval tail in ascidians is relatively simple in that it consists of a notochord which is surrounded laterally by two bands of striated muscle, dorsally by a nerve cord and ventrally by the endodermal strand which is an extension of the gut rudiment. The entire structure is surrounded by an epithelial layer (Cavey and Cloney, 1972). The nerve cord exhibits neuromuscular junctions that presumably regulate the alternating contraction of the two lateral muscle bands resulting in the flexation of the tail that allows the tadpole to swim. (Cavey and Cloney, 1972). Ascidian muscle is unique in its similarity to vertebrate cardiac muscle morphology as both muscle types remain unicellular and do not

fuse to form multinucleated myotubes. However the muscle of the ascidian larva resembles vertebrate skeletal muscle in function as it utilizes myofibrils to effect voluntary muscle contractions (Cavey and Cloney, 1972, Terakado, 1975).

This study involved an investigation of the temporal aspects of muscle differentiation in the ascidian larva and focused on the time of appearance of actin and myosin with their subsequent polymerization into thin and thick filaments respectively. The rapid development of ascidian larvae is reflected by an equally rapid differentiation of the tail musculature as less than five hours are required for the synthesis of all the required macromolecules and their assembly into a functioning contractile apparatus. Figures XI to XIV indicate the order of appearance and assembly of actin and myosin from 12 hrs post-insemination to the time the larva hatches. At 12 hrs of development, it can be seen that actin is present in small quantities in the tail muscle cells but myosin has not yet been synthesized (Fig. XI). Although the actin monomer (G-actin) is present, this protein is not assembled into F-actin in the form of thin filaments until 14.5 hrs of development (Fig XIII). Thus it appears that the actin monomer is accumulated prior to its polymerization into filaments. Because actin is the major protein component of ascidian muscle (Beach and Jeffery, 1992) it is possible that a large pool of G-actin must be accumulated to some threshold level prior to its polymerization into filaments. Once the assembly of these filaments has been initiated, a rapid increase in their density is observed as progressively more fibers are established at the periphery of the cell. It is interesting to note that prior to assembly, the actin monomers are dispersed throughout the cytoplasm of the cell and are

then presumably transported to the cell periphery where they are assembled into filaments. It seems likely that the filaments are assembled and then transported to the cell periphery rather than being assembled prior to their peripheral localization because the filaments were only observed adjacent to the sarcolemma and never throughout the cytoplasm. A similar situation was observed in a study of the ascidian *Perophora orientalis* (Terakado, 1972). Myosin exhibits a similar pattern of expression in that the monomer first appears throughout the cytoplasm at 12.5 hrs of development and is not assembled into thick filaments until 16.5 hrs post-fertilization (Fig. XIV). However, myosin appears to accumulate to reasonably high levels immediately while the accumulation of actin appears to be more gradual. It is interesting to note that the thick filaments appear to be assembled immediately into intact sarcomeres as the presence of free thick filaments was never observed. Perhaps the actin filaments are assembled first and act as a template for the concurrent polymerization of myosin filaments and their assembly into sarcomeres although there is no evidence to support this hypothesis.

The actin filaments consist of two strings of actin intertwined to form a thin helical filament to which the associated proteins tropomyosin and troponin will adhere (Raven and Johnson, 1989, Epstein and Fischman, 1991). Myosin filaments are more complex in that they consist of strands of two heavy and four light chains of myosin which appear as short rods with a globular head and long rods respectively (Epstein and Fischman, 1991). Numerous myosin monomers will adhere to form a thick filament but may require the presence of actin filaments to mediate their assembly. The association of actin and myosin filaments results in the creation of a

myofibril, the basic unit of muscle contraction. Many of these myofibrils are then assembled into sarcomeres which give the cells their striated appearance (Epstein and Fischman, 1991). The newly formed sarcomeres of *B. villosa* are functional immediately because visible movements of the tail were observed at the time of appearance of these supramolecular structures.

Because the differentiation of ascidian muscle requires the rapid synthesis and assembly of a variety of proteins, complex spatial and temporal controls must exist. The requirement for high molar ratios of actin to myosin in myofilaments (Beach and Jeffery, 1992) and the extremely rapid differentiation of muscle in ascidians would necessitate either highly active promoters of actin genes or multiple copies of these genes which are expressed simultaneously. The latter situation appears to be the case in ascidians in that the genomes of both *Styela clava* (Beach and Jeffery, 1990) and *S. plicata* (Beach and Jeffery, 1992) appear to contain multiple actin genes which code for the same isoform of muscle actin. The expression of these genes appears to be under dual control as both maternally stored transcripts and zygotically transcribed mRNAs have been detected. Extremely low levels of muscle actin mRNA have been detected in unfertilized eggs (Jeffery, 1989) and a large burst of zygotic transcription occurs just after gastrulation (Tomlinson et al, 1987a). As to whether or not the maternally derived transcripts are translated into protein remains unclear but this mRNA can be translated *in vitro* (Tomlinson et al, 1987a). Thus the regulation of expression of muscle-specific proteins during ascidian development appears to be quite complex. Actin is synthesized prior to the appearance of myosin and so these genes

are not necessarily expressed in a coordinate manner under the control of a single promoter despite the interdependent relationship of the protein products.

The appearance of actin prior to myosin in differentiating muscle cells does not seem to be unique to ascidians. In chick embryos, thin actin filaments appear prior to thick myosin filaments (Obinata et al, 1966, Lawrence et al, 1989). The pattern of expression of the actin and myosin genes also appear to be different from one another. Actin appears to accumulate gradually while myosin is absent until a sudden burst of transcription and/or translation occurs and the protein is immediately present at high levels (Lawrence et al, 1989). The current study seems to indicate that this situation also exists in ascidians as actin is initially faintly detectable and then accumulates while myosin is absent and then suddenly appears at substantial levels. Mouse embryos seem to exhibit a similar order of appearance of muscle proteins in that actin mRNA appears before myosin (Lyons, 1992) as do the proteins (Cox and Buckingham, 1992).

The relative timing of the first appearance of actin and myosin appears to reflect the overall time required for muscle differentiation in the embryo. Muscle in the somites of fetal mice takes about 15 days to fully develop (Lyon, 1992) and actin expression proceeds that of myosin by 24 hrs. Compare this to the situation in ascidians, where muscle differentiation only requires about five hours and actin is expressed a mere 30 min prior to myosin. Amphibians seem to be an exception in that both the frog *Rana pipiens* (Huang, 1967) and the salamander (Hay, 1965) assemble thin actin and thick myosin filaments simultaneously. It has not been determined if the synthesis of the constituent monomers follows the same pattern.

5. SUMMARY AND CONCLUSIONS

The development of ascidian embryos is thought to be mediated via the presence of cytoplasmic determinants localized to specific regions of the egg and subsequently segregated into specific cell lineages during early cleavage. This study has examined the role of the myoplasm in the determination of the muscle cell lineage in *Boltenia villosa* embryos by redistributing this cytoplasmic domain into a non-muscle lineage by compression of the embryo during third cleavage. The 8-cell compressed embryos developed muscle-specific features in four out of the eight blastomeres as opposed to two cells in control 8-cell embryos. The ectopic expression of these features, which include the expression of muscle actin mRNA, myosin heavy chain, sarcomeric actin and myofibrils, is due to the presence of myoplasm in an additional two blastomeres. Thus it appears that factor(s) localized in the myoplasm of ascidian eggs play a role in the determination of the primary muscle cell lineage. The molecular nature of these factors has yet to be established but the comparative analysis of the macromolecular components of eggs of anural and urodele species is a promising avenue for identification of a cytoplasmic determinant.

An investigation of the temporal aspects of muscle differentiation in the ascidian tadpole larva suggests that actin and myosin are not necessarily expressed in a coordinate manner as actin is synthesized prior to myosin. Furthermore, G-actin is not polymerized immediately into thin filaments as the monomer appears to be present prior to being assembled in parallel arrays at the cell periphery. Myosin appears to be expressed in a similar pattern except that it is polymerized into thick filaments which are

immediately assembled into functional sarcomeres. The rapid differentiation of muscle cells in the ascidian larva is reflected in the short time required for the synthesis and assembly of the macromolecules constituting a sarcomere.

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