Development 101, 751–765 (1987)
Printed in Great Britain © The Company of Biologists Limited 1987

Differential expression of a muscle actin gene in muscle cell lineages of ascidian embryos

CRAIG R. TOMLINSON*, REBECCA L. BEACH and WILLIAM R. JEFFERY

Center for Developmental Biology, Department of Zoology, University of Texas, Austin, Texas 78712, USA

* Present address: Department of Biochemistry and Molecular Biology, University of Texas System Cancer Center, M. D. Anderson Hospital, Houston, Texas 77030, USA

Summary

Specific probes were used to examine the accumulation of muscle actin mRNA during embryonic development of the ascidian *Styela*. Clones of a muscle actin gene were obtained from an adult mantle cDNA library. Four lines of evidence indicate that these clones correspond to a muscle actin gene. First, their coding regions share 11 of 14 diagnostic amino acid positions with mammalian smooth and skeletal muscle actins. Second, subclones that contain only the 3′ noncoding region of the gene select mRNA coding for muscle actin, while subclones that include the coding region of the gene select mRNA coding for muscle and nonmuscle actins. Third, a probe that contains only the 3′ noncoding region detects a single band, corresponding to a 2 kb transcript, while a probe that includes the coding region detects the 2 kb transcript and at least one other band, presumably a cytoplasmic actin transcript. Fourth, the 3′ noncoding region probe detects transcripts only in muscle cells and their precursors, while the coding region probe detects transcripts in muscle and nonmuscle cells. The muscle actin transcript is present at very low levels in eggs and early embryos, begins to accumulate between the early gastrula and tailbud stages, and by the tadpole stage attains a level about 25-fold higher than in the egg. *In situ* hybridization showed that embryonic muscle actin transcripts are restricted to the muscle cell lineages. These transcripts were initially observed in primary muscle lineage cells (descendants of the B4.1 blastomeres) at the early gastrula stage and continued to be present in these cells throughout embryonic development. In contrast, muscle actin transcripts did not appear in secondary muscle lineage cells (descendants of b4.2 and A4.1 blastomeres) until the mid-tailbud stage, and were not detected in mesenchyme cells, the presumptive adult muscle cell precursors, at any time during embryonic development. The results suggest that muscle actin gene expression is subject to spatial and temporal regulation in the muscle cell lineages.

Key words: differential gene expression, muscle actin mRNA accumulation, muscle cell lineages, ascidian development.

Introduction

Muscle cell development in ascidian embryos is a classic example of the specification of cell fate by factors localized in the egg cytoplasm (reviewed by Whittaker, 1979; Jeffery, 1985; Uzman & Jeffery, 1986). About 40 muscle cells are present in the larval tail of oviparous ascidian species (Katz, 1983). Most of these cells originate from two vegetal-posterior (B4.1) blastomeres of the 8-cell embryo. As first recognized in the ascidian *Styela* (Conklin, 1905), the B4.1 blastomeres obtain most of the yellow cytoplasm, a localized cytoskeletal domain in the egg (Jeffery & Meier, 1983). The yellow cytoplasm initially resides in the periphery of the unfertilized egg, but it undergoes a series of dramatic rearrangements soon after fertilization, resulting in the formation of a yellow crescent in the vegetal-posterior region of the uncleaved zygote (reviewed by Jeffery, 1984). Using the pigmented cytoplasm as a marker, Conklin (1905) constructed a fate map of the *Styela* embryo in which the yellow crescent was shown to be distributed

primarily to two cells of the 2-, 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. After a few more divisions, the descendants of these eight cells differentiate into larval tail muscle.

Conklin's original observations relied on the presence of yellow pigmentation for distinguishing muscle cells and their progenitors in the embryo (Conklin, 1905). More recently, other tissue-specific components, including acetylcholinesterase (AchE) (Durante, 1956; Whittaker, 1973), AchE mRNA (Perry & Melton, 1983; Meedel & Whittaker, 1984), muscle actin (Tomlinson, Bates & Jeffery, 1987), myosin (Meedel, 1983; Nishikata, Mita-Miyazawa, Deno & Satoh, 1987) and myofibrils (Crowther & Whittaker, 1983), have been used as markers for ascidian muscle cells. Using some of these markers, it has been established that isolated B4.1 lineage blastomeres can differentiate into muscle cells autonomously (Whittaker, Ortolani & Garinella-Ferruzza, 1977; Crowther & Whittaker, 1984, 1985; Deno, Nishida & Satoh, 1984; Nishikata *et al.* 1987). Furthermore, when cytoplasm from B4.1 blastomeres is redistributed to other blastomeres during third cleavage, some of the recipient cells subsequently develop AchE (Whittaker, 1980, 1982). These results suggest that muscle cell development is controlled by cytoplasmic factors segregated into the B4.1 blastomeres with the yellow cytoplasm. Morgan (1934) first suggested that localized egg cytoplasmic factors may function by regulating differential gene expression in cell lineages.

Recently, the idea that ascidian muscle cells are derived entirely from the B4.1 blastomeres has been revised according to the results of new fate maps made by microinjecting horseradish peroxidase into blastomeres of 8-, 16- and 32-cell embryos (Nishida & Satoh, 1983, 1985). The recent fate maps indicate that muscle cells have multiple sources in ascidian embryos. Although the muscle cells in the anterior and middle portions of the larval tail are descended from B4.1 blastomeres, those located in the posterior portion of the tail arise from descendants of the vegetal-anterior (A4.1) and animal-posterior (b4.2) blastomeres of the 8-cell embryo. In isolation, descendants of A4.1 and b4.2 cells express muscle cell markers at relatively low levels (b4.2 cells) or not at all (A4.1 cells) (Deno *et al.* 1984, 1985; Nishikata *et al.* 1987; Meedel, Crowther & Whittaker, 1987). Since muscle cell development in descendants of A4.1 and b4.2 blastomeres does not appear to be as autonomous as in B4.1 lineage cells, it has been suggested that they are specified by different mechanisms (Meedel *et al.* 1987). When considering the embryonic origin of muscle cells in ascidians, it is also relevant that the larval tail is resorbed during metamorphosis (reviewed by Berrill, 1947); adult muscle is thought to be derived from mesenchyme cells located in the head of the tadpole (Katz, 1983). The mesenchyme cells are also descendants of B4.1 blastomeres.

The multiple origins of ascidian muscle cells require a reassessment of the role of egg cytoplasmic factors in specifying muscle cell development. Since these factors may act by controlling gene expression, our approach has been to isolate muscle-specific actin probes to examine the temporal and spatial accumulation of muscle actin transcripts. In this investigation, we show that muscle actin mRNA accumulates differentially in muscle cell lineages of the *Styela* embryo.

Materials and methods

Biological materials

Styela plicata was obtained from Pacific Biomarine Inc. (Venice, CA) or Marinus Inc. (Long Beach, CA). Some experiments were also conducted with *Styela clava* (collected at Woods Hole, MA), a species from which large numbers of synchronously developing embryos can be obtained. Animals were maintained in tanks containing Instant Ocean artificial sea water or running sea water at 13–15°C. Gametes and embryos were handled and cultured in Millipore-filtered sea water (MFSW) as described previously (Jeffery, Tomlinson & Brodeur, 1983). Gastrulation was initiated at 3·5 h and neurulae were present by 5 h after fertilization in embryos cultured at 17°C. The early, mid-, and late tailbud stages were defined as periods from 5–7 h, 7–10 h, and 10–14 h postfertilization, respectively. Larval hatching occurred at approximately 14 h after fertilization.

The plasmid pBR322 was used for cloning the cDNA library. *Escherichia coli* strain RR1 was used as the host for transformation of the cDNA library. A chimaeric plasmid, consisting of 1·6 and 1·8 kb *Hin*dIII fragments of a *Drosophila melanogaster* actin gene (DmA2) inserted into the *Hin*dIII site of pBR322 (Fyrberg, Kindle, Davidson & Sodja, 1980), was used for the initial screening of the mantle cDNA library. *E. coli* HB 101 transformants (Cohen, Chang & Hsu, 1972) were selected for ampicillin resistance and tetracycline sensitivity. Plasmid amplification was achieved with 170 $\mu g\,ml^{-1}$ chloramphenicol. Subcloning was performed in the DNA vector M13 mp8. *E. coli* strain JM103 was transfected by DNA subcloned into M13. The plasmid Bluescribe (Stratagene, San Diego, CA) was used for DNA sequencing and synthesizing RNA probes for various actin cDNA subclones. Other plasmids grown in *E. coli* were selected by tetracycline or ampicillin resistance and amplified as described above.

Isolation of DNA

Plasmid DNA was isolated by the cleared lysate procedure (Clewell & Helsinki, 1970) and purified by banding to equilibrium in CsCl. The ethidium bromide and CsCl were removed (Davis *et al.* 1980), and DNA was precipitated with ethanol and stored as described above.

Isolation of mantle poly(A)$^+$ RNA

The mantle (body wall) of adult animals, an organ composed primarily of muscle, was surgically removed, dissected clean of other tissues and minced into fine pieces. The mantle pieces were homogenized in 10 vol. of homogenization buffer (6 M-guanidine chloride, 0·02 M-sodium acetate, and 0·001 M-dithiothreitol) by 20 strokes of a motor-driven Teflon pestle in a Dounce homogenizer and total RNA was extracted by the guanidine–HCl method (Deeley *et al.* 1977). The ethanol-precipitated RNA was centrifuged at 12000 *g* for 20 min (−10°C), washed twice with 70 % ethanol, dried under vacuum and resuspended in H_2O. Poly(A)$^+$ RNA was isolated by oligo (dT)-cellulose chromatography as described previously (Tomlinson *et al.* 1987). RNA samples dissolved in a small volume of H_2O were stored in liquid N_2.

Preparation and screening of the mantle cDNA library

Mantle poly(A)$^+$ RNA was used to synthesize cDNA in a reaction mixture containing AMV-reverse transcriptase (J. Beard, Life Sciences Inc., St Petersburg, FL) and an oligo (dT) primer (Collaborative Research Inc., Lexington, MA). The cDNA was treated with alkali to hydrolyse the RNA template, neutralized and the 3′ termini of the cDNAs were tailed with dCTP using terminal transferase (Land *et al.* 1981). The second strand was synthesized with AMV-reverse transcriptase and the large fragment of *E. coli* DNA polymerase I using an oligo (dG) primer. The double-stranded cDNA was tailed at its 3′ termini with dCTP using terminal transferase and inserted into the *Pst*I site of oligo (dG)-tailed pBR322 (Roychoudhury & Wu, 1980). *E. coli* RR1 transformants were selected for tetracycline resistance and ampicillin sensitivity. The resultant mantle cDNA library was screened as described by Maniatis *et al.* (1978) with the 1·8 kb *Hin*dIII fragment of DmA2, nick translated with ^{32}P-dCTP (3000 Ci mmole^{-1}, ICN Radiochemicals, Irvine, CA) (Rigby, Dieckmann, Rhodes & Berg, 1977).

Sequencing of DNA

DNA fragments from selected cDNA clones restricted with *Pst*I and *Hae*III were subcloned into the *Pst*I and *Hin*cII sites of M13mp8, respectively, and transfected into *E. coli* JM103. Actin DNA was also subcloned into the plasmid Bluescribe and used to transform *E. coli* strains JM101 and JM109. The double-stranded Bluescribe actin clones were denatured (Chen & Seeburg, 1985) and hybridized to a 17-mer DNA primer (Stratagene) specific for Bluescribe. Sequences of the M13 and Bluescribe subclones were determined by the dideoxy chain termination procedure (Sanger, Nicklen & Coulson, 1977) as modified by Biggen, Gibson & Hong (1983). The sequencing reactions were loaded on 8 % polyacrylamide gels.

Preparation of RNA probes

Complementary and reverse orientation RNA probes were synthesized from the Bluescribe subclones using T3 or T7 RNA polymerase. Probes made with [^{32}P]CTP (400–800 Ci mmole^{-1}; Amersham, Arlington Heights, IL) were synthesized according to the accompanying protocol (Stratagene), except that the reaction contained 2 mM of each unlabelled nucleotide. Tritium-labelled RNA probes were made using 100 μCi of each nucleotide.

Northern blot hybridization

For Northern analysis, RNA was resolved on 1 % agarose–formaldehyde gels (Maniatis, Fritsch & Sambrook, 1982) and blotted overnight onto Hybond-N (Amersham) using 0·025 M-sodium phosphate (pH 7·0) as the transfer buffer. Filters were prehybridized in 50 % formamide, 4×SET (1×SET is 0·15 M-NaCl, 0·03 M-Tris–HCl, pH 8·0, 0·002 M-EDTA), 1×Denhardt's solution (Denhardt, 1966) and 0·2 % sodium pyrophosphate, 1 % SDS, 100 μg ml^{-1} sheared, heat-denatured salmon sperm DNA at 42°C for 2–4 h. Radioactive probe was added to filters in prehybridization mix and incubated at 42°C overnight. The blots were washed twice in 2×SSC, 0·1 % SDS, 0·2 % sodium pyrophosphate for 15 min at room temperature and twice in 1×SSC, 0·1 % SDS, 0·2 % sodium pyrophosphate for 30 min at 45°C for low stringency. For high stringency, blots were subjected to an additional wash in 0·1×SSC, 0·1 % SDS, 0·2 % sodium pyrophosphate for 30 min at 45°C.

Hybridization selection, in vitro *translation, and two-dimensional gel electrophoresis*

Positive hybridization selection was carried out according to the method of Durica, Schloss & Crain (1980). 10 μg of cloned DNA was spotted onto nitrocellulose filters and incubated at 58°C for 1–2 h in 50 μl prehybridization buffer (50 % formamide, 0·6 M-NaCl, 0·1 M-Pipes, pH 6·4, 100 μg ml^{-1} poly(A)). The filters were hybridized to approximately 90 μg of total mantle RNA in 50 μl of hybridization buffer (50 % formamide, 0·6 M-NaCl, 0·1 M-Pipes, pH 6·4) at 65°, 60°, 55°, 50° and 45°C for 30 min each. The filters were washed six times in 1 ml 0·2 M-NaCl, 0·1 M-Pipes, pH 6·4, 0·5 % SDS at 60°C, three times in 1 ml 0·2 M-NaCl, 0·1 M-Pipes, pH 6·4 at 60°C, and two times in 1 ml of 0·01 M-Tris–HCl, pH 8·0, 0·002 M-EDTA at 20°C to remove unhybridized RNA. The hybridized RNA was eluted by boiling in 100 μg of H_2O for 1·5 min, ethanol precipitated and translated in an mRNA-dependent wheat germ lysate supplemented with 50 μCi ml^{-1} [^{35}S]methionine (1290 Ci mmole^{-1}; Amersham) (Morrison *et al.* 1979). The labelled protein products were resolved by two-dimensional polyacrylamide gel electrophoresis (Tomlinson *et al.* 1987). The gels were impregnated with fluor (Bonner & Laskey, 1974) and exposed to X-ray film (XRP, Kodak, Rochester, NY) for 2–8 days at −70°C.

In situ *hybridization*

The *in situ* hybridization procedure was modified from those developed by Hafen, Levine, Garber & Gehring (1983) and Cox, De Leon, Angerer & Angerer (1983). Eggs, embryos and adults (stripped of their tunics) were fixed for 20 min in ice-cold 95 % ethanol: acetic acid (3:1). In addition, some embryos were fixed in ice-cold 2 % paraformaldehyde in 0·1 M-phosphate buffer (pH 7·0) for 20 min or ice-cold 5 % formalin–MFSW for 5 min. No qualitative differences were seen in the distribution of signal in embryos fixed by these three methods. The fixed specimens were dehydrated by successive treatments with ice-cold 70 %, 80 %, 90 %, 95 %, and 100 % ethanol (twice)

for 10 min each, treated with ice-cold 100 % ethanol: 100 % toluene (3:1, 1:1, 1:3; 10 min each), cleared in 100 % toluene for 10 min at 20°C, treated successively with 100 % toluene: 100 % paraplast (3:1, 1:1, 1:3; 20 min each) at 58°C, and embedded in 100 % paraplast for at least 48 h at 58°C. The specimens were sectioned at 8 μm and attached to subbed glass slides. Deparaffinized slides were treated with 1 μg ml^{-1} proteinase K in 0·01 M-Tris–HCl, 0·05 M-EDTA (pH 8·0) for 30 min at 37°C, washed in 0·1 M-phosphate buffer (pH 7·2) for 2 min at 20°C, postfixed in 4 % paraformaldehyde in 0·1 M-phosphate for 30 min at 20°C, washed in 0·1 M-phosphate buffer (pH 7·2) for 2 min at 20°C, treated with freshly prepared 0·25 % acetic anhydride, 0·1 M-triethanolamine for 10 min at 20°C, dehydrated in an ethanol series and air dried. About 1×10^6 cts min^{-1} of tritiated RNA probe was applied to each slide in 60 μl of hybridization buffer (2×SSC, 50 % formaldehyde; 1× Denhardt's solution, and 10 % dextran sulphate), and hybridization was carried out for approximately 12 h at 45 °C. The slides were washed in 250 ml of 4×SSC for 30 min at 20°C, treated with 20 μg ml^{-1} pancreatic RNase A in 0·01 M-Tris–HCl, 0·5 M-NaCl (pH 7·0) for 30 min at 37°C, and washed successively in 250 ml of 2×SSC for 30 min at 20°C and 4 l of 1×SSC for 30 min at 45°C for low stringency conditions. For high stringency conditions, slides were subjected to an additional wash in 4 l of 0·1×SSC for 45 min at 45°C. The washed slides were dehydrated with 95 % ethanol, air dried and autoradiographed as described previously (Jeffery *et al.* 1983). The autoradiographs were stained through the emulsion with Harris haemotoxylin–eosin. The *in situ* hybridizations were conducted using complementary and homologous RNA probes. In each experiment, the homologous probes showed no grains above background.

Identification of embryonic cells

Different embryonic cells were identified in whole and sectioned embryos by several means. First, cells were identified according to their position in the embryo relative to that of regional markers, such as the blastopore and archenteron in gastrulae and neurulae and the tail in tailbud stage embryos. The location of various cell types in *Styela* embryos is described by Conklin (1905). Second, various muscle cell lineages were identified by their yellow pigment granules; primary muscle cells have the most pigment granules, secondary muscle lineage cells have intermediate amounts of pigment granules and mesenchyme cells have low levels of pigment granules (Fig. 8). Pigment granules in various embryonic cells were visualized by extraction of embryos with the nonionic detergent Triton X-100 as described by Jeffery & Meier (1983). The extracted embryos were collected by low-speed centrifugation and fixed in 5 % formalin–MFSW prior to photography. The distribution of yellow pigment granules was also examined in serially sectioned embryos. Alternate sections were subjected to *in situ* hybridization or stained with Milligan's trichrome modified for use with ascidian embryos according to Jeffery (in preparation). This procedure stains the pigment granule–mitochondrial complexes red, permitting identification of muscle cell lineages by pigment granule concentration.

Results

Isolation and characterization of muscle actin cDNAs

A portion of the coding region of a *Drosophila* actin gene (Fyrberg *et al.* 1980) was used to screen a cDNA library prepared from *S. plicata* adult mantle poly(A)$^+$ RNA. The sequences of three cDNA clones, SpMA2, SpMA3 and SpMA7, overlapped significantly, suggesting that they are part of the same actin gene (Fig. 1). To identify the actin subtype to which these cDNA clones corresponded, the derived amino acid sequence of part of their coding regions was compared to the amino acid sequences of mammalian cytoplasmic and muscle actins (Vanderkerchove & Weber, 1978, 1979). The results are shown in Table 1. The sequenced portion of the coding region shares 11 of 14 diagnostic amino acid positions with mammalian smooth and skeletal muscle actins, but only two positions with mammalian cytoplasmic actins, suggesting that the clones correspond to a muscle actin gene.

The identity of the actin cDNAs was verified by examining the specificity of subclones containing portions of the coding and 3′ noncoding regions of the gene. Although the coding sequences of different members of the actin gene family are conserved (reviewed by Firtel, 1981), the 5′ and 3′ noncoding sequences usually diverge, and can be used as gene-specific probes (Shani *et al.* 1981). Fig. 1 shows the deduced structure of part of the *S. plicata* muscle actin gene and the subclones used in the experiments described below. The subclone SpMA3C, a 666-base pair *Hae*III restriction fragment of SpMA3, spans a

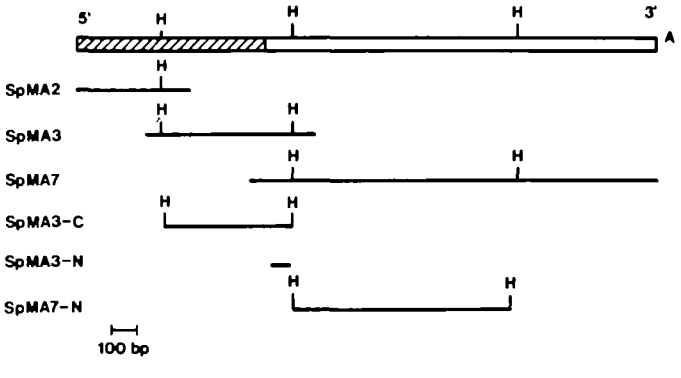

Fig. 1. A diagram illustrating the structure of the partially sequenced *S. plicata* muscle actin gene (top), compiled from the sequences of overlapping cDNAs (SpMA2, SpMA3 and SpMA7), and the positions of various subclones (SpMA3C, SpMA3N and SpMA7N) used as gene-specific probes. The striped box represents approximately 90 % of the coding region, the open box represents the entire 3′ noncoding region, and the solid lines represent cDNA clones and subclones used as probes. H, *Hae*III restriction sites. bp, base pairs. A, poly(A) tail.

Table 1. *Comparison of diagnostic amino acid positions in the coding region of SpMA with mammalian muscle actins*

Amino acid number*	Type†
103 (Val)	Cy
129 (Val)	Mus
153 (Leu)	Mus
162 (Asn)	Mus
176 (Met)	Mus
201 (Val)	Mus
225 (Gln)	Cy
259 (Thr)	Mus
266 (Ile)	Mus
271 (Ser)	N
278 (Tyr)	Mus
286 (Ile)	Mus
296 (Asn)	Mus
357 (Thr)	Mus

* Amino acid positions that differentiate mammalian muscle from cytoplasmic actin (Vanderkerchove & Weber, 1978, 1979).

† The correspondence of SpMA amino acids to mammalian cytoplasmic actin (Cy), muscle actin (Mus), or neither (N)

large portion of the coding region and a small segment of the 3′ noncoding region of the gene. Subclones SpMA3N, a 55-base pair *Hae*III–*Mnl*I, Bal-31 nuclease-truncated fragment from SpMA3, and SpMA7N, a 567-base pair *Hae*III fragment from SpMA7, contain different portions of the long 3′ noncoding region of the gene. The following experiments using these subclones strongly support the sequence data and indicate that the cDNAs correspond to a muscle actin gene.

Initial experiments were conducted in which the subclones were used to select mRNA from total mantle RNA, and this mRNA was subsequently translated in a cell-free translation system. Previous studies indicate that there are three major actin isoforms in *Styela* embryos and adults, two basic cytoplasmic actins and an acidic muscle-specific actin (Tomlinson *et al.* 1987). When the translation products from the hybridization selection experiments were analysed by two-dimensional gel electrophoresis, SpMA3N and SpMA7N selected mRNA coding for a polypeptide which migrated to the same position in gels as muscle-specific actin (see arrows in Fig. 2). In contrast, SpMA3C selected mRNA that coded for polypeptides with electrophoretic positions characteristic of muscle-specific actin and one of the cytoplasmic actins (see inset in Fig. 2A). These results indicate that subclones consisting of the 3′ noncoding region of the gene select only muscle actin mRNA, while the subclone containing the coding region selects both cytoplasmic and muscle actin mRNA.

A second series of experiments was conducted in which RNA transcribed from either SpMA7N or SpMA3C was used to probe Northern blots containing total *S. plicata* mantle RNA. A single band representing a transcript of approximately 2 kb was detected when the blots were probed with SpMA7N RNA (Fig. 3A). The 2 kb band also was observed when the same blot was probed with SpMA3C RNA and washed at high stringency (see Materials and methods for Northern blot washing conditions) (Fig. 3B). In contrast, when the blot was probed with SpMA3C RNA and washed at low stringency, at least

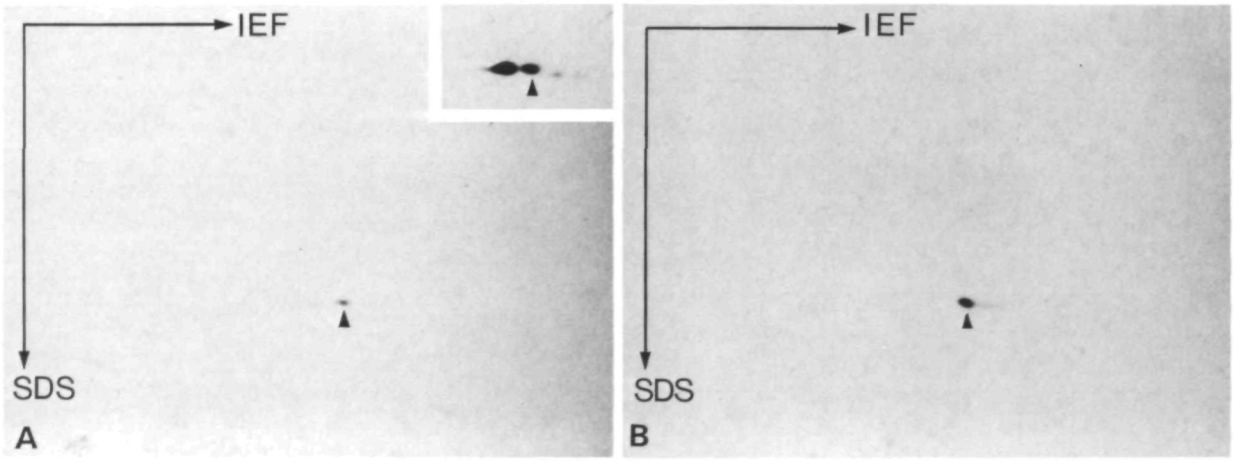

Fig. 2. Positive hybridization selection of mantle poly(A)$^+$ RNA by DNA from subclones SpMA3N (A), SpMA3C (inset of A), and SpMA7N (B). RNA was selected and translated *in vitro*. The translation products were separated by two-dimensional gel electrophoresis and fluorographed. A and B represent entire gels containing translation products from mRNA selected by SpMA3N and SpMA7N, respectively. The inset in A represents the actin region of a gel containing translation products from mRNA selected by SpMA3C. The arrow represents the position of the muscle-specific actin isoform, as determined by co-electrophoresis of cold mantle proteins. IEF: isoelectric focusing dimension. SDS: sodium dodecyl sulphate dimension.

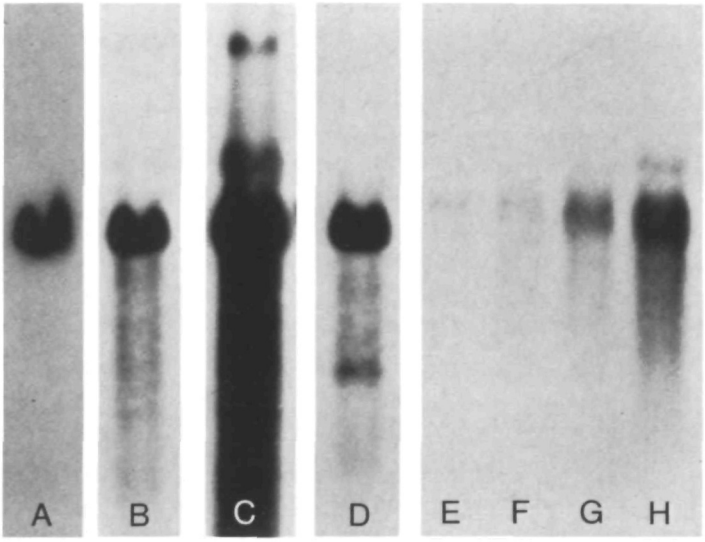

Fig. 3. Identification of muscle actin transcripts in Northern blots containing adult mantle and embryo RNA. (A) *S. plicata* mantle RNA probed with SpMA7N and washed at high stringency. The single band represents a transcript of about 2 kb. (B–C) *S. plicata* mantle RNA probed with SpMA3C and washed at high (B) or low (C) stringency. The blot shown in C was overexposed to illustrate the position of higher molecular weight bands. (D) *S. clava* mantle RNA probed with SpMA3C and washed at high stringency showing a predominant 2 kb band. (E–H) *S. clava* egg (E), early gastrula (F), tailbud (G), and tadpole RNA (H) probed with SpMA3C and washed at high stringency showing accumulation of the 2 kb transcript during development. Each lane contains 10 μg of RNA.

one other band, presumably representing a cytoplasmic actin transcript, was apparent (Fig. 3C).

In a third series of experiments, the SpMA3N and SpMA3C RNA probes were used to examine the distribution of actin transcripts in adults by *in situ* hybridization. Previous studies indicate that, while actin is present in every adult tissue, muscle-specific actin occurs only in the mantle, branchial sac and alimentary tract (Tomlinson *et al.* 1987). A section through part of the mantle and branchial sac of an adult is shown in Fig. 4A. In this section, which was probed with SpMA3N RNA and washed at low stringency (see Materials and methods for *in situ* hybridization washing conditions), grains are concentrated over specific regions of the mantle and branchial sac. The mantle contains a thin epidermis, consisting of a single layer of columnar epithelial cells, and a thick muscular dermis, consisting of longitudinal and circular bands of striated muscle fibres with numerous associated myocytes. At higher magnification, the grains present in the mantle can be seen
to be located in the dermis; only background levels of grains are present in the epidermis (Fig. 4B). The most heavily labelled cells of the dermis are myocytes, presumably because these cells are in the process of differentiating into muscle cells. SpMA3N RNA also detected transcripts in muscle cells located within the stigmata of the branchial sac (Fig. 4E) and in the lining of the stomach (Fig. 4F), but there were no labelled cells present in the epithelial layers of these organs. Consistent with the results of the Northern blots (Fig. 3C), when sections of mantle were probed with SpMA3C RNA and washed at low stringency, grains were detected in both the epidermal and dermal cells (Fig. 4C). Transcripts were detected exclusively in muscle cells in similar sections washed at high stringency (Fig. 4D).

In summary, DNA sequencing, hybridization selection, Northern blot and *in situ* hybridization experiments indicate that the cDNAs correspond to a muscle actin gene. It should also be noted that the specificity of SpMA3C RNA for muscle actin transcripts in Northern blot and *in situ* hybridization experiments depends on the stringency of the post-hybridization washes; muscle actin transcripts can be detected specifically with SpMA3N, SpMA7N or SpMA3C probes if washing is conducted at high stringency. In subsequent experiments, sections of embryos were hybridized *in situ* with the SpMA3C RNA probe and washed at high stringency to determine the temporal and spatial distribution of muscle actin transcripts.

Temporal and spatial accumulation of muscle actin mRNA in embryos

As described above, SpMA3C detects a 2 kb transcript when hybridized to Northern blots of *S. plicata* mantle RNA. As shown in Fig. 3D, SpMA3C also detected a transcript of approximately 2 kb in mantle RNA obtained from the related ascidian *S. clava*. Since large numbers of synchronously developing embryos can be obtained from *S. clava*, we used the *S. plicata* probe to examine muscle actin mRNA accumulation during *S. clava* development by Northern blot analysis. The results showed that muscle actin transcripts are present at very low levels in unfertilized eggs and early gastrulae, but accumulate to higher levels in tailbud stage embryos and hatched tadpoles (Fig. 3E–H). Densitometry of these Northern blots indicated that the 2 kb transcript exists in tadpoles at a level about 25-fold higher than in eggs. The detection of the muscle actin transcripts in unfertilized eggs confirms previous reports that both maternal and zygotic muscle actin mRNA exist in *Styela* embryos (Jeffery, Bates, Beach & Tomlinson, 1986; Tomlinson *et al.* 1987).

To determine whether muscle actin transcripts are restricted to particular embryonic cells, sections of

mid-tailbud-stage embryos were subjected to *in situ* hybridization with SpMA3C RNA and washed at high stringency (Fig. 5A). Many of these sections include the head and tail of a single embryo because the tail curls around the head as it is extended during embryogenesis. Three large muscle cells are located on each side of the central notochord in the tail of the bilaterally symmetric embryo (see Fig. 5B for orientation). Dorsal spinal cord cells and ventral endoderm cells and mesenchyme, endoderm, and epidermal cells are also evident in the head and tail, respectively. It can be seen that only the six tail muscle cells are labelled after *in situ* hybridization with SpMA3C (Fig. 5A). These results indicate that

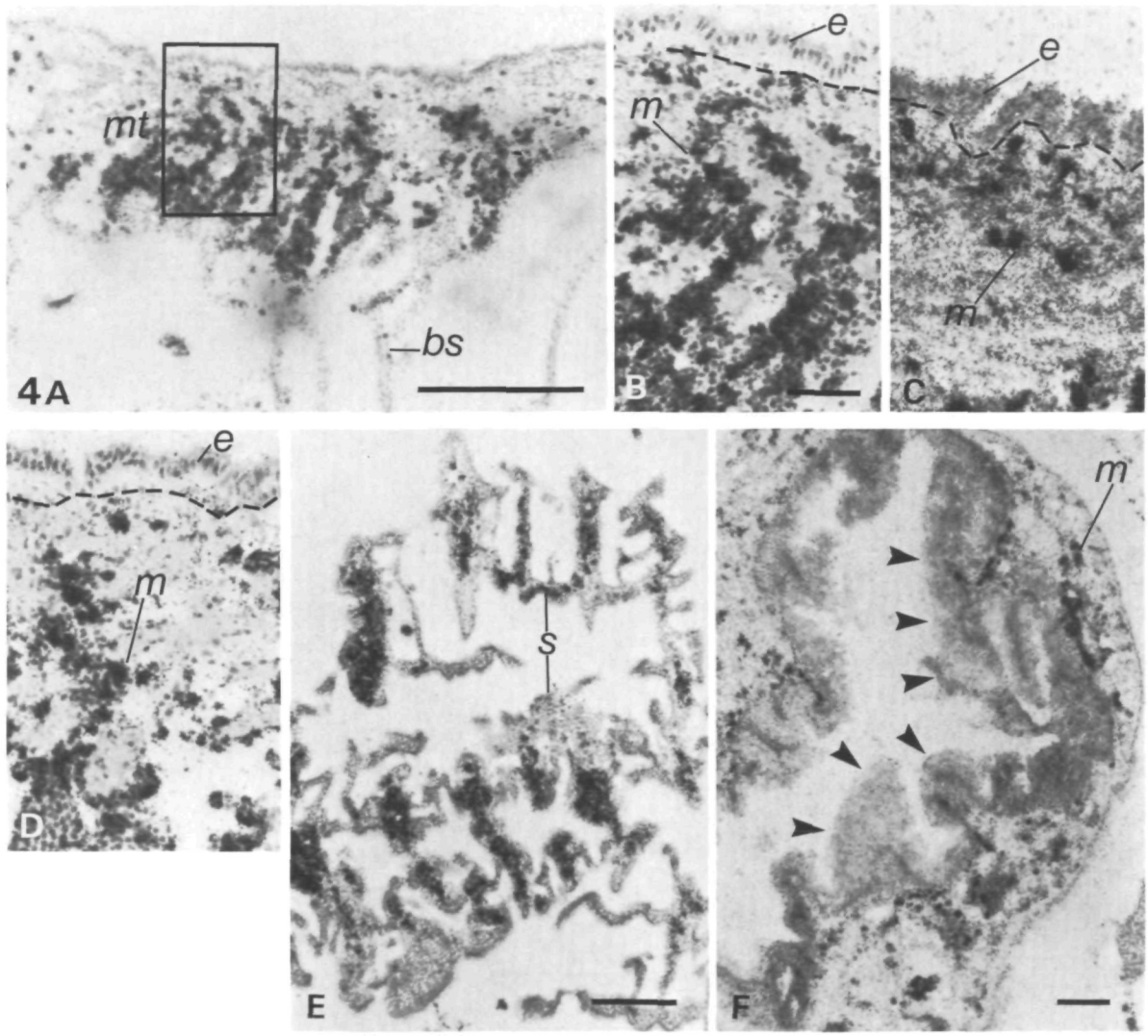

Fig. 4. *In situ* hybridization of sectioned adults with SpMA3N (A–B,E–F) or SpMA3C (C–D) RNA probes. (A) A low magnification photograph of a section through part of the mantle (*mt*) and branchial sac (*bs*) hybridized with SpMA3N RNA and washed at high stringency. The boxed area represents the region of the section shown at higher magnification in B. The magnification bar represents 100 μm. (B) A higher magnification photograph of the portion of the section outlined by the box in A. Grains are present in dense clusters over myocytes (*m*) in the dermis but columnar epidermal cells (*e*) with apical nuclei are unlabelled. The magnification bar represents 20 μm. (C) A section through the mantle hybridized with SpMA3C and washed at low stringency. Grains are clustered over myocytes (*m*) in the dermis and the epidermal (*e*) cells are unlabelled. Magnification is the same as in B. (D) A section through the mantle hybridized with SpMA3C and washed at high stringency. Grains are clustered over myocytes (*m*) in the dermis but the epidermal (*e*) cells are unlabelled. Magnification is the same as in B. In B–D, the border between the epidermis (upper region) and dermis (lower region) is indicated by dashed lines. (E) A section through the branchial sac hybridized with SpMA3N and washed at high stringency showing labelled muscle cells within the stigmata (*s*). The magnification bar represents 40 μm. (F) A section through the stomach hybridized with SpMA3N and washed at high stringency showing labelled muscle cells (*m*). The intestinal mucosa (arrowheads) is unlabelled. The magnification bar represents 10 μm.

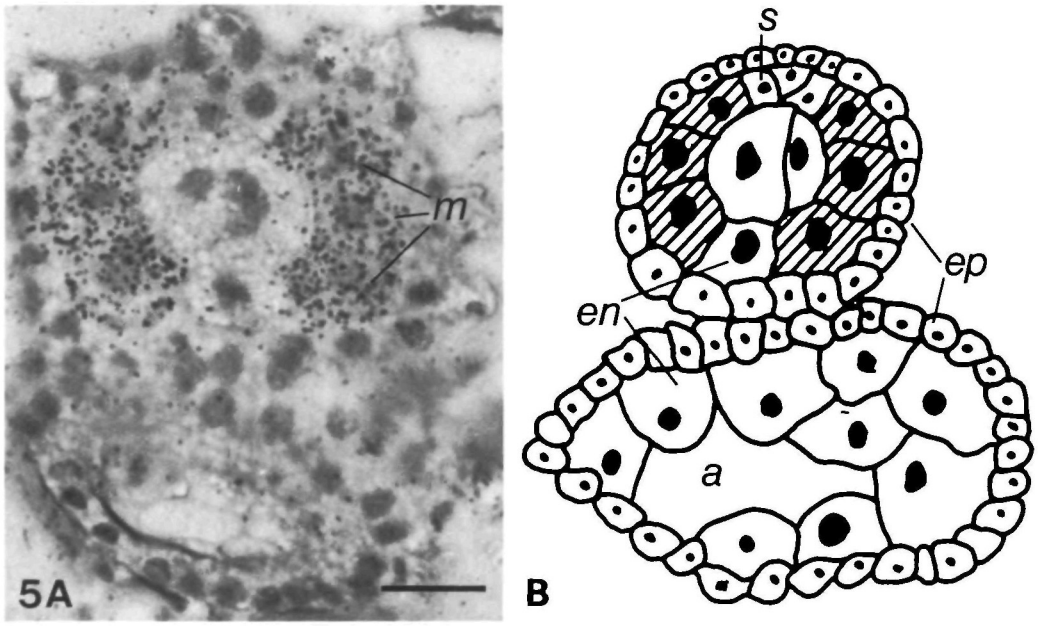

Fig. 5. (A) *In situ* hybridization of a cross-sectioned mid-tailbud-stage embryo with SpMA3C RNA. Grains above background are present only in primary muscle lineage cells (*m*) on either side of the central notochord. The magnification bar represents 20 μm. (B) A diagram of a mid-tailbud embryo in cross-section showing the position of the primary muscle lineage cells (striped) with respect to other cell types. Post-hybridization washes were conducted at high stringency. *n*, notochord cells; *ep*, epidermal cells; *en*, endodermal cells; *s*, spinal cord cells; *a*, archenteron. The dark spheres represent nuclei.

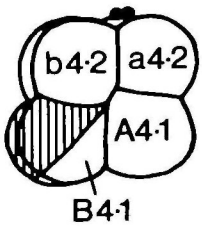

Fig. 6. A diagram of an 8-cell ascidian embryo showing the location of the a4.2, b4.2, A4.1, and B4.1 blastomere pairs. The striped zone represents the yellow crescent cytoplasm.

muscle actin transcripts are restricted to the muscle cell lineage in tailbud embryos.

Expression of the muscle actin gene in muscle cell lineages

There are three different sources of muscle cells in ascidians. Most larval tail muscle cells are descended from B4.1 blastomeres of the 8-cell embryo (Conklin, 1905) (Fig. 6). Tail muscle cells that originate from the B4.1 blastomeres are designated the primary muscle lineage. It has recently been shown that a variable number of muscle cells located at the posterior extremity of the tadpole tail are derived from A4.1 and b4.2 blastomeres of the 8-cell embryo (Nishida & Satoh, 1983, 1985) (Fig. 6). These cells belong to the secondary tail muscle lineage. During metamorphosis, the larval tail is withdrawn into the head and adult muscle is thought to arise from the mesenchyme cells (Katz, 1983). Mesenchyme cells are also progeny of B4.1 blastomeres (Fig. 6).

In the experiments shown in Fig. 7, the SpMA3C RNA probe was used to examine the distribution of muscle actin transcripts at various stages of development. At the 32-cell stage, no significant accumulations of grains were observed in any of the blastomeres (Fig. 7A). Gastrulation begins between the 64- and 128-cell stage, when vegetal cells invaginate to form the blastopore (Conklin, 1905; Satoh, 1978). The first concentrated grains were detected exclusively in primary muscle lineage cells at the beginning of gastrulation, about 4 h after fertilization (Fig. 7B). At this stage, the primary muscle cells can be distinguished by relatively large nuclei and their position at the posterior lip of the blastopore. In addition, these cells contain large numbers of myoplasmic pigment granules, as determined by extraction of embryos with Triton X-100 (Fig. 8). Signal continued to be apparent in primary muscle lineage cells as gastrulation proceeded (Fig. 7C–D). The mesenchyme cells, identified as yellow pigment-depleted cells located at the lateral lips of the blastopore (Fig. 8B), remained unlabelled throughout gastrulation. At the late gastrula stage, primary muscle lineage and mesenchyme cells enter the interior of the embryo and become aligned in two rows along the

midline of the neurula, ventral and posterior to the neural plate and notochord cells. In frontal (Fig. 7E) or parasagittal (Fig. 7F) sections, it can be seen that primary muscle lineage cells, but not mesenchyme cells, accumulate muscle actin transcripts in neurulae. To determine whether muscle actin transcripts are

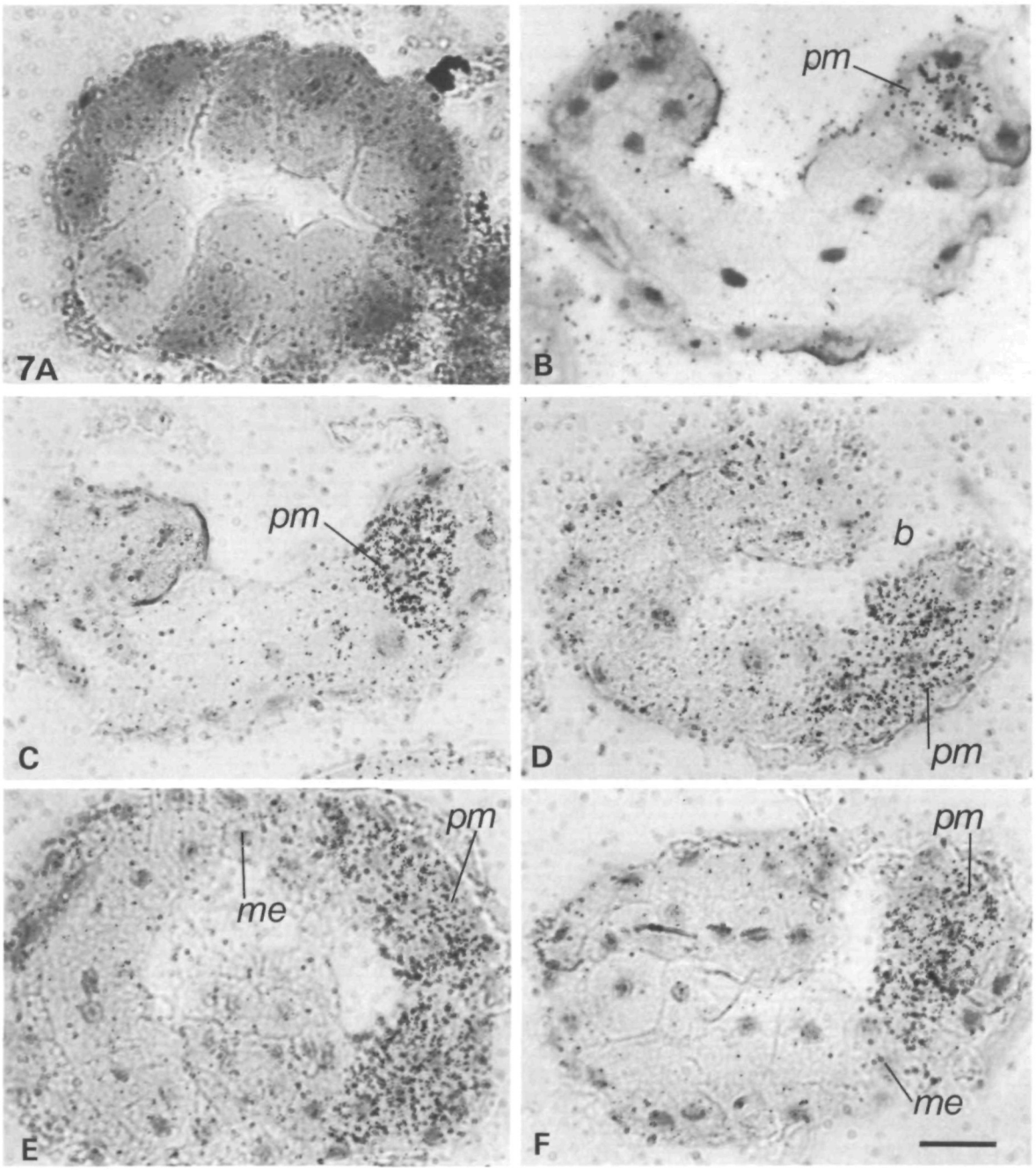

Fig. 7. *In situ* hybridization of sectioned 32-cell, gastrula, and neurula-stage embryos with SpMA3C RNA. (A) A section through the animal–vegetal axis of a 32-cell-stage embryo. (B) A section through the animal–vegetal (dorsal–ventral) axis of an early gastrula showing label in the primary muscle lineage cells (*pm*) located at the posterior lip of the forming blastopore. (C–D) Sections through the animal–vegetal axes of later-stage gastrulae showing accumulation of actin mRNA in the primary muscle lineage cells (*pm*). (E) A frontal section through the anterior–posterior axis of a late gastrula showing two rows of labelled primary muscle lineage cells (*pm*) and unlabelled mesenchyme cells (*me*) anterior to the primary muscle lineage cells. (F) A sagittal section through the anterior–posterior axis of a neurula showing a row of labelled primary muscle lineage cells (*pm*) posterior to unlabelled mesenchyme cells. In sections through the animal–vegetal (dorsal–ventral) axis, the vegetal pole (dorsal side) is facing up. Post-hybridization washes were conducted at high stringency. *b*, blastopore. The magnification bar in A represents 20 μm; magnification is the same in each frame.

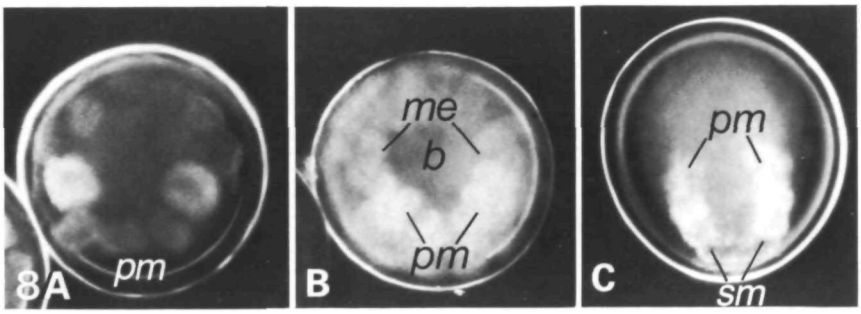

Fig. 8. Embryos extracted with Triton X-100 to show the location of yellow pigment granules. (A) A 32-cell embryo viewed from the vegetal pole showing the relative concentrations of Triton X-100-resistant pigment granules (bright areas) in primary muscle lineage cells (*pm*) in the posterior–vegetal region of the embryo and non-B4.1-derived cells in the anterior–vegetal region of the embryo. (B) A gastrula viewed from the vegetal pole showing concentrated pigment granules in primary muscle lineage cells (*pm*) at the posterior lip of the blastopore (*b*) and less concentrated pigment granules in mesenchyme cells (*me*) at the lateral lips of the blastopore. (C) An early-tailbud embryo viewed from the dorsal side showing concentrated pigment granules in primary muscle lineage cells (*pm*) of the anterior and middle portions of the tail and less concentrated pigment granules in secondary muscle cells (*sm*) at the posterior tip of the tail. In each frame, the posterior pole of the embryo faces down. The magnification bar represents 50 μm; magnification is the same in each frame.

restricted to primary muscle lineage cells, gastrulae and neurulae were serially sectioned and each section was subjected to *in situ* hybridization. The results indicate that only primary muscle lineage cells contain muscle actin transcripts. Thus, the accumulation of muscle actin mRNA is restricted to primary muscle lineage cells in gastrulae and neurulae.

After neurulation, primary muscle lineage cells cease dividing (Nishida & Satoh, 1985) and become part of the tail, which gradually extends from the posterior region of the embryo. In contrast, the mesenchyme cells continue to divide (Berrill, 1935) and become located in the head, immediately anterior to the rows of tail muscle cells. The early history of the secondary muscle lineage cells is not well known. Scanning electron microscopy suggests that these cells are located on the dorsal side of the embryo near the posterior margin of the neural plate and may enter the blastopore in the wake of primary muscle lineage cells (see fig. 5 in Nishida, 1986). The early tailbud is the first stage at which cells of the secondary muscle lineage can be distinguished with certainty. They are located interior to epidermal cells at the posterior tip of the tail; and contain fewer yellow pigment granules than primary muscle lineage cells (Fig. 8C). Although Conklin did not include secondary muscle lineage cells in his fate map of the *Styela* embryo, he did note a difference in morphology of cells at the tip of the tail. The cells at the tip of the tail were labelled as mesenchyme (see figs 8, 13–15, 18, 20 in Conklin, 1906), presumably because of their light yellow colour, but are spatially distinct from the population of mesenchyme cells that enter the larval head region. Although primary muscle lineage cells are labelled at the early tailbud stage, there is still no accumulation of muscle actin mRNA in mesenchyme or secondary muscle lineage cells, which can be identified at the tip of the tail (Fig. 9A). As the tail continues to extend in mid-tailbud embryos, however, secondary muscle lineage cells begin to accumulate muscle actin transcripts (Fig. 9B). To be certain that our sections of mid-tailbud embryos included the posterior tip of the tail, serial sections were made of mid-tailbud embryos, each section was subjected to *in situ* hybridization with SpMA3C RNA and the number of notochord cells was counted. The total number of notochord cells is 40 in *Styela* (Grave, 1944). Therefore, sections that exhibited about 40 notochord cells were assumed to include the tip of the tail. Examples of such sections are shown in Fig. 9C–E; in all cases, these embryos exhibited labelling in muscle cells extending to the tip of the tail. We estimate that muscle actin mRNA begins to accumulate to detectable levels in secondary muscle lineage cells by about 7–8 h after fertilization, or 3·5–4 h following the first detection of these transcripts in primary muscle lineage cells.

In contrast to primary and secondary muscle cells, mesenchyme cells still lacked muscle actin transcripts in mid-tailbud embryos (Fig. 9A,B). At the hatched tadpole stage, the mesenchyme cells also lacked detectable muscle actin mRNA (Fig. 9E), although labelling was still apparent in the tail muscle cells. The results of the *in situ* hybridization studies are summarized in Table 2. They indicate that zygotic muscle actin mRNA first appears in the primary muscle lineage cells at the beginning of gastrulation and continues to be present in these cells through the tadpole stage; muscle actin mRNA accumulation is delayed in the secondary muscle lineage cells until the

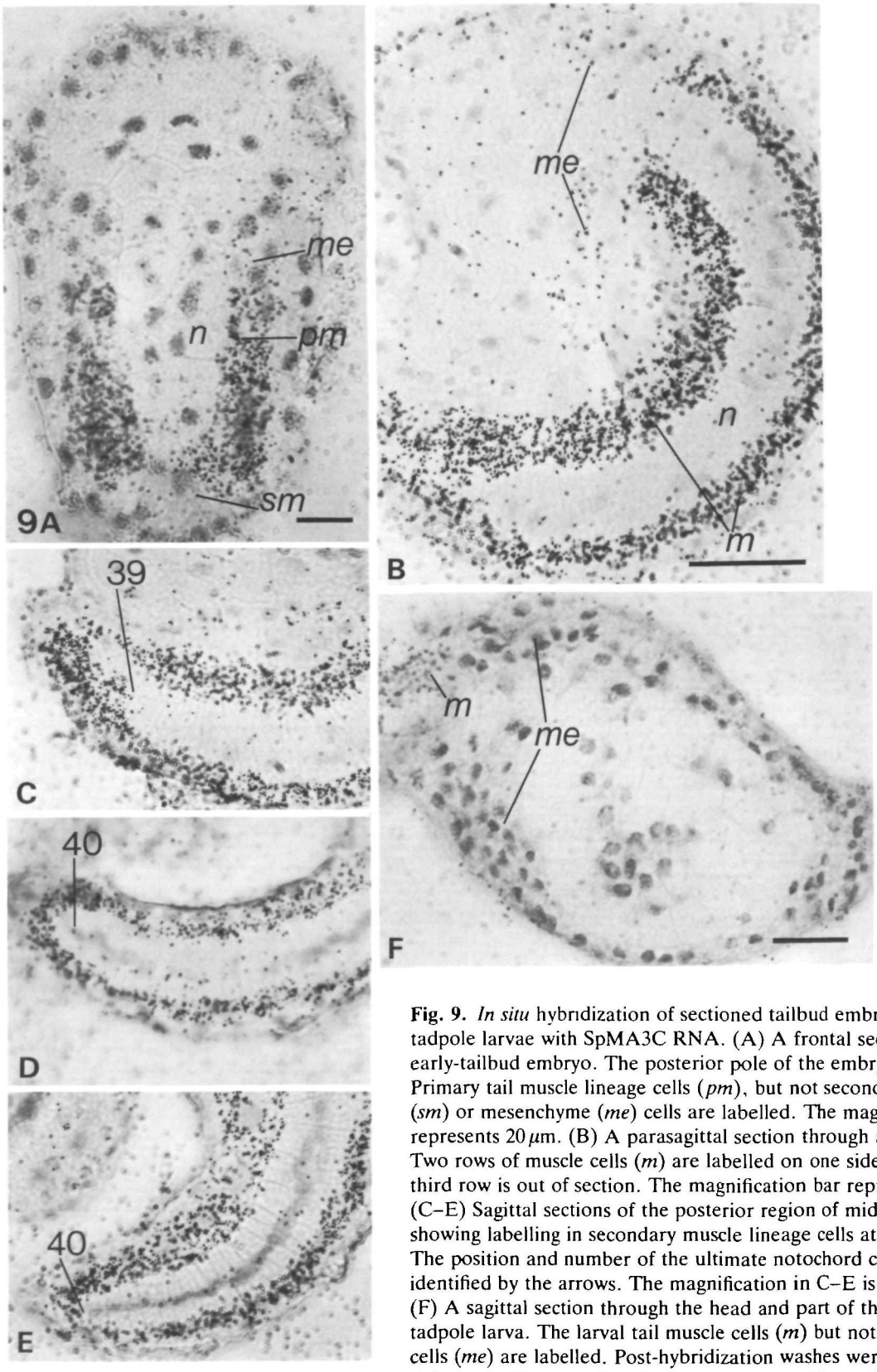

Fig. 9. *In situ* hybridization of sectioned tailbud embryos and hatched tadpole larvae with SpMA3C RNA. (A) A frontal section through an early-tailbud embryo. The posterior pole of the embryo is facing down. Primary tail muscle lineage cells (*pm*), but not secondary muscle lineage (*sm*) or mesenchyme (*me*) cells are labelled. The magnification bar represents 20 μm. (B) A parasagittal section through a mid-tailbud embryo. Two rows of muscle cells (*m*) are labelled on one side of the embryo; the third row is out of section. The magnification bar represents 25 μm. (C–E) Sagittal sections of the posterior region of mid-tailbud embryos showing labelling in secondary muscle lineage cells at the tip of the tail. The position and number of the ultimate notochord cell in each section is identified by the arrows. The magnification in C–E is the same as in B. (F) A sagittal section through the head and part of the tail of a hatched tadpole larva. The larval tail muscle cells (*m*) but not the mesenchyme cells (*me*) are labelled. Post-hybridization washes were conducted at high stringency. The magnification bar represents 15 μm. (A–E) Fixation in ethanol:acetic acid. (F) Fixation in formalin–sea water. *n*, notochord.

Table 2. *Comparison of the timing of muscle actin gene expression in* Styela *muscle cell lineages*

Cell lineage	Origin (blastomeres) at 8-cell stage	Functional stage	First expression
Primary	B4 1	Larva	Early gastrula
Secondary	A4.1 and b4.1	Larva	Mid-tailbud
Mesenchyme	B4.1	Adult	Postmetamorphosis

mid-tailbud stage and muscle actin mRNA does not accumulate to detectable levels in the mesenchyme cells throughout embryonic development.

Discussion

cDNA clones corresponding to an ascidian muscle actin gene have been characterized and used as specific probes to examine the spatial distribution of muscle actin transcripts in embryos, larvae and adults. Four lines of evidence indicate that these cDNA clones correspond to a muscle actin gene. First, their coding region shares 11 of 14 diagnostic amino acid positions with mammalian muscle actins (Vanderkerchove & Weber, 1978, 1979). Second, subclones consisting of the 3′ noncoding region select mRNA coding for muscle actin, while subclones including the coding region select mRNA coding for both cytoplasmic and muscle actin. Third, an RNA probe transcribed from a 3′ noncoding region subclone detects a 2 kb transcript, while a probe made from a coding region subclone detects at least two different actin transcripts at low stringency, but only the 2 kb transcript at high stringency. Finally, probes made from the 3′ noncoding region subclone detect transcripts exclusively in muscle cells, while a probe made from the coding region subclone detects transcripts in both muscle and nonmuscle cells. The isolation of an ascidian muscle actin gene with sequence homology to mammalian muscle actins is consistent with *N*-terminal partial sequencing studies indicating that *Styela* actins are more closely related to vertebrate than invertebrate muscle actins (Vanderkerchove & Weber, 1984).

The purpose of isolating muscle actin cDNAs was to use them as highly specific markers for gene expression in the muscle cell lineages. Markers previously developed for this purpose in ascidians have been AchE (Durante, 1956; Whittaker, 1973), myosin (Meedel, 1983; Nishikata *et al.* 1987), and myofibrils (Crowther & Whittaker, 1984). The use of cloned probes corresponding to a specific transcript is a more direct measure of specific gene expression and is not complicated by the possibility of regulation at the level of translation. Muscle actin also appears to be a more specific marker of differentiated muscle cells than AchE, for instance, whose histochemical detection appears to be dependent on fixation conditions (Meedel & Whittaker, 1979). Under certain conditions AchE can be detected both in tail muscle and mesenchyme cells, whereas muscle actin transcripts accumulate exclusively in tail muscle cells and their precursors during early development.

Although there appears to be a low background of maternal muscle actin transcripts in the egg (Jeffery *et al.* 1986; Tomlinson *et al.* 1987), zygotic muscle actin mRNA first becomes detectable in the primary muscle lineage cells of *Styela* embryos at early gastrula stage, only about 3·5 h after fertilization, and eventually accumulates to levels 25-fold higher than those characteristic of the egg. As determined by translation of RNA injected into *Xenopus* eggs (Meedel & Whittaker, 1984), zygotic AchE transcripts also first appear at the gastrula stage in the ascidian *Ciona*. It is likely that the early gastrula stage is a transition during ascidian development when specific genes are first expressed in particular cell lineages.

Primary muscle lineage cells originate from B4.1 cells of the 8-cell embryo (Conklin, 1905; Ortolani, 1955), while secondary muscle lineage cells are derived from the descendants of b4.2 and A4.1 blastomeres (Nishida & Satoh, 1983, 1985). Consistent with their heterogeneous origin, we have demonstrated that muscle actin transcripts accumulate at different stages of development in different muscle cell lineages. Muscle actin mRNA did not accumulate in secondary muscle cells until the mid-tailbud stage and were never present in mesenchyme cells, which also originate from the B4.1 cells of the 8-cell embryo (Conklin, 1905). The mesenchyme cells are thought to differentiate into adult muscle and other mesodermal derivatives after metamorphosis (Katz, 1983). Since the actin cDNAs were obtained from an adult mantle cDNA library, it is certain that the muscle actin gene corresponding to these clones is expressed in adults. A third episode of muscle actin gene expression may occur in mesenchyme cells or their descendants during or after metamorphosis, but this remains to be investigated.

The results indicate that different muscle cell lineages express the muscle actin gene at different times in development. This brings up the possibility that mechanisms controlling muscle cell specification are different in the various muscle cell lineages. One possibility is that factors localized in the egg cytoplasm are responsible for muscle cell development in the primary muscle lineage cells, whereas inductive interactions are involved in specifying muscle cells in the secondary muscle cell lineage. Induction of secondary muscle cells is consistent with the relative inability of isolated A4.1 and b4.2 blastomeres to develop autonomously (Deno *et al.* 1985; Nishikata *et*

al. 1987; Meedel & Whittaker, 1987). Experiments are now in progress with the muscle actin cDNA probes to determine whether isolated blastomeres of early cleavage-stage embryos express the muscle actin gene autonomously. Another possibility is that factors localized in the egg cytoplasm are responsible for muscle cell development in both primary and secondary muscle cell lineages, but their efficiency in promoting muscle actin gene expression depends on their concentration in each lineage (Whittaker, 1982; Nishida & Satoh, 1983).

Although it is well known that factors responsible for muscle cell determination are present in the cytoplasm of ascidian eggs (Whittaker, 1980, 1982), their precise cytoplasmic localization remains to be determined. An intriguing possibility is that these factors are present in the yellow-pigmented cytoplasm, which enters the muscle cell lineages during embryogenesis. Although most of the yellow cytoplasm forms a crescent in the posterior-vegetal region of the zygote and enters the B4.1 blastomeres during cleavage, the illustrations accompanying Conklin (1905), vital dye mapping studies with *Phallusia mammillata* embryos (Zalokar & Sardet, 1984) and our detergent extraction studies indicate that appreciable numbers of yellow pigment granules also enter non-B4.1 blastomeres. If factors associated with the yellow cytoplasm are responsible for specifying cells in both primary and secondary muscle lineages, the delayed expression of the muscle actin gene in the latter may be due to an initial segregation of fewer of these factors to the progenitors of the secondary muscle cells. This interpretation of the results is also consistent with the absence of muscle actin gene expression in the mesenchyme cells, descendants of B4.1 blastomeres that obtain even less of the yellow cytoplasm than the secondary muscle lineage cells. Since mesenchyme cells continue to divide during embryogenesis, while primary and secondary muscle cells cease dividing shortly after gastrulation (Berrill, 1935; Nishida & Satoh, 1985), it is also possible that a combination of threshold levels of factors present in the yellow cytoplasm and the cessation of cell division is required for muscle actin gene expression.

In summary, the results of this investigation indicate that muscle actin gene expression is temporally regulated in various muscle cell lineages of ascidian embryos. The muscle actin cDNAs we have isolated and characterized should be useful tools in further studies of the role of egg cytoplasmic factors and induction in ascidian muscle cell development.

This research was supported by grants from the NIH (HD-13970) and NSF (DCB-84116763). We thank M. E. White for making some of the RNA probes, P. Kemp for technical assistance and S. Daugherty for photography.

References

Berrill, N. J. (1935). Studies in tunicate development. III. Differential reduction and acceleration. *Phil. Trans. Roy. Soc. (London) Ser.* B **225**, 225–326.

Berrill, N. J. (1947). Metamorphosis in ascidians. *J. Morph.* **81**, 249–267.

Biggen, M. D., Gibson, T. J. & Hong, G. F. (1983). Gradient gels and ^{35}S-label as an aid to rapid DNA sequence determination. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3963–3965.

Bonner, W. M. & Laskey, R. A. (1974). A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

Chen, E. Y. & Seeburg, P. H. (1985). Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. *DNA* **4**, 165–170.

Clewell, D. B. & Helsinki, D. R. (1970). Properties of a supercoiled deoxyribonucleic acid–protein relaxation complex and strand specificity of the relaxation event. *Biochem.* **9**, 4428–4439.

Cohen, S. N., Chang, A. C. & Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2110–2114.

Conklin, E. G. (1905). The organization and cell lineage of the ascidian egg. *J. Acad. natn. Sci. Philad.* **13**, 1–119.

Conklin, E. G. (1906). Does half an ascidian egg give rise to a whole larva? *Wilhelm Roux Arch. EntwMech. Org.* **21**, 727–753.

Cox, K. H., De Leon, D. V., Angerer, L. M. & Angerer, R. C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Devl Biol.* **101**, 485–502.

Crowther, R. J. & Whittaker, J. R. (1983). Developmental autonomy of muscle fine structure in muscle lineage cells of ascidian embryos. *Devl Biol.* **96**, 1–10.

Crowther, R. J. & Whittaker, J. R. (1984). Differentiation of histospecific ultrastructural features of cleavage-arrested early ascidian embryos. *Wilhelm Roux's Arch. devl Biol.* **194**, 87–98.

Davis, R. W., Botstein, D. & Roth, J. R. (1980). *A Manual for Genetic Engineering. Advanced Bacterial Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Deeley, R. G., Gordon, J. I., Burns, A. T. H., Mullinix, K. P., Bina-Stein, M. & Goldberger, R. F. (1977). Primary activation of the vitellogenin gene in the rooster. *J. biol. Chem.* **252**, 8310–8319.

Denhardt, D. T. (1966). A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**, 641–646.

Deno, T., Nishida, H. & Satoh, N. (1984). Autonomous muscle cell differentiation in partial ascidian embryos according to the newly-verified cell lineages. *Devl Biol.* **104**, 322–328.

Deno, T., Nishida, H. & Satoh, N. (1985). Histospecific acetylcholinesterase development in quarter ascidian

embryos derived from each blastomere pair of the eight-cell stage. *Biol. Bull. mar. biol. Lab. Woods Hole* **168**, 239–248.

DURANTE, M. (1956). Cholinesterase in the development of *Ciona intestinalis* (Ascidia). *Experientia* **12**, 307–308.

DURICA, D. S., SCHLOSS, J. A. & CRAIN, W. R. (1980). Organization of actin gene sequences in the sea urchin: Molecular cloning of an intron-containing DNA sequence for a cytoplasmic actin. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5683–5687.

FIRTEL, R. A. (1981). Multigene families encoding actin and tubulin. *Cell* **24**, 6–7.

FYRBERG, E. A., KINDLE, D. L., DAVIDSON, N. & SODJA, A. (1980). The actin genes of *Drosophila*: A dispersed multigene family. *Cell* **19**, 365–378.

GRAVE, C. (1944). The larva of *Styela (Cynthia) partita*: structure, activities, and duration of life. *J. Morph.* **75**, 453–467.

HAFEN, E., LEVINE, M., GARBER, R. L. & GEHRING, W. J. (1983). An improved *in situ* hybridization method for the detection of cellular RNAs in *Drosophila* tissues sections and its application for localizing transcripts of the homeotic *Antennapedia* gene complex. *EMBO J.* **2**, 617–623.

JEFFERY, W. R. (1984). Pattern formation by ooplasmic segregation in ascidian eggs. *Biol. Bull. mar. biol. Lab., Woods Hole* **166**, 277–298.

JEFFERY, W. R. (1985). Specification of cell fate by cytoplasmic determinants in ascidian embryos. *Cell* **41**, 11–12.

JEFFERY, W. R., BATES, W. R., BEACH, R. L. & TOMLINSON, C. R. (1986). Is maternal mRNA a determinant of tissue-specific proteins in ascidian embryos? *J. Embryol. exp. Morph.* **97 Supplement**, 1–14.

JEFFERY, W. R. & MEIER, S. (1983). A yellow crescent cytoskeletal domain in ascidian eggs and its role in early development. *Devl Biol.* **96**, 125–143.

JEFFERY, W. R., TOMLINSON, C. R. & BRODEUR, R. D. (1983). Localization of actin messenger RNA during early ascidian development. *Devl Biol.* **99**, 408–417.

KATZ, M. J. (1983). Comparative anatomy of the tunicate tadpole, *Ciona intestinalis*. *Biol. Bull. mar. biol. Lab., Woods Hole* **164**, 1–27.

LAND, H., GREZ, M., HAUSER, H., LINDENMAIER, W. & SCHUTZ, G. (1981). 5′ terminal sequences of eukaryotic mRNA can be cloned with high efficiency. *Nucleic Acids Res.* **9**, 2251–2266.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

MANIATIS, T., HARDISON, R. C., LACY, E., LAUER, J., O'CONNELL, C., QUON, D., SIM, D. K. & EFSTRATIADIS, A. (1978). The isolation of structural genes from libraries of eukaryotic DNA. *Cell* **15**, 687–701.

MEEDEL, T. H. (1983). Myosin expression in the developing ascidian embryo. *J. exp. Zool.* **227**, 203–211.

MEEDEL, T. H. & WHITTAKER, J. R. (1979). Development of acetylcholinesterase during embryogenesis of the ascidian *Ciona intestinalis*. *J. exp. Zool.* **210**, 1–10.

MEEDEL, T. H. & WHITTAKER, J. R. (1984). Lineage segregation and developmental autonomy in expression of functional muscle acetylcholinesterase mRNA in the ascidian embryo. *Devl Biol.* **105**, 479–487.

MEEDEL, T. H., CROWTHER, R. J. & WHITTAKER, J. R. (1987). Determinative properties of muscle lineages in ascidian embryos. *Development* **100**, 245–260.

MORGAN, T. H. (1934). *Embryology and Genetics.* New York: Columbia University Press.

MORRISON, M. R., BRODEUR, R., PARDUE, S., BASKIN, F., HALL, C. L. & ROSENBERG, R. N. (1979). Differences in the distribution of poly(A) size classes in individual messenger RNAs from neuroblastoma cells. *J. biol. Chem.* **254**, 7675–7683.

NISHIDA, H. (1986). Cell division pattern during gastrulation of the ascidian, *Halocynthia roretzi*. *Dev. Growth Differ.* **28**, 191–201.

NISHIDA, H. & SATOH, N. (1983). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. I. Up to the eight-cell stage. *Devl Biol.* **99**, 382–394.

NISHIDA, H. & SATOH, N. (1985). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. II. The 16- and 32-cell stages. *Devl Biol.* **110**, 440–454.

NISHIKATA, T., MITA-MIYAZAWA, I., DENO, T. & SATOH, N. (1987). Muscle cell differentiation in ascidian embryos analysed with a tissue-specific monoclonal antibody. *Development* **99**, 163–171.

ORTOLANI, G. (1955). The presumptive territory of the mesoderm in the ascidian germ. *Experientia* **11**, 445–446.

PERRY, H. E. & MELTON, D. A. (1983). A rapid increase in acetylcholinesterase mRNA during ascidian embryogenesis as demonstrated by microinjection into *Xenopus laevis* oocytes. *Cell Differ.* **13**, 233–238.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C. & BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. molec. Biol.* **113**, 237–251.

ROYCHOUDHURY, R. & WU, R. (1980). Terminal transferase-catalysed addition of nucleotides to the 3′ termini of DNA. *Meth. Enzymol.* **65**, 43–62.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467.

SATOH, N. (1978). Cellular morphology and architecture during early morphogenesis of the ascidian egg: An SEM study. *Biol. Bull. mar. biol. Lab. Woods Hole* **155**, 608–614.

SHANI, M., NUDEL, U., ZEVIN-SONKIN, D., ZAKUT, R., GIVOL, D., KATCOFF, D., CARMON, Y., REITER, J., FRISCHUAL, A. M. & YAFFE, D. (1981). Skeletal muscle actin mRNA. Characterization of the 3′ untranslated region. *Nucleic Acids Res.* **9**, 529–589.

TOMLINSON, C. R., BATES, W. R. & JEFFERY, W. R. (1987). Development of a muscle actin specified by

maternal and zygotic mRNA in ascidian embryos. *Devl Biol.* **123**, 470–482.

UZMAN, J. A. & JEFFERY, W. R. (1986). Cytoplasmic determinants for cell lineage specification in ascidian embryos. *Cell Differ.* **18**, 215–224.

VANDERKERCHOVE, J. & WEBER, K. (1978). Mammalian cytoplasmic actins are the products of at least two genes and differ in primary structure in at least 25 different positions from skeletal muscle actins. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1106–1110.

VANDERKERCHOVE, J. & WEBER, K. (1979). The complete amino acid sequence of actins from bovine aorta, heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. *Differentiation* **14**, 123–133.

VANDERKERCHOVE, J. & WEBER, K. (1984). Chordate muscle actins differ distinctly from invertebrate muscle actins. The evolution of different vertebrate muscle actins. *J. molec. Biol.* **179**, 391–413.

WHITTAKER, J. R. (1973). Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue specific enzyme development. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2096–2100.

WHITTAKER, J. R. (1979). Cytoplasmic determinants of tissue-specific differentiation in the ascidian egg. In *Determinants of Spatial Organization* (ed. S. Subtelny & I. R. Konigsberg), pp. 29–51. New York: Academic Press.

WHITTAKER, J. R. (1980). Acetylcholinesterase development in extra cells caused by changing the distribution of myoplasm in ascidian embryos. *J. Embryol. exp. Morph.* **55**, 343–354.

WHITTAKER, J. R. (1982). Muscle lineage cytoplasm can change the developmental expression in epidermal lineage cells of ascidian embryos. *Devl Biol.* **93**, 463–470.

WHITTAKER, J. R., ORTOLANI, G. & GARINELLA-FERRUZZA, N. (1977). Autonomy of acetylcholinesterase differentiation in muscle lineage cells of ascidian embryos. *Devl Biol.* **55**, 196–200.

ZALOKAR, M. & SARDET, C. (1984). Tracing of cell lineage in embryonic development of *Phallusia mammillata* (Ascidia) by vital staining of mitochondria. *Devl Biol.* **102**, 195–205.

(*Accepted 27 August 1987*)