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Identification of a cytoskeletal protein localized in the myoplasm of ascidian eggs: localization is modified during anural development

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Summary

The myoplasm of ascidian eggs is a localized cytoskeletal domain that is segregated to presumptive larval tail muscle cells during embryonic development. We have identified a cytoskeletal protein recognized by a vertebrate neurofilament monoclonal antibody (NN18) which is concentrated in the myoplasm in eggs and embryos of a variety of ascidian species. The NN18 antigen is localized in the periphery of unfertilized eggs, segregates with the myoplasm after fertilization, and enters the larval tail muscle cells during embryonic development. Western blots of one-dimensional and two-dimensional gels showed that the major component recognized by NN18 antibody is a $58\times10^3 M_r$ protein (p58), which exists in at least three different isoforms. The enrichment of p58 in the Triton X-100-insoluble fraction of eggs and its reticular staining pattern in eggs and embryos suggests that it is a cytoskeletal protein. In subsequent experiments, p58 was used as a marker to determine whether changes in the myoplasm occur in eggs of anural ascidian species, i.e. those exhibiting a life cycle lacking tadpole larvae with differentiated muscle cells. Although p58 was localized in the myoplasm in eggs of four urodele ascidian species that develop into swimming tadpole larvae, this protein was distributed uniformly in eggs of three anural ascidian species. The eggs of two of these anural species contained the actin lamina, another component of the myoplasm, whereas the third anural species lacked the actin lamina. There was no detectible localization of p58 after fertilization or segregation into muscle lineage cells during cleavage of anural eggs. NN18 antigen was uniformly distributed in pre-vitellogenic oocytes and then localized in the perinuclear zone during vitellogenesis of urodele and anural ascidians. Subsequently, NN18 antigen was concentrated in the peripheral cytoplasm of post-vitellogenic oocytes and mature eggs of urodele, but not anural, ascidians. It is concluded that the myoplasm of ascidian eggs contains an intermediate filament-like cytoskeletal network which is missing in anural species that have modified or eliminated the tadpole larva.

Key words: ascidian eggs, myoplasm, cytoskeleton, cytoplasmic localization, anural development.

Introduction

The eggs of many invertebrates contain localized cytoplasmic regions that are thought to be important in embryonic development (see Jeffery, 1988 for review). One of the best examples of a localized region is the myoplasm of ascidian eggs. The myoplasm is initially present in the cortex of unfertilized eggs and during ooplasmic segregation is translocated first into a cap in the vegetal pole region and then into a crescent in the posterior region of fertilized zygotes (Conklin, 1905; Sawada and Schatten, 1988; Jeffery and Bates, 1989; Sardet *et al.* 1989). During cleavage, the myoplasmic crescent is segregated into presumptive muscle blastomeres and eventually into the larval tail muscle cells. Although the myoplasm has multiple roles in embryonic development (see Jeffery and Swalla, 1990*b* for review), a primary function of this region may be to mediate autonomous determination of larval muscle cells. Several lines of evidence suggest that factors important in muscle cell development may be present in the myoplasm. First, only cells that contain myoplasm can develop muscle cell markers (Whittaker *et al.* 1977; Crowther and Whittaker, 1983; Deno *et al.* 1984). Second, when cytoplasm from presumptive muscle cells is diverted (Whittaker, 1980, 1982) or microinjected (Deno and Satoh, 1984) into non-muscle cells during cleavage, some of these cells develop muscle cell markers. Finally, microinjection of monoclonal antibodies against myoplasmic components into eggs blocks

subsequent expression of muscle cell markers (Nishikata *et al.* 1987). The myoplasmic factors involved in ascidian muscle cell development have yet to be identified.

The myoplasm is a localized cytoskeletal domain consisting of a submembrane actin lamina, which drives myoplasmic translocation during ooplasmic segregation (Jeffery and Meier, 1984), and an underlying network of filaments, with associated pigment granules, mitochondria and mRNA (Sawada and Osanai, 1981; Jeffery and Meier, 1983; Jeffery, 1984). The nature of the filamentous network has not been established, although morphological evidence suggests that it may be composed of intermediate filaments. Characterization of isolated myoplasmic crescents indicates that a specific set of egg polypeptides are present in the myoplasm (Jeffery, 1985). Progress in determining the developmental role of these and other myoplasmic components, however, has been hampered by the lack of functional assays. For example, genetic analysis, which has been important in revealing the role of localized gene products in *Drosophila* embryos (see Nüsslein-Volhard *et al.* 1987), is rudimentary in ascidians (Sabbadin, 1982).

An alternative approach for testing the developmental significance of substances localized in the myoplasm is to determine their existence and localization in closely related ascidian species that exhibit radical differences in their mode of development. Most ascidians exhibit urodele development, in which the egg develops into a swimming tadpole larva with a tail containing a notochord and muscle cells. In contrast, a limited number of ascidian species exhibit anural development, in which the development of a tadpole larva with differentiated notochord and muscle cells has been eliminated (see Jeffery and Swalla, 1990*a* for review). Anural development appears to have evolved several different times in uniform habitats where there is no selective advantage for a tadpole larva (Berrill, 1931). It has been suggested that mitochondria do not exhibit their usual localization in the myoplasm of the anural ascidian *Molgula arenata* (Whittaker, 1979*a*). Although muscle lineage cells are formed in anural ascidian embryos, they do not differentiate into tail muscle cells (Berrill, 1931; Whittaker, 1979*b*; Swalla and Jeffery, 1990*b*). Recent studies on the urodele ascidian *Molgula oculata* and the anural ascidian *Molgula occulta*, sympatric species that form viable interspecific hybrids, suggest that certain urodele larval features, such as notochord and brain pigment cells, can be expressed during development of anural eggs fertilized with sperm from the urodele species (Swalla and Jeffery, 1990*b*). In contrast, muscle cell differentiation is not rescued in the hybrid larvae. Thus, myoplasmic components that control development of muscle cells may be absent or modified in anural ascidian eggs.

In the present investigation, we have identified a 58×10^3 M_r cytoskeletal protein recognized by a vertebrate anti-neurofilament antibody that is localized in the myoplasm of urodele ascidian eggs. In contrast, this protein is uniformly distributed in eggs of several species of anural ascidians, suggesting that anural development is accompanied by changes in the organization of the myoplasmic cytoskeleton.

Materials and methods

Ascidians

The species used in this study were *Ascidia ceratodes* (supplied by Sea Life Supply, Monterey, CA); *Styela clava* and *Molgula manhattensis* (collected at Woods Hole, MA); *Molgula citrina* and *Molgula provisionalis* (collected in the Cape Cod canal near Bourne, MA); *Molgula oculata* and *Molgula occulta* (collected from sand flats at Roscoff, France); and *Bostrichobranchus pilularis* (supplied by Gulf Specimen Co., Panacea, FL). The animals were maintained either in running sea water or in aquaria containing Instant Ocean.

Gametes, insemination and embryo culture

Ascidians are self-sterile or self-fertile hermaphrodites. Embryos were obtained from oviparous species (*S. clava*, *A. ceratodes*, *M. oculata*, *M. occulta*, *M. manhattensis*, and *M. provisionalis*) by artificial fertilization. Preparation of gametes and insemination were carried out as described previously (see Tomlinson *et al.* 1987 for *S. clava*; Swalla and Jeffery, 1990*b* for *M. oculata* and *M. occulta*; and Costello and Hendley, 1971 for *M. citrina*). *A. ceratodes* gametes were obtained from the oviducts and sperm ducts of ripe dissected animals. Eggs were washed several times in a large volume of Millipore filtered sea water (MFSW), treated with acidic MFSW (pH 6.0) for 30–60 min to remove gelatinous material surrounding the eggs, and washed several times in MFSW before insemination. A drop of dry sperm was diluted in 10 ml of sea water and several drops of this suspension were used to inseminate eggs in about 100 ml of MFSW. Gametes were dissected from the gonads of *M. provisionalis*, a self-fertile species, incubated for 30 min, and then washed with several volumes of MFSW. Some experiments used mixed cultures containing gametes from several different *M. provisionalis* individuals. Eggs and developing embryos were dissected from the brood sacs of *M. citrina* and *B. pilularis*, viviparous species which usually contain all stages of pre-metamorphic development. Embryos of oviparous species were cultured in MFSW at 14°C (*A. ceratodes*), 17°C (*S. clava*, *M. provisionalis*, and *M. manhattensis*), or 18°C (*M. oculata* and *M. occulta*).

Preparation of cytoskeletal residues

Cytoskeletal residues were prepared from *S. clava* eggs according to Jeffery and Meier (1983). Embryos were washed several times in cytoskeletal extraction buffer (CEB), which consisted of 10 mM piperazine-N,N-bis [2-ethanesulfonic acid], 300 mM sucrose, 100 mM KCl, 5 mM magnesium acetate, 1 mM EGTA, 10 μM leupeptin (pH 8.0), and then extracted in CEB containing 0.5 % Triton X-100 (Sigma Chemical Co., St Louis, MO) for 60 min on ice. After extraction, the supernatant was decanted, and the detergent-insoluble pellet containing cytoskeletal residues was washed several times in CEB, then either fixed with 100 % methanol followed by 100 % ethanol (20 min each; −20°C) and embedded for immunocytochemistry or homogenized to prepare extracts for gel electrophoresis and Western blot analysis.

Immunocytochemistry

Eggs and embryos were collected by centrifugation, the

supernatant was decanted and the pellet was fixed in 100% methanol (−20°C) for 20 min, 100% ethanol (−20°C) for 20 min, and embedded in polyester wax (Steedman, 1957) as described by Norenburg and Barrett (1987). Sections were cut at 8 μm, de-waxed in absolute ethanol, hydrated, and stained with a number of different antibodies, including anti-neurofilament $160\times10^3 M_r$ monoclonal antibody (1:25 dilution; clone NN18; ICN Immunobiologicals, Lisle, IL), monoclonal anti-neurofilament $68\times10^3 M_r$ (1:25 dilution; clone NR-4; ICN), a *Halocynthia roretzi* anti-notochord monoclonal antibody (1:25 dilution; Mita-Miyazawa *et al.* 1987), and anti-actin monoclonal antibody (1:500 dilution; ICN). After staining, the specimens were rinsed in PBS and treated with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories Inc., Downingtown, PA). After rinsing several times in PBS, the specimens were mounted in glycerol, and examined and photographed using a Leitz epifluorescence microscope.

Protein extraction

Eggs or embryos were collected by centrifugation. The pellets were washed three times in 15 ml of ice cold homogenization buffer (HB) containing 0.01 M Tris–HCl, 0.01 M NaCl, 0.001 M $MgCl_2$ (pH 7.2). After washing, the samples were homogenized in 5 ml of ice-cold HB containing 10 μM leupeptin (Sigma Chemical Co., St. Louis, MO). Homogenates were prepared from cytoskeletal residues by the same method. The homogenates were stored at −70°C.

Polyacrylamide gel electrophoresis

One-dimensional (1D) gel electrophoresis was conducted according to Laemmli (1970). Protein samples, prepared as described above, were denatured and loaded on 12% polyacrylamide gels with 4% stacking gels. Two-dimensional (2D) gel electrophoresis was carried out as described by O'Farrell (1975). Protein samples were treated with a mixture of 50 $\mu g\,ml^{-1}$ each of DNAase I and pancreatic RNAase A for 5 min at 4°C, brought to 0.05 M lysine and 9 M urea, and mixed with an equal volume of 5% 2-mercaptoethanol, 2% Nonidet P-40, 2% ampholines (2% pH range 3.5–10), 0.2% SDS, and 9.8 M urea. The samples were loaded on 4% polyacrylamide isoelectric focusing gels for electrophoresis in the first dimension. After electrophoresis at 400 V for 15 h, the gels were removed from their tubes, equilibrated in 0.063 M Tris–HCl, 2.5% SDS, 5% β-mercaptoethanol, and 10% (w/v) glycerol (pH 6.8) for 1 h, and loaded onto 12% polyacrylamide slab gels containing 0.1% SDS. Electrophoresis in the second dimension was carried out at 80 V through the stacking gel and at 100 V through the separating gel. Gels were stained with silver nitrate according to Wray *et al.* (1981).

Western blots

Protein was transferred from 1D or 2D gels to nitrocellulose filters (0.45 μM; Schleicher & Schuell, Keene, NH) according to Towbin *et al.* (1979) using a Hoefer Transphor unit (Hoefer Scientific Instruments, San Francisco, CA) at 0.2 A for 3–4 h at 4°C. Nonspecific binding sites were blocked by incubation of filters for 30 min at room temperature in 0.14 M NaCl, 0.05 M Tris–HCl, pH 7.2, 1% BSA, 0.1% Tween 20. Filters were subsequently rinsed in 0.14 M NaCl, 0.05 M Tris–HCl (pH 7.2) 0.1% Tween 20 (TBST) and incubated for 30 min with NN18, NR4, or anti-notochord antibodies (1:500 dilutions) in TBST, 30 min with alkaline phosphatase-conjugated anti-mouse IgG (1:7500 dilution) (Promega, Madison, WI), and the alkaline phosphatase reaction product was developed according to the protocol supplied with a Protoblot (Promega) system.

Results

Antibody NN18 stains the myoplasm of eggs and embryos

Previous studies showed that the myoplasm of ascidian eggs contains cytoskeletal elements resembling intermediate filaments (Jeffery and Meier, 1983). Therefore, we tested the ability of intermediate filament (IF) antibodies to stain the myoplasm in sections of eggs and embryos. The antibodies used for staining included those that recognize specific types of vertebrate IFs as well an antibody that reacts with all classes of vertebrate and invertebrate IFs (Pruss *et al.* 1981). Although some of these antibodies stained specific cytoplasmic inclusions (Jeffery *et al.* 1990) or follicle and test cells, only NN18, a monoclonal antibody against the middle-molecular weight (MMW) neurofilament protein (Debus *et al.* 1983; Shaw *et al.* 1984), specifically stained the myoplasm (Fig. 1). NN18 also stained the test cells and follicle cells but, since these cells stained with control antibodies (Fig. 1A and data not shown) and generally are autofluorescent (Deno, 1987), this reaction was considered to be non-specific. Although fixation and embedding removed most of the weak autofluorescence of myoplasm reported in living ascidian eggs (Deno, 1987), controls to evaluate the possibility of autofluorescence were included with each NN18 staining experiment. For example, Fig. 1A shows staining of an unfertilized *A. ceratodes* egg with a *H. roretzi* notochord monoclonal antibody (Mita-Miyazawa *et al.* 1987) that did not cross-react with the ascidian species used in the present investigation. Note that with identical exposure times the control *A. ceratodes* unfertilized egg (Fig. 1A) is negative. In contrast, NN18 staining was concentrated in the myoplasm of unfertilized eggs (Fig. 1B), fertilized eggs (Fig. 1C–E), and embryos (Fig. 1F–K). NN18 stained a fine reticular network that appeared to be excluded from nuclei of early cleaving embryos (Fig. 1F,G) and tail muscle lineage cells (Fig. 1H–K). Lower levels of NN18 staining were observed throughout the egg cytoplasm (Fig. 1B–E), in the cytoplasm of non-muscle lineage cells of cleaving embryos (Fig. 1G–I), and especially in the neural tube of tailbud embryos (Fig. 1J). NN18 staining was undetectable after metamorphosis and in adult somatic tissues (data not shown). Although Fig. 1 shows NN18-staining in *A. ceratodes* eggs and embryos, this antibody showed similar staining patterns, including concentration of antigen in the myoplasm, throughout early development in every urodele ascidian species examined (Figs 6A, 7A,C,E; Fig. 8C,D and data not shown). The results show that NN18 antibody stains a cytoplasmic component concentrated in the myoplasm of eggs and the cytoplasm of larval tail muscle cells in urodele ascidians.

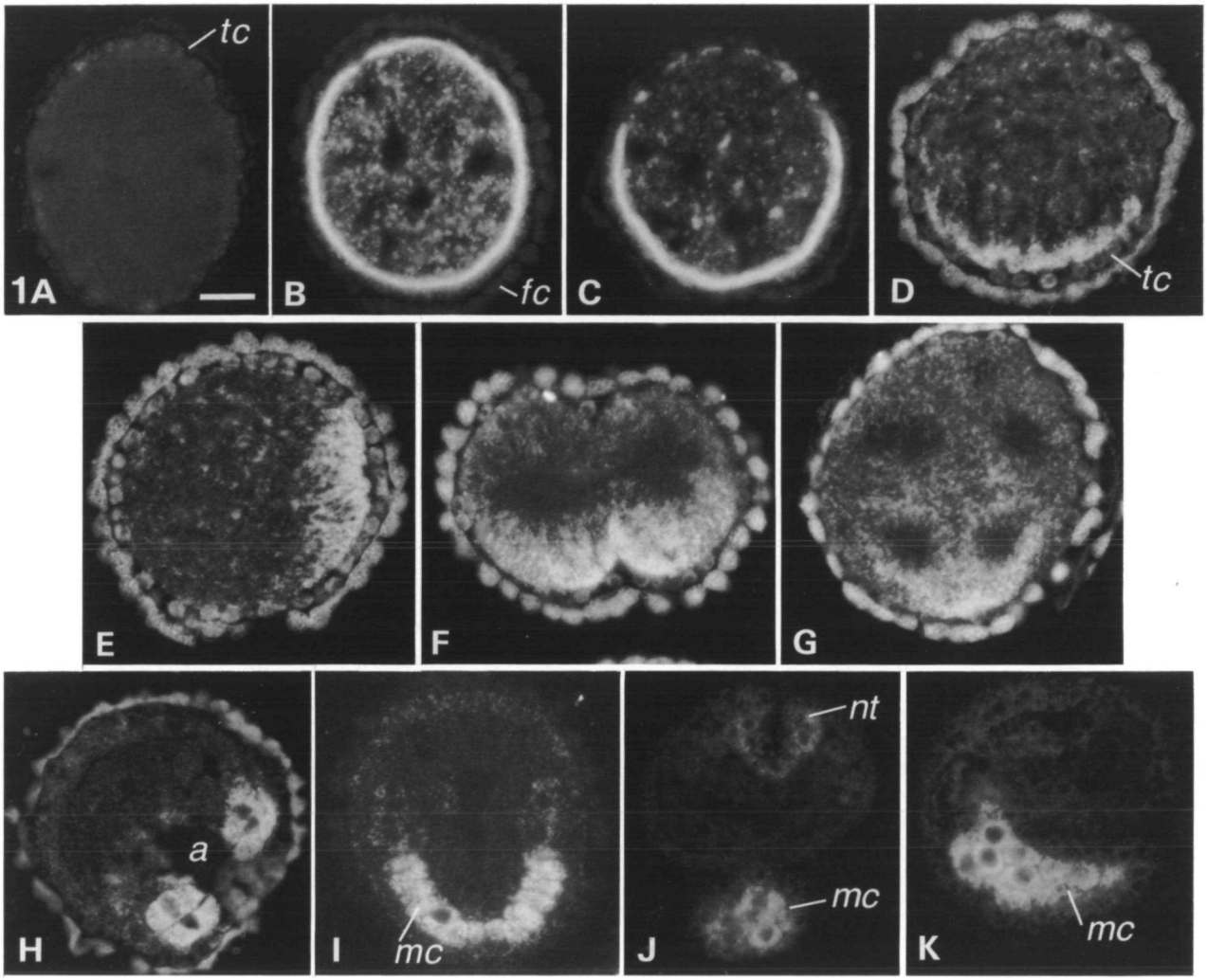

Fig. 1. Distribution of NN18 antigen during *A. ceratodes* development. (A) A section of an unfertilized egg stained with a *H. roretzi* anti-notochord antibody. Non-specific staining is shown in the test cells (tc), but there is no staining in the egg cytoplasm. (B–K) Sections of eggs and embryos stained with NN18. (B) An unfertilized egg showing NN18 staining concentrated in the cortical myoplasm. fc: follicle cells. (C–E) Fertilized eggs showing concentration of NN18 staining material in the vegetal pole region (bottom) during the first phase of ooplasmic segregation (C–D) and in the posterior region (right) during the second phase of ooplasmic segregation (E). (F–G) 2- (F) and 4- (G) cell embryos showing staining in the posterior region of the embryo (bottom). (H) A gastrula sectioned through the frontal plane showing staining in presumptive muscle cells; (a) archenteron. (I) An early tailbud-stage embryo showing staining in the developing tail muscle cells (mc). (J–K) Cross (J) and parasagittal (K) sections of later tailbud embryos showing staining in the presumptive muscle cells (mc) and neural tube (nt). Photographs were taken with identical exposures. Scale bar is 20 μm; magnification is the same in each frame.

The NN18 antigen is a 58×10^3 M_r polypeptide

The antigen(s) recognized by NN18 was identified by Western blotting. As shown in Fig. 2, NN18 stained a 58×10^3 M_r band in 1D gels containing proteins isolated from *A. ceratodes* eggs and embryos at various stages of development. The 58×10^3 M_r band was present at similar levels throughout embryonic development (Fig. 2), but decreased to undetectable levels during metamorphosis (data not shown). Additional bands did not stain at the neurula or tailbud stages (Fig. 2G–K) suggesting that the same antigen is recognized in the muscle and neural cells of *A. ceratodes* embryos. A component of similar mobility to the *A. ceratodes* 58×10^3 M_r band was stained in eggs of six other ascidian species, including those that exhibit urodele (Figs 3A–C and 5A) and anural development (Fig. 3D,E). In several of these species, NN18 also stained higher or lower relative molecular mass bands on Western blots. Some of these bands, including the high relative molecular mass band in *S. clava* eggs (Fig. 5A), were also stained by antibodies that did not react with the 58×10^3 M_r band (data not shown), indicating they are nonspecific reactants. Other components, including several high relative molecular mass bands visible in eggs of several different *Molgula* species (Fig. 3; also see Fig. 4D) and the low molecular mass band in *M.*

Fig. 2. Identification of NN18 antigen by Western blots of 1D gels containing *A. ceratodes* eggs and embryos. From left to right the lanes represent (A) unfertilized eggs, (B) 1-cell embryos, (C) 2-cell embryos, (D) 4-cell embryos, (E) 32-cell embryos, (F) gastrulae, (G) neurulae, (H–J) early-, mid-, late-tailbud embryos, and (K) swimming tadpoles. The arrowhead indicates the position of p58. Equivalent amounts of protein were loaded on each lane.

provisionalis eggs (Fig. 3E), which could be a breakdown product of the $58\times10^3 M_r$ component, did not react with other antibodies. These minor components appear to react with NN18 specifically, but their significance is unknown. The results indicate that the primary antigen recognized by NN18 antibody is a $58\times10^3 M_r$ protein (p58) which is present in eggs of a wide variety of ascidian species.

p58 was further characterized by Western blotting 2D gels (Fig. 4). In embryos of the urodele ascidians *S. clava* (Fig. 4B) and *A. ceratodes* (Fig. 4C) and eggs of the anural ascidian *M. occulta* (Fig. 4D), p58 consists of at least three different isoforms with pIs ranging between about 6.0 and 6.5. By comparing the position of NN18 staining components detected by Western blots (Fig. 4B) with silver-stained spots in gels containing the same proteins (Fig. 4A), the p58 isoforms were identified as a group of polypeptides known to be restricted to isolated myoplasmic crescents (Jeffery, 1985). Thus, the Western blots are consistent with the egg and embryo immunofluorescence in showing that NN18 stains a component concentrated in the myoplasm.

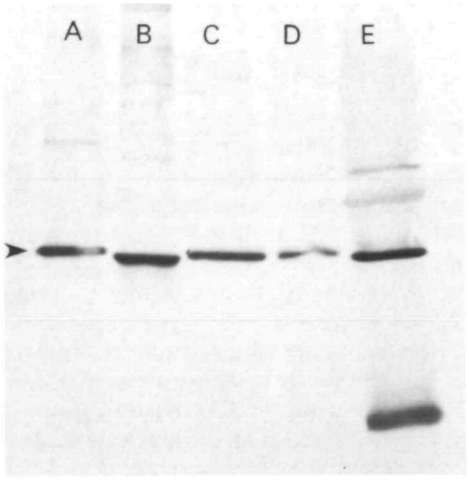

Fig. 3. Identification of NN18 antigen by Western blots of 1D gels containing egg proteins from different ascidian species. (A) *M. citrina* eggs. (B) *M. manhattensis* eggs. (C) *M. oculata* eggs. (D) *M. occulta* eggs. (E) *M. provisionalis* eggs. The arrowhead indicates the position of p58. Equivalent amounts of protein were loaded on each lane.

In summary, the results indicate that the myoplasm of ascidian eggs contains a $58\times10^3 M_r$ protein that is recognized by a vertebrate MMW neurofilament antibody.

p58 is a cytoskeletal protein

The reticular nature of NN18 staining suggests that p58 may be a component of the cytoskeleton. To test this possibility, p58 distribution was examined in *S. clava* eggs treated with the non-ionic detergent Triton X-100. Fig. 5 shows a Western blot of a gel containing proteins extracted from whole eggs and the insoluble and supernatant fractions of detergent-extracted eggs. Most of the p58 present in whole eggs (Fig. 5A) was recovered in the insoluble fraction (Fig. 5B) after detergent treatment, suggesting that this protein is enriched in the cytoskeleton. These results were confirmed by experiments in which sections of whole (Fig. 6A) and Triton X-100 (Fig. 6B) extracted *S. clava* eggs were stained with NN18 and photographed with identical exposures. The results show that NN18 antigen is concentrated in the myoplasm of egg cytoskeletal residues (Fig. 6B). In fact, extraction intensified immunofluorescent staining, perhaps by increasing accessibility to the antigen. Thus, p58 appears to be a cytoskeletal protein.

p58 is not localized in anural ascidian eggs and embryos

The absence of tail muscle cell differentiation in anural ascidian embryos may be due to changes in the organization of the myoplasm (Whittaker, 1979*a*; Swalla and Jeffery, 1990*b*). Both urodele and anural ascidians contain p58, as shown by Western blotting (Fig. 3). However, immunofluorescence with NN18 showed dramatic differences in p58 localization in eggs and embryos of urodele and anural ascidians. Eggs of several phylogenetically diverse urodele ascidians and their closest anural counterparts were examined to

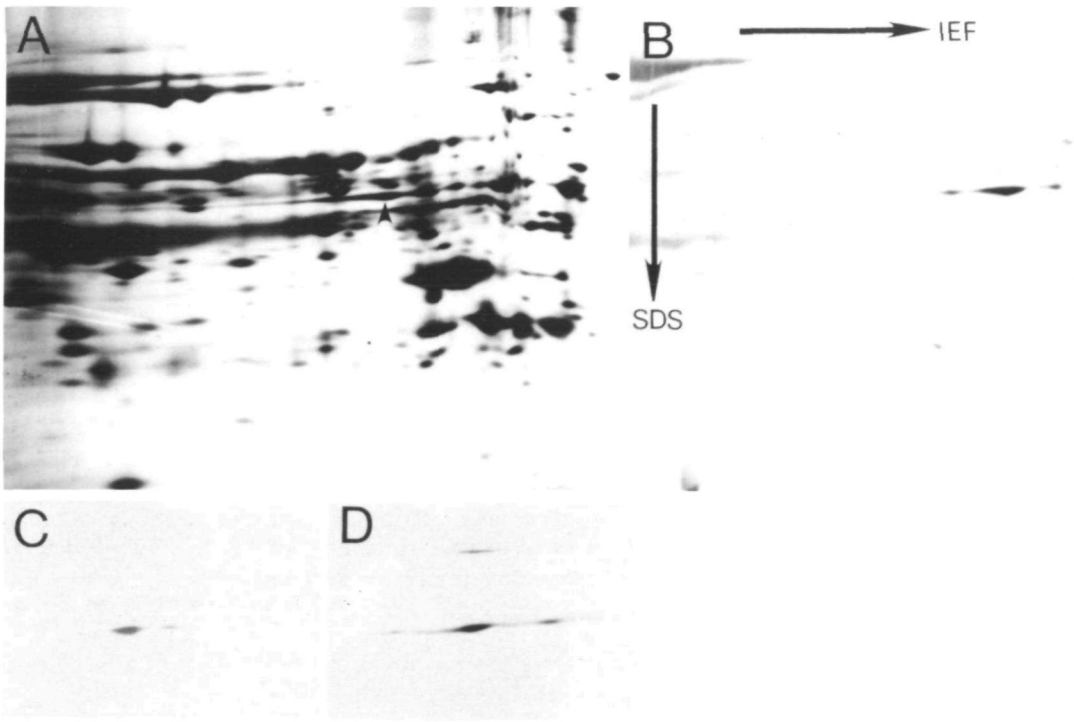

Fig. 4. Identification of NN18 antigen in 2D gels. (A) A silver stained gel containing *S. clava* tailbud proteins. The arrowhead indicates the position of the middle NN18-staining isoform. (B) A Western blot of a gel containing *S. clava* tailbud proteins and run in parallel to the gel shown in (A). IEF: isoelectric focusing direction. SDS: direction of electrophoresis through SDS gel. (C) A Western blot of a gel containing *A. ceratodes* gastrula proteins. (D) A Western blot of a gel containing *M. occulta* egg proteins. Only the NN18 staining region of the gel is shown in C–D. Equivalent amounts of protein were loaded on each lane.

Fig. 5. Identification of the NN18 antigen in *S. clava* cytoskeletal residues. Western blot of a 1D gel containing proteins extracted from whole eggs (A) and Triton X-100-insoluble (B) and the supernatant (C) fractions. Equivalent volumes of protein were loaded on lanes A, B and C. The position of p58 is shown by the arrowhead.

minimize species-specific differences (Fig. 7). NN18 staining was then examined during embryonic development (Fig. 8) of *M. oculata* and *M. occulta*, closely related species that exhibit urodele and anural development respectively (Monniot, 1969; Swalla and Jeffery, 1990*b*). Unfertilized *M. oculata* eggs showed concentrated NN18 staining in the myoplasm (Fig. 7A), however, in *M. occulta* eggs, NN18 staining was diffuse (Fig. 7B), suggesting that p58 is distributed throughout the cytoplasm. In *M. occulta*, NN18 staining was uniformly distributed at the 2-cell stage (Fig. 8A) and was not concentrated in the posterior region containing the muscle lineage cells of an eight hour embryo (Fig. 8B), suggesting that p58 does not segregate into the myoplasm or muscle lineage cells of anural embryos. However, some concentrated NN18 staining was observed in neural tissue of *M. occulta* embryos (Fig. 8B), consistent with the fact that anural embryos form a neural tube (Swalla and Jeffery, 1990*b*). In contrast, *M. oculata* embryos showed a staining pattern typical of other urodele species (compare Fig. 8C,D with Fig. 1F,K); NN18 antigen was concentrated in the vegetal posterior at the 2-cell stage (Fig. 8C) and in the tail muscle and neural tissues at the tailbud-stage (Fig. 8D), eight hours after fertilization. In further experiments, the distribution of NN18 staining material was examined in unfertilized eggs of *M. citrina* and *B. pilularis*, viviparous species with much larger eggs and slower development than *M. oculata* and *M. occulta*. Eggs of the urodele species (*M. citrina*) showed intense cortical NN18 staining (Fig. 7C), which was absent in eggs of the anural species (*B. pilularis*) (Fig. 7D). Finally, unfertilized eggs of the urodele *M. manhattensis* and the related anural species *M. provisionalis* (Van Name, 1945; Plough, 1978) were stained. *M. manhattensis* eggs showed NN18 staining concentrated in the periphery (Fig. 7E), whereas *M. provisionalis* eggs failed to show peripheral staining (Fig. 7F). These results indicate that, although p58 is present in eggs of

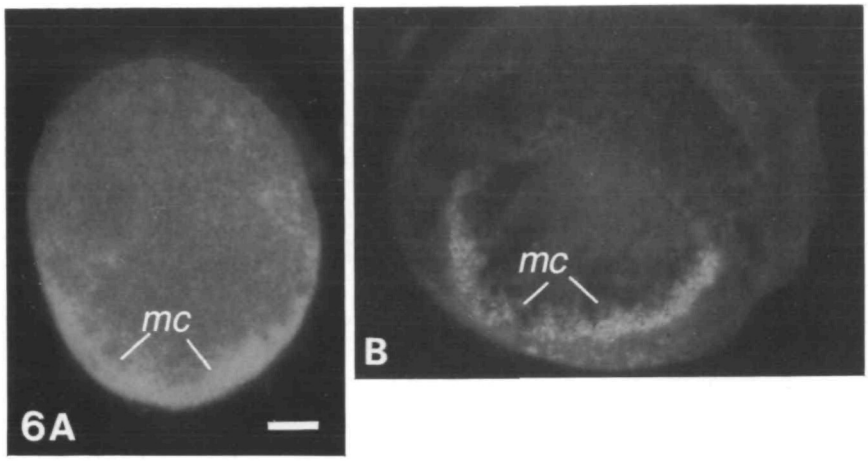

Fig. 6. Distribution of NN18 antigen in sections of whole (A) and Triton X-100 extracted (B) *S. clava* eggs. Photographs were taken at identical exposures. mc, myoplasmic cap. The scale bar is 20 μm; magnification is the same in each frame.

anural ascidians, it is not concentrated in the myoplasm.

The actin lamina, another component of the myoplasmic cytoskeleton (Sawada and Osanai, 1981; Jeffery and Meier, 1983), was examined in experiments similar to those described above. Even though eggs of the anural species *M. occulta* and *B. pilularis* lack NN18 staining, they contain a peripheral actin lamina (Fig. 9A–B), suggesting that they retain some of the myoplasmic cytoskeletal organization. In contrast, *M. provisionalis* eggs appear to lack both peripheral actin (Fig. 9C) and NN18 staining (Fig. 7F). This result was not due to inability of the antibody to detect actin in *M. provisionalis* because positive staining was noted after metamorphosis in epidermal ampullae, structures known to contain actin filament bundles (Bates and Mallett, 1990), in experiments in which developing juveniles were mixed with unfertilized eggs before fixation (data not shown). These results indicate differential degeneration of the myoplasmic cytoskeleton has occurred in eggs of different anural ascidian species.

Localization of p58 during oogenesis and maturation

To investigate the ontogeny of p58 localization in urodele and anural ascidians, *M. oculata* and *M. occulta* oocytes and mature eggs were stained with NN18. Based on their size and yolk content, ascidian oocytes have been divided into stages I (pre-vitellogenic), II (vitellogenic), and III (post-vitellogenic) (Jeffery and Capco, 1978). When ripe stage III *Molgula* oocytes are released into sea water they undergo maturation (Whittaker, 1979*b*; Sawada and Schatten, 1988). NN18 antigen was distributed throughout the cytoplasm in stage I oocytes of both species (Fig. 10A,E). In stage II oocytes of both species, NN18 staining was concentrated in the perinuclear region, from which it extended out toward the cortex in a reticular pattern (Fig. 10B,F). In contrast, NN18 staining in post-vitellogenic Stage III *M. occulta* oocytes was uniform and reduced in intensity relative to younger oocytes (Fig. 10C–D). Stage III *M. oculata* oocytes showed NN18 staining enhanced in the periphery and reduced in the internal cytoplasm, particularly the perinuclear region (Fig. 10G); and peripheral concentration of antigen continued to be observed after oocyte maturation (Fig. 10H). These results suggest that the myoplasmic cytoskeletal domain of anural ascidian eggs may be modified by failure of p58 to accumulate in the oocyte periphery after vitellogenesis.

Discussion

In the present investigation, we have identified a cytoskeletal protein that is concentrated in the myoplasm of ascidian eggs. The spatial distribution of p58 is similar to that of a myoplasmic antigen recognized by IIH10G4, a monoclonal antibody produced from isolated myoplasmic crescents of ascidian eggs (Nishikata *et al.* 1987). Although NN18 and IIH10G4 antibodies both react with a myoplasmic component that is subsequently concentrated in tail muscle and neural cells of tailbud embryos, their possible identity is uncertain because characterization of the IIH10G4 antigen by Western blotting has not been reported (Nishikata *et al.* 1987). The concentration of p58 in the myoplasm, demonstrated by immunofluorescence and Western blot experiments in the present investigation, is confirmed by a previous study in which components of the same electrophoretic mobility as p58 were shown to be enriched in isolated myoplasmic crescents (Jeffery, 1985).

The ascidian cytoskeletal protein localized in the myoplasm was detected by staining with NN18, a monoclonal antibody that recognizes the MMW neurofilament protein in a variety of vertebrate species (Debus *et al.* 1983; Shaw *et al.* 1984). The reaction of this antibody with p58 suggests that the antigen shares an epitope with a specific class of vertebrate IFs. In somatic cells, IFs are commonly observed to form a cage-like structure around the nuclear envelope (Franke, 1971), which may serve as an IF assembly site (Georgatos and Blobel, 1987; Vikstrom *et al.* 1989). The reticular distribution, enrichment in the Triton X-100 insoluble fraction, and perinuclear distribution in vitellogenic oocytes of the NN18 staining material supports the possibility that p58 is an ascidian IF protein. Consistent with this interpretation, screening

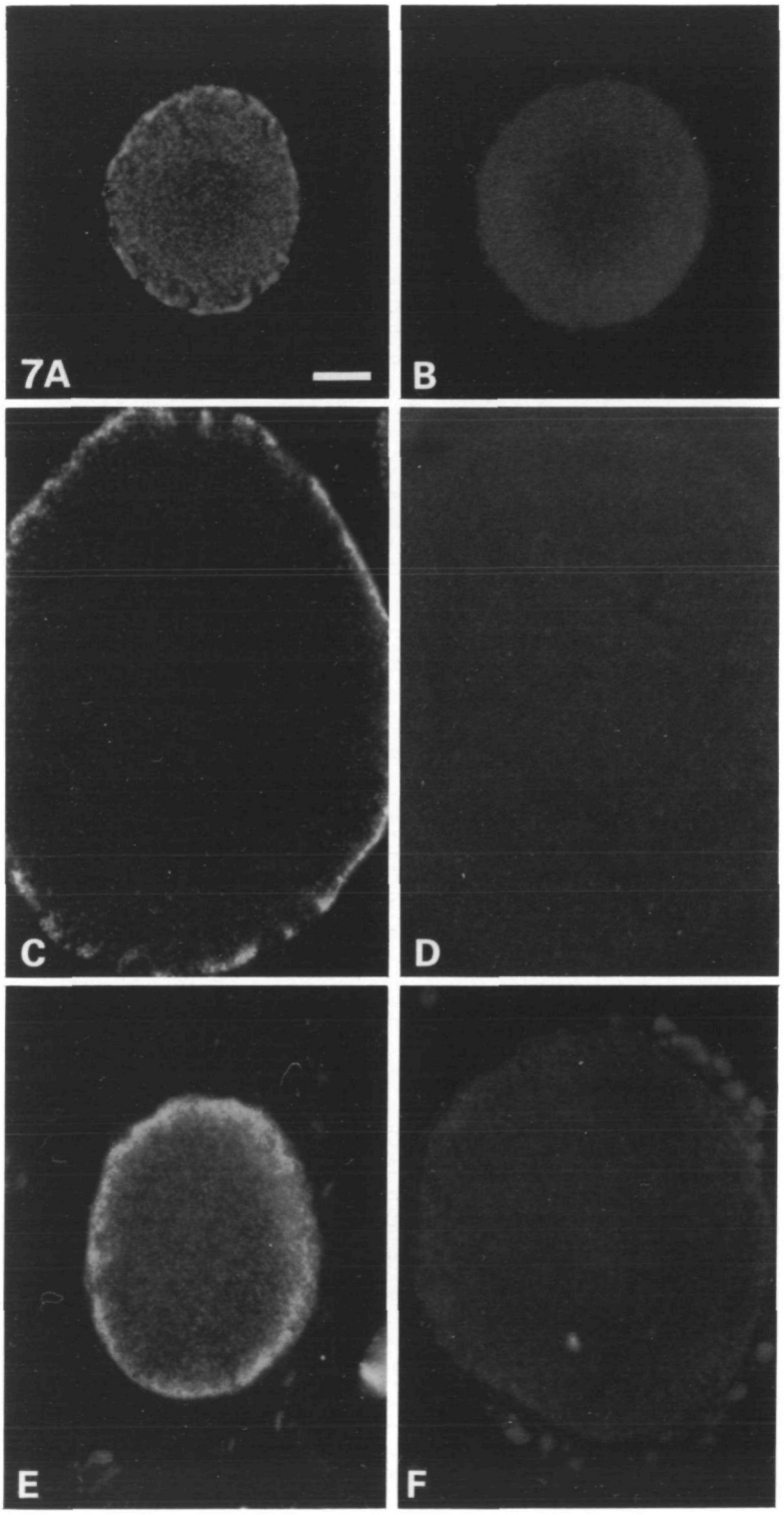

Fig. 7. Distribution of NN18 antigen in sectioned unfertilized eggs of urodele (A, C, E) and anural (B, D, F) ascidians. NN18 staining is localized in the cortical myoplasm in eggs of the urodele ascidians *M. oculata* (A), *M. citrina* (C), and *M. manhattensis* (E). NN18 staining is not localized in the egg periphery of the anural ascidians *M. occulta* (B), *B. pilularis* (D), and *M. provisionalis* (F). The scale bar is 20 μm; magnification is the same in each frame.

an ascidian expression library with NN18 antibody has yielded a cDNA clone showing significant identity in protein sequence to the N-terminal head domain of porcine MMW neurofilament protein (Swalla and Jeffery, 1990*a*), the original NN18 antigen (Debus *et al.* 1983). Because p58 could not be detected in adult tissues, however, it may represent an IF system that is restricted to eggs and early embryos (Klymkowsky *et al.* 1989).

Previous studies indicate that the myoplasm consists of two parts: an actin lamina, which lies immediately beneath the egg plasma membrane, and a more internal filamentous network, which is morphologically similar to IFs (Jeffery and Meier, 1983). Myoplasmic organ-

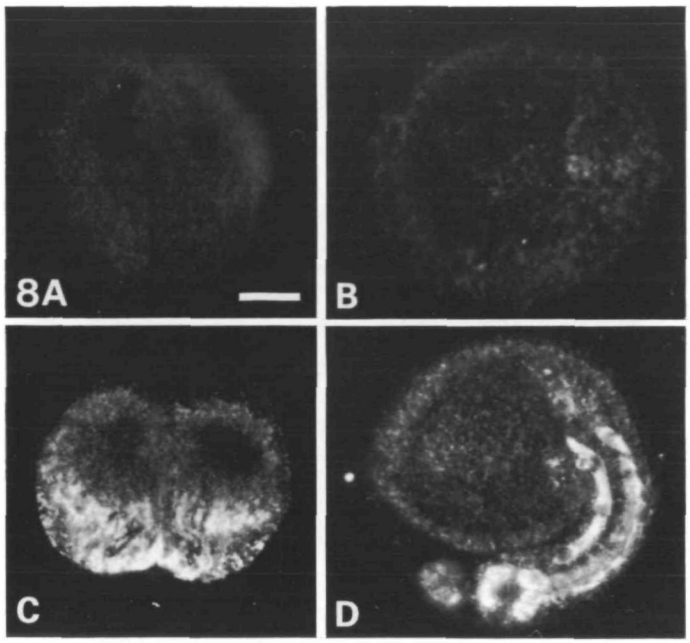

Fig. 8. Distribution of NN18 antigen in embryos of the anural ascidian *M. occulta* (A, B) and the urodele ascidian *M. oculata* (C, D). NN18 staining is concentrated in the vegetal hemisphere of 2-cell *M. oculata* embryos (C) but not in 2-cell *M. occulta* embryos (A). NN18 staining is concentrated in the tail muscle cells of *M. oculata* tailbud embryos (D) but not in the posterior region of 8 h *M. occulta* embryos (B). In B and D, the anterior side of each embryo faces left. NN18 staining is noted in a few neural cells located at the midline in the posterior region of the *M. occulta* embryo (B). The scale bar is 20 μm: magnification is the same in each frame.

elles, including mitochondria and pigment granules, are attached to the internal network, which promotes their localization and segregation during early development (see Jeffery and Swalla, 1990*b* for review). The broad distribution of NN18 staining in the egg cortex (relative to the actin lamina) and the presumed relationship between p58 and IF proteins, suggests that p58 is a part of the internal myoplasmic network. Antibody staining at the ultrastructural level, however, will be necessary to establish whether this protein is a component of the elaborate filamentous network that underlies the actin lamina.

The existence of ascidian species in which the tadpole larva is eliminated and larval muscle differentiation is suppressed provides a unique approach to investigate the significance of myoplasmic components. Previous studies have shown that genes encoding contractile proteins are not expressed during embryogenesis in anural ascidians (Swalla and Jeffery, 1990*b*). Furthermore, contractile protein expression is not among the urodele features that can be rescued by fertilizing eggs of the anural ascidian *M. occulta* with sperm from the urodele ascidian *M. oculata* (Swalla and Jeffery, 1990*b*), suggesting that components involved in larval muscle cell development are absent or modified in *M. occulta* eggs. Therefore, it is significant that p58 is not localized

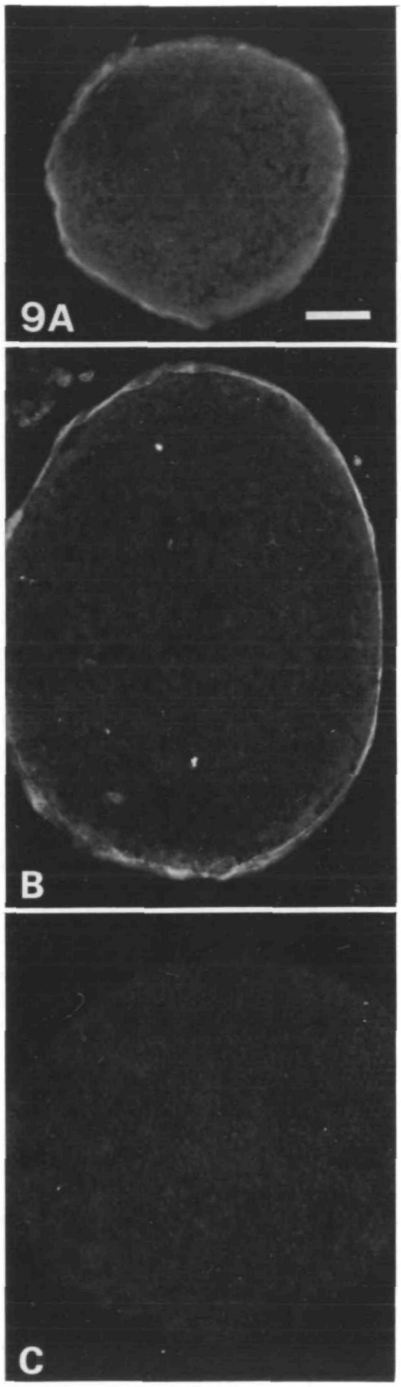

Fig. 9. Distribution of actin in sectioned unfertilized eggs of anural ascidians stained with actin antibody. A peripheral actin lamina is present in eggs of *M. occulta* (A) and *B. pilularis* (B), but not in eggs of *M. provisionalis* (C). The scale bar is 20 μm; magnification is the same in each frame.

in eggs of *M. occulta*, *M. provisionalis*, and *B. pilularis*, phylogenetically-diverse species of anural ascidians that probably evolved independently (Berrill, 1931). These results bring up the possibility that the cytoskeletal matrix containing p58 may serve as a scaffold for

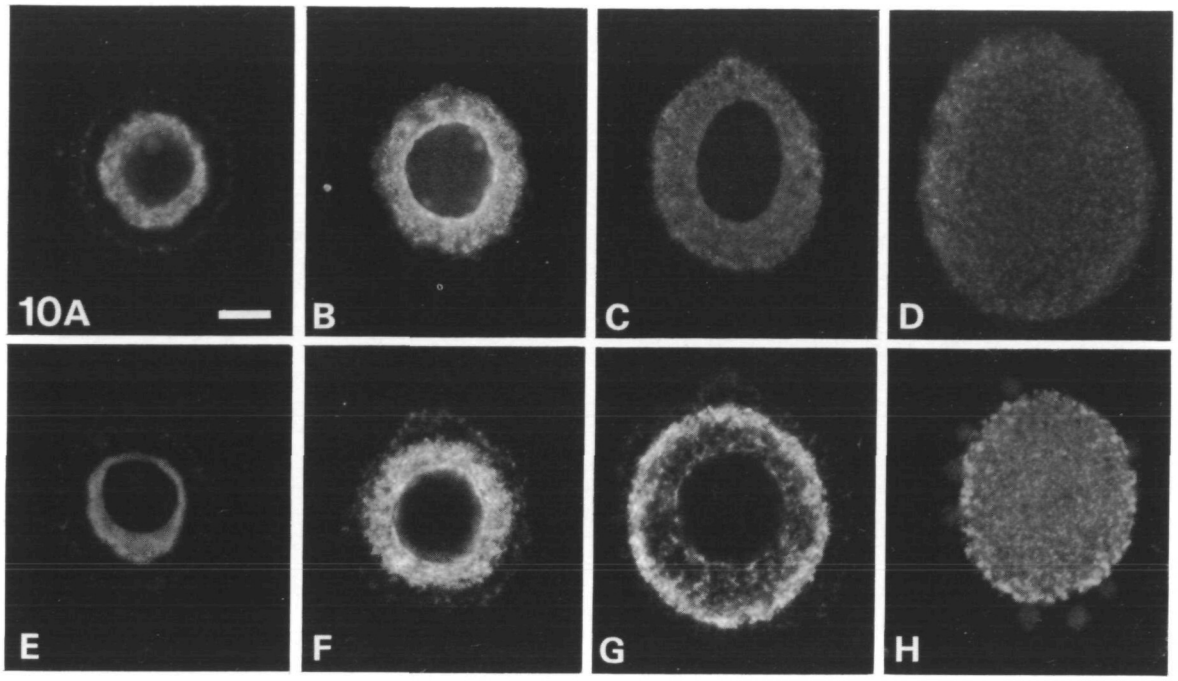

Fig. 10. Distribution of NN18 antigen in sectioned oocytes and mature eggs of the anural ascidian *M. occulta* (A–D) and the urodele ascidian *M. oculata* (E–H). (A, E) Stage I oocytes. (B, F) Stage II oocytes. Note that NN18 antigen is concentrated in a perinuclear ring in stage II oocytes (B, F) of both species. (C, G) Stage III oocytes. (D, H) Mature eggs. Note that a peripheral concentration of NN18-staining material is present in *M. oculata* (G, H), but not *M. occulta* (C, D), stage III oocytes and mature eggs. The germinal vesicle is the unstained sphere within the oocyte cytoplasm. The scale bar is 20 μm; magnification is the same in each frame.

localization and segregation of muscle determinants during ascidian development.

NN18 antigen is distributed throughout the cytoplasm of previtellogenic oocytes of urodele and anural ascidians, but exists as a perinuclear localization with extensions radiating toward the oocyte periphery during vitellogenesis. Although the mechanism of p58 localization during oogenesis is unknown, an intriguing possibility is that cytoskeletal filaments, assembled near the nuclear envelope, polymerize toward the oocyte plasma membrane where they interact with the actin lamina, which is already present in previtellogenic oocytes (Jeffery, unpublished). This scenario is consistent with the topology of vimentin IF assembly in somatic cells, which is thought to proceed vectorially from the nuclear envelope to the plasma membrane (Georgatos and Blobel, 1987; Vikstrom *et al.* 1989). Subsequent concentration of cytoskeletal components in the myoplasm of urodele ascidian oocytes during the post-vitellogenic period might involve terminating perinuclear assembly, severing existing attachments from the assembly zone, and peripheral translocation of the IF network.

Our results show that localization of NN18 antigen in the egg periphery is first apparent in post-vitellogenic oocytes. In anural ascidians, the terminal stages of p58 localization appear to be defective, resulting in uniform distribution of this protein in the cytoplasm of unfertilized eggs. Then, during subsequent development of anural embryos, p58 does not segregate preferentially into the presumptive muscle lineage cells, as it does during development of urodele embryos. Although the mechanisms underlying changes in p58 distribution in anural ascidian eggs are unknown, randomization is unlikely to be due to lack of attachment sites in the oocyte membrane skeleton because the actin lamina was observed in eggs of two of the three anural species examined. More reasonable possibilities are that peripheral translocation of the IF network or ankryn-like proteins (Lazarides and Woods, 1989), which could link internal cytoskeletal elements to the membrane skeleton, may be altered in anural ascidian eggs.

Our results imply that changes in organization of the myoplasmic cytoskeleton, including the actin lamina and the p58-containing cytoskeletal network, have occurred in ascidians that have eliminated the conventional urodele mode of development. Anural ascidians are divided into two groups based on whether they exhibit a hatched larval stage (indirect anural development) or metamorphose directly into an adult (direct anural development) (Jeffery and Swalla, 1990*a*). Anural species that inhabit sandy or muddy bottoms, which exhibit indirect or direct development, are thought to be more recently evolved than those species that inhabit the shoreline and exhibit direct development (Berrill, 1931). Therefore, it is interesting that the myoplasm of *M. provisionalis*, a shoreline species, lacks both the actin lamina and p58 localization, whereas *M. occulta* and *B. pilularis*, bottom-dwelling species,

contain the actin lamina but lack p58 localization in the myoplasm. These results suggest that progressive degeneration of the myoplasm has accompanied the evolution of anural development.

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