J. Embryol. exp. Morph. ***97 Supplement,*** *1–14 (1986)*
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Is maternal mRNA a determinant of tissue-specific proteins in ascidian embryos?

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INTRODUCTION

The generation of different cell types during embryonic development is thought to be mediated by the combined activity of cytoplasmic factors (determinants), which are localized in the egg, and inductive interactions, which occur between different embryonic cells and tissues. Ascidians, animals that exhibit rapid and exceptionally autonomous development (reviewed by Jeffery, 1985), appear to employ cytoplasmic determinants to generate embryonic cell diversity. Although determinants have not been identified in ascidians or other animals, it is hypothesized that they function in at least two different ways. First, as initially pointed out by Morgan (1934), determinants may be regulatory factors which promote differential gene expression in specific cell lineages. Consistent with this possibility, inhibitors of transcription, added prior to gastrulation, block the appearance of some ascidian tissue-specific enzymes and morphological markers whose expression is regulated by the activity of cytoplasmic determinants (Whittaker, 1973; Crowther & Whittaker, 1984). Second, determinants may be localized factors which promote cell diversification independent of zygotic gene expression. This class of determinants is implicated in specifying crucial embryonic events that occur before the onset of zygotic transcription. Evidence for the second class of determinants in ascidians was first obtained from interspecific hybridization studies. These studies showed that haploid andromerogones, which developed from anucleate eggs of one ascidian species fertilized with sperm of another species, exhibited larval adhesive papillae that were morphologically identical to those of the maternal species (Minganti, 1959).

This paper reviews the class of cytoplasmic determinants that function independently of embryonic gene expression in ascidian embryos. The review focuses on the possibility that maternal mRNA molecules serve as determinants for the expression of alkaline phosphatase in the endodermal cell lineage and the expression of muscle actin in the muscle cell lineage.

Key words: ascidian embryo, maternal mRNA, proteins, determinant, alkaline phosphatase, actin, nucleus, *Styela plicata*.

ASCIDIAN DEVELOPMENT

Ascidian eggs exhibit localized cytoplasmic regions that are differentially distributed to the various cell lineages during early embryogenesis. These cytoplasmic regions are thought to reflect the spatial distribution of cytoplasmic determinants. In some ascidian eggs, especially those of the genus *Styela*, the various cytoplasmic regions exhibit different pigmentations. This attribute enabled Conklin (1905) to follow precisely the fate of five different cytoplasmic regions during early development of *Styela partita*. The position of the different regions is established in the uncleaved egg by a dramatic series of cytoplasmic rearrangements which occur between fertilization and first cleavage (reviewed by Jeffery, 1984). During the early cleavages, the cytoplasmic regions are partitioned into certain embryonic cells, and they each have entered a specific cell lineage by the 64-cell stage. Since this review focuses on endodermal and muscle cell markers, the development of these tissues is examined in detail.

Most of the endoplasm, a grey, yolk-filled region localized in the vegetal hemisphere of *S. partita* eggs, enters the endodermal cells during early development (Fig. 1A). During the early cleavages, the endoplasm is partitioned to two cells of the 2-cell embryo, four cells of 4- and 8-cell embryos, six cells of 16- and 32-cell embryos, and ten cells of the 64-cell embryo. The descendants of these ten cells form presumptive gut cells in the larva. The myoplasm, a yellow crescent-shaped region localized in the vegetal–posterior region of uncleaved *S. partita* eggs, enters the mesenchyme and larval tail muscle cells during early development (Fig. 1B). During the early cleavages, the portion of the myoplasm that eventually enters the muscle cells is partitioned to two cells of 2-, 4- and 8-cell embryos, four cells of the 16-cell embryo, six cells of the 32-cell embryo, and eight cells of the 64-cell embryo (Fig. 1B). The descendants of these eight cells, along with minor contributions from cells that originate from other embryonic regions (Nishida & Satoh, 1983), form the larval tail musculature.

ENDODERMAL ALKALINE PHOSPHATASE DETERMINANTS

Alkaline phosphatase (AP), an enzyme which is restricted to the larval endodermal tissue and the adult gut of ascidians, is first expressed in the embryo at gastrulation (Minganti, 1954; Whittaker, 1977). Histochemical analysis has shown that AP activity develops primarily in the ten endodermal cells of 64-cell *Ciona intestinalis* embryos (Whittaker, 1973). When *Ciona* embryos are cleavage-arrested by cytochalasin B treatment and cultured until the time at which controls form hatched larvae, AP activity appears only in blastomeres corresponding to the maximal cell number expected from the known endodermal cell lineage: two, four, four, six, six, and ten cells of cleavage-arrested 2-, 4-, 8-, 16-, 32- and 64-cell embryos, respectively (Fig. 1A) (Whittaker, 1977). This result suggests that AP determinants are segregated into the endodermal cells during the early cleavages.

Inhibitor experiments conducted with the histochemical studies indicated that the appearance of endodermal AP activity is blocked by puromycin, but not by

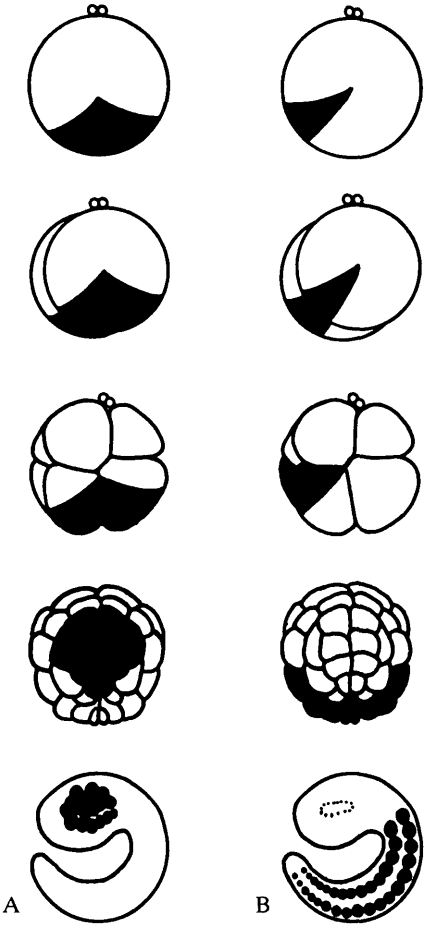

Fig. 1. Distribution of endoplasm (A) and myoplasm (B) during ascidian development. Top to bottom: 1-cell stage, 2-cell stage, 8-cell stage, 64-cell stage, tadpole larva. The darkened areas represent the cytoplasmic regions or cells that contain endoplasm (A) or myoplasm (B).

actinomycin D or other inhibitors of transcription (Whittaker, 1977). These results suggest that AP is synthesized *de novo* in the endodermal cells and is specified by determinants that do not require embryonic gene transcription for their function. It has been hypothesized that the AP determinants may either be localized and differentially segregated maternal mRNA molecules coding for AP, or translational activators of uniformly distributed AP mRNA molecules (Whittaker, 1977). According to these hypotheses, the translational activation of maternal AP mRNA in gastrulae would trigger AP expression in the endodermal cells.

Because the necessary molecular probes are unavailable, the existence and spatial distribution of AP mRNA has not been determined in eggs or early embryos. The requirement of the embryonic genome for AP expression has been tested, however, by assaying AP activity in nucleate and anucleate fragments prepared from fertilized *Styela plicata* eggs (Bates & Jeffery, 1986). The rationale

for these experiments is as follows. If AP expression is controlled entirely by maternal cytoplasmic factors, such as AP mRNA, then AP synthesis would be expected in both nucleate and anucleate fragments. In contrast, muscle AchE expression, which is known to require embryonic gene expression (Whittaker, 1973), would be expected in nucleate, but not in anucleate fragments. Nucleate and anucleate fragments of roughly equivalent volumes were prepared between the first stage of ooplasmic segregation and the first cleavage by extruding egg cytoplasm out of a small hole made in the follicular envelope (FE) (Fig. 2). The extruded cytoplasm was separated from the portion of the egg inside the FE by cutting across the narrow cytoplasmic constriction near the edge of the FE. The nucleate and anucleate fragments were cultured until controls reached the larval stage, and then were assayed for AP and AchE activity by histochemistry. The microsurgical operation itself did not affect enzyme activity because whole extruded eggs were able to develop levels of AP and AchE activity similar to those of unoperated controls. Most of the nucleate fragments developed into small larvae which exhibited AchE activity in their tail muscle cells and AP activity in their endodermal region (Fig. 3A). In contrast, none of the anucleate fragments developed AchE or AP activity (Fig. 3B). The absence of AchE activity in the anucleate fragments is expected because of the requirement of transcription for

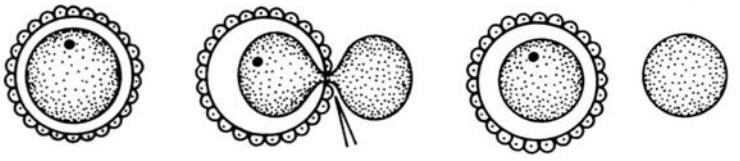

Fig. 2. A schematic diagram of the method used to prepare nucleate and anucleate fragments from ascidian eggs.

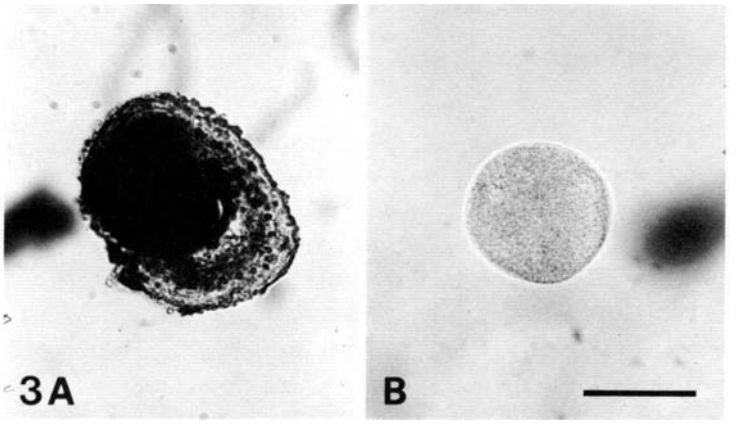

Fig. 3. Alkaline phosphatase expression in nucleate and anucleate fragments from fertilized eggs. (A) A larva that developed from a nucleate fragment showing dark AP staining in the endoderm region of the head. (B) An anucleate fragment, cultured until the time at which the corresponding nucleate fragment hatched, showing the absence of AP staining. Bar, 100 μm.

AchE expression (Whittaker, 1973). The inability of the anucleate fragments to express AP, however, is inconsistent with the possibility that AP expression depends solely on the translational activation of maternal AP mRNA.

There are several reasons why AP may not have been expressed in the anucleate fragments. First, the anucleate fragments may not be capable of protein synthesis. This is unlikely because the anucleate fragments continue to incorporate labelled amino acids into protein during the time that controls develop into larvae. Second, the anucleate fragments may be unable to express AP because they do not cleave. This is also an unlikely explanation because cleavage-arrested, 1-cell embryos are known to express AP (Whittaker, 1977). Third, since the anucleate fragments consist of about half the egg volume and contain various proportions of the different cytoplasmic regions, it is possible that they contain too little endoplasm to express AP. To test this possibility, anucleate fragments enriched in endoplasm were assayed for AchE and AP activity (Bates & Jeffery, 1986). These fragments were prepared from fertilized eggs in which extrusion was carried out so that most of the endoplasm entered the anucleate fragment. The endoplasmic anucleate fragments, which sometimes contained 90% of the total egg volume, did not develop AP or AchE activity, although many of the much smaller nucleate fragments cleaved and developed AchE and AP. These results suggest that the lack of AP expression in anucleate fragments is not due to the absence of protein synthesis or cleavage, or to a reduced proportion of endoplasm.

Another explanation for the lack of AP expression in the anucleate fragments is that the AP determinants could be localized in or around the female pronucleus. If the AP determinants were localized in the nucleoplasm, or in the perinuclear cytoplasm attached to the nuclear membrane, they would always be located in the nucleate fragment after extrusion, regardless of the size or ooplasmic composition of the anucleate fragment. The possibility that AP determinants are absent from the anucleate fragments was evaluated by inseminating anucleate fragments prepared from unfertilized eggs and subsequently examining their ability to express AP (Bates & Jeffery, 1986). Many of the inseminated anucleate fragments cleaved and some developed to the larval stage as andromerogones. AP activity was present in the andromerogones that arrested prior to gastrulation and was localized in the endodermal region of andromerogones that reached the larval stage.

The results of the egg fragmentation studies do not support the hypothesis that ascidian AP determinants function independently of embryonic gene transcription. The simplest interpretation of the results is that AP determinants, like determinants that specify AchE, tyrosinase and a number of morphological features in ascidian larvae (Whittaker, 1973; Crowther & Whittaker, 1984), function by activating AP gene transcription. The appearance of AP at gastrulation, when the first zygotic mRNA transcripts are detected in ascidians (Meedel & Whittaker, 1978), supports this explanation. Unfortunately, this interpretation conflicts with the observation that AP expression is resistant to actinomycin D treatment. A possible resolution of the conflict may be that AP gene transcription

is less sensitive to actinomycin D than AchE or tyrosinase gene transcription. The fact that actinomycin D does not completely inhibit transcription in ascidian embryos (Whittaker, 1977) is consistent with this explanation. It is also conceivable, however, that the AP determinants require nuclei for purposes other than transcription. One possibility is that nuclear events may regulate the timing of AP determinant activation. This possibility is supported by aphidicolin experiments which suggest that rounds of DNA replication until the 64-cell stage are required for AP expression (Satoh, 1982).

MUSCLE ACTIN DETERMINANTS

AchE and myofibrils, two specific markers for ascidian larval muscle cells, first appear at the neurula stage (Whittaker, 1973; Crowther & Whittaker, 1983). The development of AchE and myofibrils in cultured muscle progenitor blastomeres isolated from early embryos suggests that these markers are specified primarily by intrinsic cellular factors, probably cytoplasmic determinants, rather than by inductive interactions (Whittaker, Ortolani & Farinella-Ferruzza, 1977; Crowther & Whittaker, 1983). Cleavage-arrest experiments, in which AchE and myofibrils were shown to develop only in the maximal number of blastomeres expected from the known muscle cell lineage (Whittaker, 1973; Crowther & Whittaker, 1983), suggest that the determinants specifying these factors segregate into the muscle progenitor cells during the early cleavages. Presumably, the segregation of determinants into the muscle cell lineage is due to their localization in the myoplasm.

The AchE determinants probably function by the activation of AchE genes in the muscle cell lineage. This possibility is supported by the sensitivity of AchE expression to actinomycin D (Whittaker, 1973, 1977), the restriction of AchE expression to nucleate egg fragments (Bates & Jeffery, 1986) and the initial appearance of AchE transcripts exclusively in the muscle progenitor cells of cleavage-arrested embryos (Meedel & Whittaker, 1984). Although sensitivity to actinomycin D (Terakado, 1973) suggests that myofibril assembly is also dependent on embryonic gene transcription, it is conceivable that the constituent proteins of the myofibril may be assembled from a maternal protein pool or from embryonic proteins whose synthesis is directed by maternal transcripts. The latter possibility is supported by *in situ* hybridization studies with cloned DNA probes which showed that actin mRNA, but not histone mRNA, is localized in the myoplasm of *S. plicata* eggs and differentially partitioned to the myoplasmic blastomeres during early development (Jeffery, Tomlinson & Brodeur, 1983). If the localized actin messages include muscle actin transcripts, they may serve as determinants, just as maternal AP mRNAs are proposed as determinants for endodermal AP synthesis.

In many animals there are two classes of actin; a ubiquitous class, which is present in the cytoskeleton, and a restricted class, which is located in the myofibrils of muscle cells (Garrels & Gibson, 1976). Each actin class can contain more than one isoform, and the actins are usually encoded by a multigene family (Firtel, 1981). Two-dimensional gel electrophoresis indicates that there are three major

actin isoforms in *Styela* (Tomlinson & Jeffery, 1986). The major actin isoforms, however, are not present in every adult tissue. Two basic actin isoforms, which probably correspond to the vertebrate β and γ cytoskeletal actins, are present in body wall muscle, branchial sac, gut and gonads. An acidic actin isoform, which probably corresponds to a vertebrate α muscle actin, is present only in the branchial sac and body wall musculature. Several minor actin isoforms also exist in *Styela*, but their relationship to the major actin species is unknown.

Eggs and early embryos contain almost exclusively cytoskeletal actins, whereas larvae contain all three major actin isoforms (Tomlinson & Jeffery, 1986). The embryonic actin isoforms appear to be identical to those of adults. In labelling experiments, the cytoskeletal actins are synthesized at every stage of embryogenesis, while labelled muscle actin is first detected between gastrulation and the early neurula stage (Figs 4, 5). Muscle actin could be synthesized earlier in small amounts, but it would be difficult to detect because of the slow equilibration of exogenous labelled amino acids with the intracellular amino acid pool. When cytokinesis is inhibited with cytochalasin B, muscle actin accumulates in the myoplasmic blastomeres, suggesting that it is normally synthesized in the muscle cell lineage (Tomlinson & Jeffery, 1986). In summary, the results suggest that muscle actin begins to be synthesized at least by the gastrula stage and is specified by determinants that segregate into the muscle cell lineage.

Inhibitor experiments (Tomlinson & Jeffery, 1986), conducted along with the labelling studies discussed above, indicate that muscle actin synthesis is minimally affected by levels of actinomycin D that completely suppress AchE (Whittaker, 1973) and myofibril (Terakado, 1973) development in ascidian embryos (Fig. 5). These results suggest that muscle actin expression may involve determinants that do not require embryonic transcription. As proposed for endodermal AP expression, the determinants may either be maternal mRNAs coding for muscle actin which are localized in the myoplasm, segregated to the muscle cell lineage and translationally activated after gastrulation, or the determinants may be localized translational activators of uniformly distributed muscle actin mRNA.

In principle, the possibility that muscle actin synthesis is independent of embryonic transcription could be tested by the same methods employed for AP. Large numbers of anucleate fragments would be required to detect the position of labelled actin in gels, however, and anucleate fragments have proved difficult to prepare in mass. Instead, the presence of muscle actin mRNA in ascidian eggs have been determined directly using a specific cloned DNA probe (Tomlinson, Beach & Jeffery, 1986). The approach was to isolate muscle actin clones from a cDNA library (prepared from *S. plicata* adult body-wall muscle poly(A)$^+$ RNA) and use the appropriate restriction fragments as probes to detect specific actin mRNAs. The cDNA library was screened with a *Hind* III fragment containing most of the codogenic region of a *Drosophila melanogaster* actin gene (pDmA2; Fyrberg, Kindle, Davidson & Sodja, 1980). Several positive clones were then sequenced (Sanger, Nicklen & Coulson, 1977) after DNA inserts were subcloned into the *Hinc* III site of M13mp8. The sequence of the *Styela* actin cDNAs was

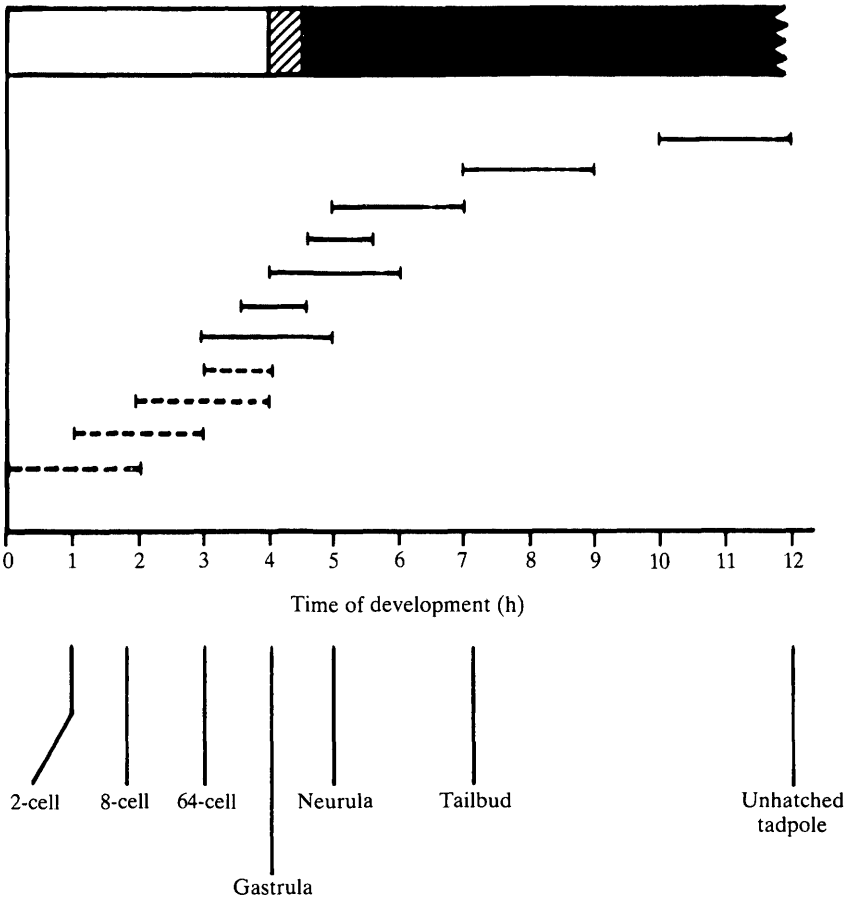

Fig. 4. A diagram illustrating the timing of muscle actin synthesis during early development. Muscle actin synthesis was determined by pulse labelling embryos at various times during early development with [^{35}S]methionine followed by electrophoresis of proteins through two-dimensional gels and autoradiography. Top bar: the open area represents a period of undetectable muscle actin synthesis. The striped area represents a period in which a low level of muscle actin synthesis was detected. The filled area represents a period in which a relatively high level of muscle actin synthesis was detected. The labelling intervals are represented as truncated dashed (no muscle actin detected) and solid lines (muscle actin detected) at the bottom of the graph.

compared to the mammalian consensus sequences for cytoskeletal, smooth muscle and skeletal muscle actins (Vanderkerckhove & Weber, 1978, 1979). A cDNA clone was selected for further analysis that shared eleven of fifteen diagnostic amino acid positions with mammalian smooth and skeletal muscle actins, but only three of fifteen positions with mammalian cytoskeletal actins.

Since the codogenic region of actin mRNA is conserved, but the non-codogenic region diverges in sequence between the various actin transcripts (Shani *et al.* 1981), cloned probes containing the codogenic region would be expected to detect all actin transcripts, while cloned probes containing only the 3′ non-codogenic region would be expected to detect a specific actin transcript. Subclone SpGA, a

666 base-pair *Hae* III DNA fragment containing a region complementary to part of the codogenic and the 3′ non-codogenic regions of the *S. plicata* muscle actin mRNA, was used as a probe for general actin mRNA. Subclone SpMA, a 55 base-pair DNA fragment, which is complementary to part of the 3′ non-codogenic region but not the codogenic region of muscle actin mRNA, was used as a specific probe for muscle actin mRNA (Fig. 6).

The specificity of these probes was supported by four lines of evidence (Tomlinson *et al.* 1986): first, in genomic Southern blots SpGA recognizes at least three DNA bands, while SpMA recognizes only one DNA band. Presumably, *S. plicata* has at least three actin genes, one of which is a muscle actin gene. Second, SpMA hybridizes specifically to a sub-set of the plaques recognized by SpGA when both probes are used to screen an *S. plicata* genomic DNA library. Third, in translation selection analysis, SpGA selects adult muscle cell mRNA coding for a least two actins, while SpMA selects mRNA coding for a single actin, the most acidic isoform. Fourth, when used to probe sections of adults by *in situ* hybridization, SpGA hybridizes to epidermis, branchial sac and body wall musculature, gut, testes and ovaries, while SpMA hybridizes only to branchial sac and body wall muscle cells (Fig. 7). Therefore, assuming SpMA is a specific probe for muscle actin transcripts, three questions can be asked: is muscle actin mRNA present in the egg?; if muscle actin mRNA is present in the egg, does it persist until the time when the muscle actin isoform first appears in the embryo? and when does embryonic muscle actin mRNA transcription begin?

The presence of muscle actin in eggs was tested by dot hybridization and S1 nuclease protection analysis (Tomlinson *et al.* 1986). As expected, dots containing

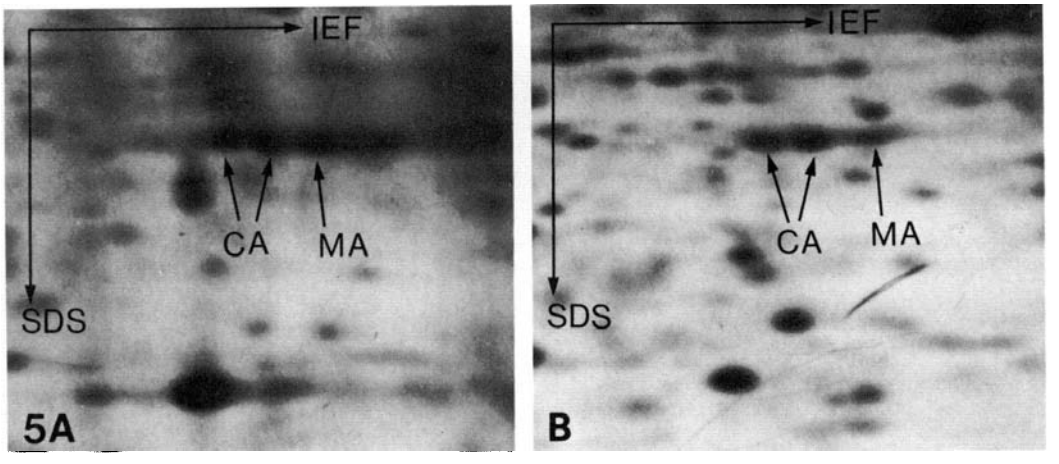

Fig. 5. Two-dimensional gel electrophoresis of embryonic proteins labelled for 6 h after fertilization in the presence (B) or absence (A) of 20 $\mu g\,ml^{-1}$ actinomycin D. A small region of the gels containing the actin isoforms is shown in each figure. SDS, direction of electrophoresis in sodium dodecyl sulphate-containing gel; IEF, direction of electrophoresis in isoelectric focusing gel; CA, cytoskeletal actins; MA, muscle actin.

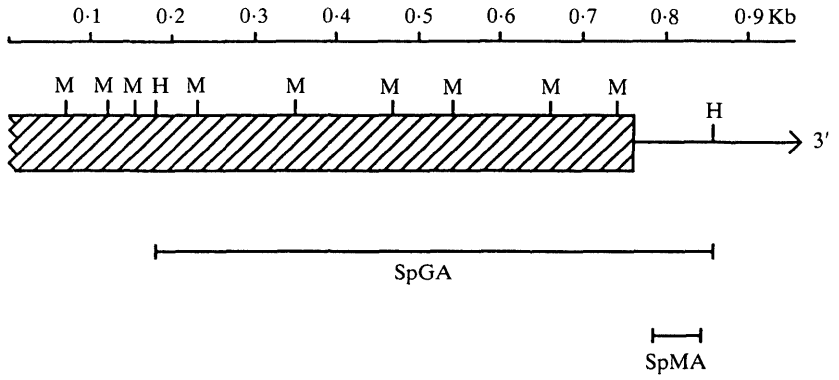

Fig. 6. A diagram of the muscle actin cDNA clone used to prepare the SpGA and SpMA probes. Striped area, Codogenic region; line terminating in an arrow, non-codogenic region; M, *Mnl* I restriction site; H, *Hae* III restriction site. SpGA is a 666 base pair, *Hae* III fragment. SpMA is a 55 base pair *Mnl* I–*Hae* III fragment that was shortened by limited digestion with nuclease *Bal* 31.

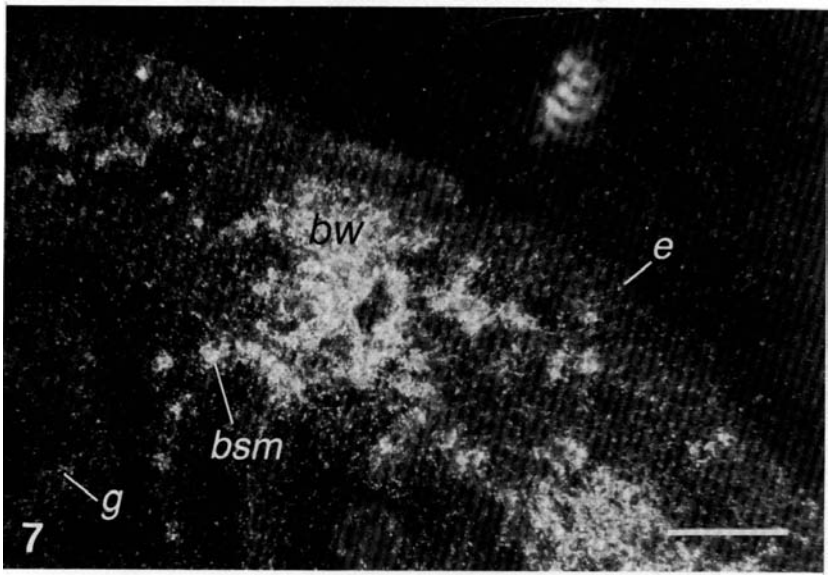

Fig. 7. *In situ* hybridization of a sectioned adult using a ^{35}S-labelled RNA probe transcribed *in vitro* from SpMA. Signal above the background is present in the body wall (*bw*) and branchial sac (*bsm*) muscle cells, but not in gut (*g*) or epidermal (*e*) cells. Bar, 2 mm.

total and poly(A)$^+$ RNA from eggs showed high signals when probed with SpGA. Substantial signals were also observed when similar dots were probed with SpMA at high stringency, suggesting that muscle actin mRNA is indeed present in eggs. This result was confirmed by using the SpGA probe in an S1 nuclease protection analysis. The SpGA probe contained 666 bases of *Styela* DNA and 54 bases of DNA from the M13 cloning vector. When SpGA was annealed to either adult muscle or egg poly(A)$^+$ RNA, then treated with S1 nuclease and subjected to

polyacrylamide gel electrophoresis, the nuclease-resistant material included fragments of about 666 bases (Fig. 8). A 666 base fragment would correspond precisely to 481 bases of the codogenic region and 185 bases of the 3′ non-codogenic region of a muscle actin transcript. These results suggest that muscle actin mRNA is present in the egg.

To determine whether muscle actin mRNA persists during embryogenesis, poly(A)$^+$ RNA was isolated from various developmental stages, and dots were probed with SpMA (Tomlinson *et al.* 1986). As shown in Fig. 9, the intensity of the signals from oocytes and unfertilized eggs decreased during the early development. At the neurula stage, however, there was a transient increase in SpMA hybridization. These data suggest that maternal muscle actin transcripts persist in

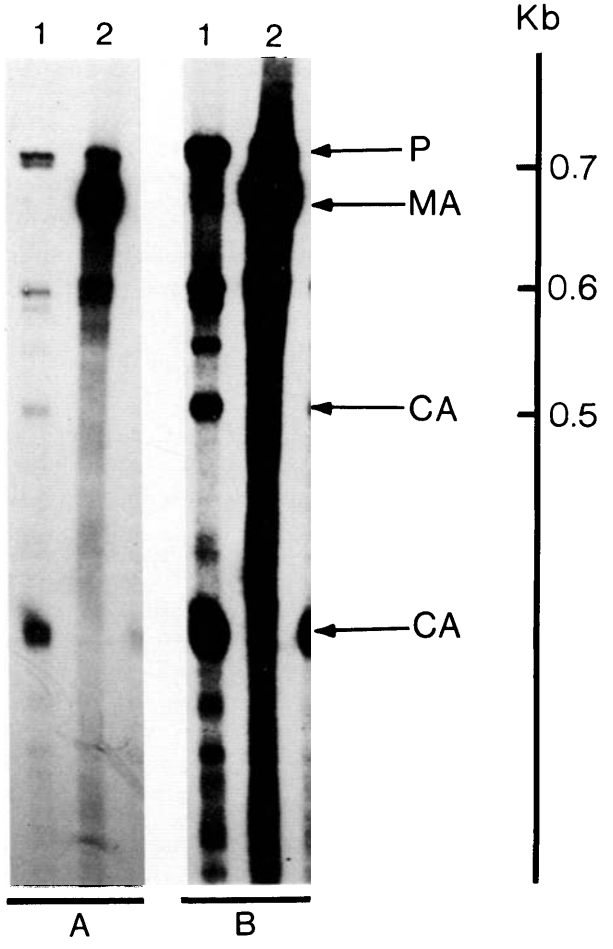

Fig. 8. S1 nuclease protection analysis of egg and adult muscle poly(A)$^+$ RNA using the SpGA probe. (A) 30 h exposure, (B) 72 h exposure. Lane 1, egg poly(A)$^+$ RNA; lane 2, muscle poly(A)$^+$ RNA; P, undigested probe; MA, protected fragments from the muscle actin transcript; CA, protected fragments from cytoskeletal or other actin transcripts; Kb, kilobases.

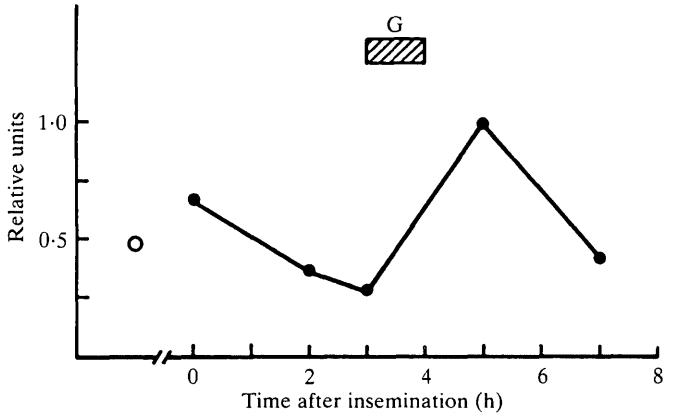

Fig. 9. Analysis of muscle actin mRNA during early development by dot hybridization with SpMA. Open circle (left), level of muscle actin mRNA in mature ovarian oocytes; closed circles (right), levels of muscle actin mRNA during embryonic development. The period of gastrulation (G) is indicated above.

the embryo at least until the time when muscle actin synthesis is first detected. The subsequent increase in muscle actin mRNA in neurulae is probably caused by the transcriptional activation of muscle actin genes. Thus, these results suggest that muscle actin synthesis prior to the neurula stage may be directed by maternal transcripts, while in subsequent developmental stages, embryonic transcripts may participate in the synthesis of this protein.

Although these results support the possibility that maternal mRNA may be a determinant for muscle actin, it remains to be demonstrated whether muscle actin transcripts present in the egg actually direct muscle actin synthesis *in vitro* (Jeffery *et al.* 1983) or *in vivo*. At present, it is also unknown whether maternal muscle actin mRNA is localized in the egg and differentially segregated into muscle progenitor cells during the early cleavages, or whether it is evenly distributed and translationally activated in the muscle progenitor cells. Presumably, these questions can be answered by examining the synthesis of actin isoforms in anucleate egg fragments and by using SpMA for *in situ* hybridization to sections of eggs and early embryos.

CONCLUDING REMARKS

The possibility that maternal mRNA may serve as a cytoplasmic determinant of tissue-specific proteins has been critically evaluated based on evidence from studies of endodermal AP and muscle actin expression in ascidian embryos. Although the independence of AP expression from embryonic gene activity is suggested by its insensitivity to inhibitors of transcription, this hypothesis is not supported by studies of AP development in anucleate egg fragments. Instead, the results suggest that AP expression requires the presence of a nucleus. The nuclear requirement does not preclude the possibility that maternal AP mRNA directs AP synthesis in the endodermal cells, but it indicates that nuclear events may act in

concert with AP mRNA translation. Because of the direct evidence for maternal muscle actin transcripts in eggs, the case for translation of muscle actin by maternal mRNA is stronger than that for AP. It is unknown, however, whether the presence of maternal muscle actin mRNA in early embryos is sufficient to direct muscle actin synthesis during early development. In conclusion, there is still too little evidence to say for certain whether maternal mRNA molecules serve as cytoplasmic determinants for tissue-specific proteins during ascidian development. Maternal mRNA coding for tissue-specific proteins, however, remains a prime candidate for at least one type of cytoplasmic determinant.

The research presented from this laboratory was supported by grants from the NIH (HD-13970), the NSF (DCB-84116763), the Muscular Dystrophy Association and The University of Texas Research Institute. We thank S. Daugherty for photographic assistance and J. Young for artwork.

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