Messenger RNA in the Cytoskeletal Framework: Analysis by In Situ Hybridization

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ABSTRACT The possibility of an association of mRNA with the cytoskeletal framework (CF) of ascidian (Styela plicata) follicle cells was examined in this study. The approach was to extract the follicle cells with Triton X-100 and determine whether mRNA persisted in the insoluble residue by two methods, in situ hybridization with poly(U) and actin DNA probes and the incorporation of radioactive isotopes into RNA. Triton X-100 extraction of follicle cells yielded a filamentous CF containing ~70% of the total poly (A) but only 9% of the total lipid, 23% of the total protein, and 28% of the total RNA. In situ hybridization with a poly (U) probe indicated that ~70% of the poly (A) was associated with the CF. In situ hybridization with a cloned actin DNA probe indicated that ~60% of the actin mRNA was associated with the CF. Autoradiography of detergent-extracted follicle cells, which had been labeled with [3H]uridine or [3H]adenosine, indicated that >90% of the newly synthesized poly (A)+RNA was preserved in the CF. Thus more newly synthesized mRNA than steady-state mRNA may be present in the Triton X-100 insoluble fraction. It is concluded that a significant proportion of the mRNA complement of ascidian follicle cells is associated with the CF.

The cytoplasm of many eukaryotic cells contains an elaborate network of filaments and associated components which has been termed the cytoskeletal framework (CF) (21). The CF includes microtubules, microfilaments, intermediate filaments, microtrabeculae (36), centrioles (20), a membrane protein lamina (2), and probably many other cellular components. It can be visualized by immunofluorescence microscopy of intact cells (19, 24, 25, 35) or by electron microscopy of detergent-extracted cells (3, 20, 27, 29, 30). Gentle disruption of eukaryotic cells with nonionic detergents permits concomitant biochemical and morphological analyses of the CF after differential centrifugation is used to separate the cell lysate into detergent soluble and insoluble fractions. An important result of these biochemical studies is that most of the polyribosomal mRNA is found to be associated with the CF (8, 20, 33).

The purpose of the present investigation was to test the possibility of mRNA interaction with the CF by cytological methods, in particular in situ hybridization. In this paper, in situ hybridization with poly(U) or a cloned actin DNA probe and radioactive isotope tracing methods have been used to demonstrate that a significant proportion of the mRNA of follicle cells is associated with the CF. The follicle cells of ascidian eggs were selected as a model system because they are known to contain a filamentous cytoskeleton (9, 10) and are extremely active in the synthesis and accumulation of poly(A)+RNA during oogenesis and early development (15). The association of mRNA with the CF may be a mechanism for controlling the polarity of mRNA distribution in eukaryotic cells.

MATERIALS AND METHODS

Biological Materials and Radioisotope Incorporation

The eggs of the ascidian Styela plicata, obtained from Pacific Biomedical Laboratories (Venice, CA), were used as a source of follicle cells. Ripe eggs with mature follicular envelopes (egg-envelope complexes) were prepared from surgically removed gonads or obtained by photoperiod-induced spawning (34). The egg-envelope complexes were washed six times by centrifugation (600 g for 30 s) through 20 vol of artificial sea water (ASW). Isolated follicle cells were prepared by resuspending the egg-envelope complexes in 20 vol of pH 6.5 ASW containing 0.005 M EDTA. After incubation in EDTA-ASW for about 15 min the follicle cells become loosened from the chorion and could be separated from the remainder of the egg-envelope complex by gentle agitation of the suspension. Centrifugation at 600 g for 30 s pellet the eggs and other parts of the envelope while the follicle cells remained in the supernatant fraction. The isolated follicle cells were washed several times by centrifugation through 20 vol of ASW (6,000 g for 10 min) before further manipulations. Radioactive incorporation experiments were carried out by adding 10 µCi/ml of [3H]uridine (25 Ci/mmol) or [3H]adenosine (33 Ci/mmol) (New England Nuclear, Boston, MA) to egg-envelope complexes or to isolated follicle cells resuspended in Millipore (0.45 µm pore size)-filtered ASW supplemented with 0.25 mg/ml streptomycin sulfate. After the termination of the incorporation period the egg-envelope complexes or the
isolated follicle cells were washed in the appropriate buffer and processed as described below.

**Detergent Extraction**

To prepare the CF of follicle cells, egg-envelope complexes or isolated follicle cells were extracted with the nonionic detergent Triton X-100. A number of different extraction conditions were tested in pilot studies, including buffers containing 0.25 to 1% Triton X-100, 0.05 to 0.6 M KCl and 0 to 0.01 M CaCl₂. The following procedure was developed as a result and was found to be necessary to prevent mRNA degradation and to preserve cytoskeletal filaments in the CF. Egg-envelope complexes or isolated follicle cells were washed with the optimal extraction buffer (0.3 M sucrose, 0.1 M KCl, 0.005 M Mg acetate, 0.001 M EGTA, 0.01 M PIPES, 10 μM leupeptin, 1 mg/ml heparin; pH 6.8), then extracted for 10 min at 4°C with extraction buffer containing 0.5% Triton X-100. The detergent-insoluble residue was collected by centrifugation, washed twice, and resuspended in extraction buffer lacking detergent.

**Biochemical Analyses**

The distribution of lipid (37), protein (22), and poly (A) (16) between the soluble and cytoskeletal framework fractions of isolated follicle cells was measured as described previously. Total RNA in phenol extracts of the soluble and cytoskeletal framework fractions was measured by its absorbance at a wavelength of 260 nm.

**Electron Microscopy**

Whole mounts of the follicle cell CF were prepared by pipetting detergent extracted egg-envelope complexes onto 300 mesh grids, allowing them to settle and then spreading them across the surface of the grid with a blunt glass rod. This results in the detachment of the follicle cell CF from the chorion and its partial flattening on the surface of the grid. The whole mounts were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 30 min, washed in buffer without fixative, postfixed for 5 min with 1% OsO₄ at room temperature, dehydrated, and critical point dried before microscopy.

**Histology**

Intact or detergent-extracted egg-envelope complexes were fixed for 1 h at 4°C in Petrenkevichs fluid, dehydrated through an ethanol series (at -20°C for in situ hybridization), cleared in toluene and embedded in paraplast. Sections, 8-μm thick, were cut and attached to glass slides. Slides prepared from specimens labeled with radioactive isotopes were treated with cold 5% trichloroacetic acid (TCA) for 10 min before autoradiography.

**In Situ Hybridization**

In situ hybridization with poly(U) was carried out essentially as described previously (4). The slides were successively treated with 100 μg/ml DNase I (Boehringer-Mannheim) in 0.1 M Tris-HCl (pH 7.5) for 1 h at 37°C; 0.9 μCi/ml [³H]poly(U) (4.65 Ci/mmol; New England Nuclear) in 0.01 M Tris-HCl, 0.2 M NaCl, 0.005 M MgCl₂ (pH 7.5) for 3 h at 50°C; 50 μg/ml pancreatic RNase A in 0.05 M Tris-HCl, 0.1 M KCl, 0.001 M MgCl₂ (pH 7.5) for 1 h at 37°C; 5% TCA for 10 min at 4°C; double distilled water, and then air dried.

The In situ hybridization technique using a cloned actin DNA probe consisted of the following steps. The Hind III restriction fragments of pBR322 containing the DmA2 actin sequence from *Drosophila melanogaster* were isolated by preparative agarose gel electrophoresis. The 1.8 kilobase pair (kb) fragment, which contains most of the coding region of an actin gene (12), was nick-translated with [³H]poly(CTP (60 Ci/mmol; New England Nuclear Corp.) to a specific activity of 1× 10⁶-10⁷ cpm/μg DNA. The 1.8 kb actin probe was dissolved in the hybridization buffer (2X SSC, 50% formamide, 10% dextran sulfate, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll (40,000 mol wt), pH 7.0), denatured by heating at 90°C for 5 min and then applied to a concentration of 10 ng/ml (20 μl per slide) to histological sections prepared as described above. The sections were treated with 1 μg/ml protease K in 0.01M Tris-HCl, 0.003 M CaCl₂, pH 7.5 for 30 min at 20°C and then to 0.01 M CaCl₂ anaes to 0.01 M CaCl₂, 0.005 M MgCl₂, pH 7.5 for 30 min at 40°C and then to 0.01 M CaCl₂, 0.005 M MgCl₂, pH 7.5 for 30 min at 4°C and then to 0.01 M Tris-HCl, 0.003 M MgCl₂, pH 7.5 for 30 min at 37°C. The slides were rinsed three times with hybridization buffer before application of the denatured actin DNA probe. An acid-washed cover slip was placed on the slide and sealed with rubber cement. Annealing was carried out for 18 h at 40°C in moist chambers constructed of plastic Petri dishes containing Whatman filters saturated with the hybridization buffer. After annealing the cover slips were removed and the slides were washed twice with hybridization buffer for 15 min at 20°C. The washed slides were treated with 0.5% TCA at 4°C for 10 min, rinsed in distilled water, and air dried before autoradiography.

**Autoradiography**

Autoradiography was carried out using Kodak NTB-2 liquid emulsion as described previously (26). The slides were exposed for 14 d at 4°C and stained through the emulsion with hematoxylin-eosin after development.

**RESULTS**

**Nature of the Cytoskeletal Framework**

The ascidian follicle cells form an epithelial layer which surrounds the egg, representing the outermost component of the egg-envelope complex (Fig. 1a). They lie immediately above the chorion (Fig. 1b) and, in conjunction with the underlying test cells, are thought to synthesize the chorionic mucopolysaccharides (9). The follicle cells of *Styela plicata* exhibit several types of inclusion granules surrounding an

![Figure 1](image-url)
attenuated protoplasmic region which contains the nucleus and cytoplasm (Fig. 1 b). The smaller granules in the basal region of the cell contain chorionic precursor materials while the contents of the large apical granule are unknown, but possibly include substances responsible for the unusual buoyancy of this cell type (18). Ultrastructural studies suggest that ascidian follicle cells contain a well developed cytoskeleton (9, 10) which includes microfilaments and microtubules (10).

When egg-envelope complexes or isolated follicle cells were extracted with Triton X-100 as described in Materials and Methods, a detergent-insoluble, filamentous residue was obtained (Fig. 2). Although the whole mounts of detergent-extracted follicle cells observed by electron microscopy contained filaments of various sizes, they were primarily composed of a network of filaments ranging from 10 to 20 nm in diameter. It should be emphasized that the filamentous network shown in Fig. 2 actually represents the insoluble remnant of the protoplasmic region of a follicle cell. The contents of the small inclusion granules are apparently released into the soluble phase during the extraction. Biochemical analyses showed that detergent-extracted follicle cells contained only 9% of the total lipid, 23% of the total protein, and 28% of the RNA. In contrast, poly(A) appeared to be preferentially conserved in the detergent-insoluble fraction when measured by hybridization with \[^{3}H\]poly(U). Thus the CF of ascidian follicle cells, like that of a number of cell types (8, 20, 23), is enriched in poly(A). The CF of follicle cells resembles the cytoskeleton of vertebrate cells seen by high voltage electron microscopy (36) or revealed after extraction with nonionic detergents (3, 20, 27, 29, 30).

**Cytoskeletal Poly(A)+ RNA Detected by In Situ Hybridization**

To examine the distribution of poly(A) in the CF, egg-envelope complexes extracted with Triton X-100 were fixed, processed for histology, and sections were subjected to in situ hybridization with poly(U), a method which has previously been shown to specifically detect the position of poly(A) in ascidian tissues (15, 17). Egg-envelope complexes, instead of isolated follicle cells, were used in this experiment because the presence of the chorion aids in the orientation of the follicle cell CF. As shown in Fig. 3 a, intact follicle cells exhibit a strong and distinctly localized autoradiographic signal after in situ hybridization with \[^{3}H\]poly(U) due to the intense synthesis and accumulation of poly(A)+RNA in the attenuated protoplasmic region (15). After extraction of the egg-envelope complex with Triton X-100 the follicle cell CF could be localized at the outer edge of the chorion in hematoxylin-stained autoradiographs. The apical granules, which became resilient after detergent treatment, and sometimes the basal granules, were also visible in the autoradiographs. As shown in Fig. 3 b and c, the protoplasmic area of the CF labeled uniformly after in situ

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**Figure 2** Electron micrographs of whole mounted spreads of the follicle cell cytoskeletal framework prepared by Triton X-100 extraction. (a) Triton X-100-extracted follicle cells spread on a grid. The dark spherical bodies represent the large inclusion bodies. The contents of the small inclusion bodies (empty area in the cytoskeletal framework) have been extracted. The filamentous areas represent the remnant of the protoplasmic regions of the follicle cells. X 1,000 (b and c) Filamentous organization of the protoplasmic region of follicle cells extracted with Triton X-100. (b) X 10,000; (c) X 60,000.

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hybridization with [3H]poly(U). As in intact cells, many fewer grains were seen over the apical or basal granules of the follicle cell CF. The interaction of [3H]poly(U) with the CF was sensitive to pretreatment of the sections with pancreatic RNase dissolved in a buffer containing 0.01 M KCl but not in a buffer containing 0.1 M KCl (Table I) suggesting that the poly(U) binding sites consisted of poly(A) (1, 4). Furthermore, the persistence of poly(A) sequences in detergent-extracted cells prepared with buffers ranging in KCl concentration from 0.05 to 0.6 M (Table I) indicates that the interaction is probably not generated by the relatively low ionic strength buffers (14) used.

**TABLE I**

| Conditions | 0.01 M KCl | 0.05 M KCl | 0.22 M KCl | 0.60 M KCl |
|------------|-----------|-----------|-----------|-----------|
| Untreated  | 39.9 ± 2.6| 33.5 ± 3.7| 43.5 ± 5.7| 31.8 ± 2.1|
| RNase, 0.01 M KCl | -- | 41.0 ± 6.2 | -- | -- |
| RNase, 0.01 M KCl | -- | 8.9 ± 2.1 | -- | -- |

* The follicle cell cytoskeletal frameworks of stage III oocytes (17) were selected for grain counting. Grains were counted over 10 μm² areas of the follicle cell protoplasmic regions and their cytoskeletal frameworks.

† Sections were treated with 50 μg/ml pancreatic RNase A in the appropriate buffer for 36 h at 37°C before in situ hybridization. CFs incubated in the same buffer but without enzyme showed grain densities similar to untreated CFs.

§ Grain counts are given ± SD.

**TABLE II**

| Molecular probe | Intact follicle cell framework | Cytoskeletal framework | % |
|----------------|-----------------------------|------------------------|---|
| Poly(U)        | 25.5 ± 4.6                  | 18.0 ± 0.9             | 72 |
| Actin DNA      | 17.6 ± 1.7                  | 10.4 ± 2.4             | 59 |

* Follicle cells or their cytoskeletal frameworks which were associated with stage III oocytes (17) were selected for grain counting. Grains were counted over 10 μm² areas of the follicle cell protoplasmic regions and their cytoskeletal frameworks.

† Grain counts are given ± SD.

in the preparation of the CF. The presence of poly(A) sequences in the detergent-extracted follicle cells suggests that the CF contains poly(A)+RNA.

To estimate the proportion of total cellular poly(A) which remains in the CF by in situ hybridization, intact and detergent-extracted follicle cells were mixed and the mixtures were fixed, histologically processed, treated with [3H]poly(U), and autoradiographed. In this way grains present over follicle cells and their CFs could be counted and compared in the same sections. Grain counts showed that the CF contained ~60-70% of the poly(U) binding activity exhibited by the intact follicle cells (Table II). These data are similar to those obtained by [3H]-poly(U) hybridization of phenol-extracted RNA from the detergent-insoluble fraction of follicle cells and suggest that a significant proportion of the total poly(A)+RNA is associated with the CF.

**Cytoskeletal Actin mRNA Detected by In Situ Hybridization**

To determine whether the RNA sequences associated with
the follicle cell CF include recognizable classes of mRNA, detergent-extracted egg-envelope complexes were subjected to in situ hybridization with a more specific probe. A DNA probe containing an actin gene was selected since actin mRNA is a prevalent message and the follicle cells are known to synthesize actin during early development (Jeffery, unpublished observation). The actin DNA probe used was restricted from pBR322 containing the *Drosophila* actin gene DmA2 (12). The nick-translated 1.8 kb actin DNA, applied to sections at saturating concentrations (Table III), showed uniform binding in situ to the protoplasmic regions of follicle cells (Fig. 4a) and their CFs (Fig. 4c). As was the case after in situ hybridization with [H]poly(U), much less activity appeared above the basal and apical inclusion granules than above the protoplasmic regions of the follicle cells. The binding of actin DNA to follicle cells or their CFs was virtually eliminated by pretreatment of the sections with RNase (Fig. 4b; Table III) but not by pretreatment with DNase or protease (Table III) indicating that the probe interacted with RNA in the specimen. Further indication of the specificity of in situ hybridization was obtained by experiments in which pBR322 (labeled to a specific activity of 1 10^6 cpm/µg DNA) was used as a probe instead of actin DNA. As indicated in Table III, pBR322 did not significantly bind to sections of follicle cells or their CFs. The results suggest that the actin DNA probe specifically hybridized with RNA in the sectioned material and that actin mRNA is among the messages associated with the CF.

**Newly Synthesized Cytoskeletal Poly(A)+RNA Detected by Radioisotope Incorporation**

Since ~90% of the RNA produced by the follicle cells during a 2-h exposure to radioactive nucleosides is poly(A)+RNA (15), the association of newly synthesized mRNA with the CF can also be tested by radioisotope incorporation. As shown in Fig. 5, significant amounts of [H]uridine or [H]adenosine are incorporated into the RNA of follicle cells or their CFs during

| Probe | Amount | Pretreatment | Cytoskeletal framework |
|-------|--------|--------------|------------------------|
| Actin | 0.5    | —            | 3.0 ± 1.2, 4.4 ± 0.7   |
| Actin | 1.0    | —            | 7.6 ± 1.5, 9.6 ± 2.8   |
| Actin | 5.0    | —            | 20.4 ± 1.6, 13.2 ± 3.7 |
| Actin | 10.0   | —            | 24.5 ± 2.2, 18.6 ± 2.4 |
| Actin | 20.0   | —            | 21.7 ± 0.8, 17.9 ± 2.9 |
| Actin | 5.0    | RNase        | 3.3 ± 0.2, 4.3 ± 1.1   |
| Actin | 5.0    | DNase        | 19.8 ± 2.1, 15.0 ± 1.8 |
| Actin | 5.0    | Proteinase   | 22.4 ± 3.3, 14.9 ± 2.4 |
| pBR322| 25.0   | —            | 1.1 ± 0.3, 4.1 ± 1.1   |

* The follicle cell cytoskeletal frameworks of stage III oocytes (17) were selected for grain counting. Grains were counted over 10 µm² areas of the follicle cell protoplasmic regions and their cytoskeletal frameworks.

† Sections were pretreated with pancreatic RNase (50 µg/ml) in 0.05 M Tris-HCl, 0.1 M KCl, 0.005 M MgCl₂, pH 7.2 for 18 h at 37°C.

DNase I (50 µg/ml) in 0.01 M Tris-HCl, 0.003 M MgCl₂ for 2 h at 37°C; and protease K (10 µg/ml) in 0.01 M Tris-HCl, 0.003 M MgCl₂ for 30 min at 20°C before in situ hybridization.

§ Grain counts are given ± SD.

**FIGURE 4** In situ hybridization of a cloned actin DNA probe to sections of follicle cells and their cytoskeletal frameworks. (a) Autoradiograph of intact follicle cells hybridized with the 1.8 kb actin DNA probe. (b) Autoradiograph of intact follicle cells in sections treated with 50 µg/ml pancreatic RNase for 25 h in 0.05 M Tris-HCl, 0.1 M KCl, 0.001 M MgCl₂, pH 7.4 at 37°C before in situ hybridization with the actin DNA probe. (c) Autoradiograph of Triton X-100-extracted follicle cells hybridized with the actin DNA probe. ×1,000.
a 2-h labeling period. As noted in the in situ hybridization experiments, the protoplasmic regions of the cell, but not the basal or apical granules, were heavily labeled. The grains in the [3H]uridine-labeled sections were virtually eliminated by pretreatment with pancreatic RNase (Fig. 5c). Many grains remained in RNase-treated sections of [3H]adenosine labeled follicle cells presumably due to incorporation into RNase-resistant poly(A) sequences (Fig. 5f). Experiments similar to those described above, in which mixtures of intact and detergent-extracted, egg-envelope complexes labeled with [3H]uridine were autoradiographed together showed that >90% of the grains present over the intact cells were also developed above
the CF. This result suggests that most of the newly synthesized poly(A)+RNA is associated with the CF. In contrast, only 60–70% of the total cellular poly(A)+RNA or actin mRNA could be detected in the CF by in situ hybridization. There are two explanations for these results. Either CF interactions are reduced and mRNA is released into the soluble fraction as transcripts age in the cytoplasm or messages associated with the CF exhibit a shorter half-life than other mRNA sequences.

**DISCUSSION**

Three lines of evidence indicate that mRNA is present in the CF of ascidian follicle cells. First, the CF binds [3H]poly(U) as evidenced by solution hybridization experiments with RNA extracted from Triton X-100 insoluble fractions and in situ hybridization experiments with histological sections of the CF. Second, a specific class of messages, those coding for actin, can be detected in the CF by in situ hybridization with an actin DNA probe. Finally, [3H]uridine- or [3H]adenosine-labeled RNA can be detected in the CF by autoradiography at a time when >90% of the transcribed RNA is polyadenylated (15).

The interaction between the CF and mRNA could occur in vivo or it may be an artifact of the detergent extraction procedure. Artifacts could arise by the entrapment of mRNA in the membrane protein lamina (2) or the filamentous actin network that encases the CF (12, 23, 32) or by the adventitious association of mRNA with the internal parts of the CF. It seems unlikely that the mRNA could be trapped in the peripheral regions of the CF since the autoradiographic signal was uniformly distributed within the protoplasmic region of the detergent extracted cell after in situ hybridization or radioisotope incorporation, exactly as it is seen in the intact cell. Moreover, it is felt that the results of the present investigation do not support an adventitious binding of mRNA to the CF.

The association of mRNA with the CF does not appear to be affected by variations in theionic strength of the extraction buffer, as would be expected for artifacts generated by electrostatic interaction of RNA and protein (14). Furthermore, there is no reason to expect that newly synthesized poly(A)+RNA sequences would interact with the CF to a greater extent than their counterparts at steady-state, if they are formed by adventitious interactions. It is unlikely, for example, that the shorter poly(A) sequences which exist in steady-state mRNA (16, 31) could account for this result because it has already been shown that the soluble and insoluble fractions of detergent-extracted mammalian cells contain RNA with poly(A) tracts of similar size (8).

In summary, the present experiments suggest that the CF of ascidian follicle cells contains mRNA. These mRNA-cytoskeletal interactions may be responsible for the polarized distribution of mRNA observed in eukaryotic cells (5–7, 11, 17, 28).

I thank Dr. Norman Davidson for providing the plasmid containing the Drosophila actin gene, Dr. Mary Ann Rankin for conducting the lipid analysis and Ms. Lynne Hunter and Ms. Dianne McCoig for technical assistance.

This work was supported by grants from the National Institutes of Health (HD-13970 and GM-25119) and the Muscula Dystrophy Association.

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