

Polyribosome Targeting to Microtubules: Enrichment of Specific mRNAs in a Reconstituted Microtubule Preparation from Sea Urchin Embryos

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Abstract. A subset of mRNAs, polyribosomes, and poly(A)-binding proteins copurify with microtubules from sea urchin embryos. Several lines of evidence indicate that the interaction of microtubules with ribosomes is specific: a distinct stalk-like structure appears to mediate their association; ribosomes bind to microtubules with a constant stoichiometry through

several purification cycles; and the presence of ribosomes in these preparations depends on the presence of intact microtubules. Five specific mRNAs are enriched with the microtubule-bound ribosomes, indicating that translation of specific proteins may occur on the microtubule scaffolding in vivo.

IN many embryos, the pattern of development that follows fertilization can be traced back to the localization of developmental determinants in the egg cytoplasm (Wilson, 1925; Davidson, 1986). The inheritance of a specific subset of mRNAs and proteins provides the macromolecular blueprints for the construction of diverse cellular forms with unique functions. In many cases, the compartmentalization of developmentally significant mRNAs is thought to occur through an association with the cytoskeleton. Similarly, in somatic cells, the cytoskeleton provides a network upon which individual mRNAs can be arranged in a three-dimensional pattern. The localized synthesis of specific proteins may have important consequences for the intrinsic regulation of cell fate and the specification of cellular domains such as axons and dendrites. All the major cytoskeletal structures, microtubules, microfilaments, and intermediate filaments, have been implicated in the targeting and transport of mRNA (reviewed by Jeffery, 1989; Fulton, 1993; Singer, 1992; Steward and Banker, 1992; Suprenant, 1993; Wilhelm and Vale, 1993).

Of the cytoskeletal elements, microtubules are especially well designed for mRNA localization. Microtubules are asymmetric with a fast-growing (plus) and slow-growing (minus) end. Within the cell, microtubules are generally organized with their minus ends embedded in a microtubule organizing center and their plus ends oriented radially towards the cell periphery (Euteneuer and McIntosh, 1981; Haimo and Telzer, 1981). Along this organized network, dynein and kinesin generate the forces necessary for microtubule-based transport towards the minus or plus ends, respectively (Gibbons and Rowe, 1965; Vale et al., 1985; Lye

et al., 1987; Paschal et al., 1987). Finally, the rapid turnover rates, provided by dynamic instability (Mitchison and Kirschner, 1984), allow microtubule arrays to be rapidly changed into dramatically new configurations during the transition from interphase to mitosis. Taken together, these unique properties of microtubules provide a highly organized and yet dynamic network for the anchoring and distribution of mRNA to a given region of an egg or embryo.

Recent evidence indicates that specific mRNAs are localized and transported within oocytes and embryos in a microtubule-dependent manner. Microtubules are involved in the transport of Vg1 RNA to the vegetal hemisphere of stage IV *Xenopus* oocytes (Yisraeli et al., 1990) and the perinuclear localization of cyclin B transcripts in syncytial *Drosophila* embryos (Raff et al., 1990). The mRNA for the anterior morphogen, *bicoid*, is transported from the nurse cells to the anterior margin of the oocyte in a microtubule-dependent process (Pokrywka and Stephenson, 1991). During the subsequent cellularization of *Drosophila* embryos, a network of microtubules sandwiches the RNA coding for the segmentation gene product *fushi tarazu* against the plasma membrane, thus acting as a physical barrier for the diffusion of a developmental determinant (Edgar et al., 1987). Finally, granules containing myelin basic protein mRNA appear to move along microtubule tracts in oligodendrocytic processes (Ainger et al., 1993).

Specific localization signals in the mRNA itself may be required for mRNA association with the cytoskeleton. For *bicoid*, Vg1, and *nanos* (posterior *Drosophila* morphogen), localization signals have been identified in the 3' untranslated region of the transcripts (Macdonald and Struhl, 1988; Gottlieb, 1992; Gravis and Lehman, 1992; Mohry and Melton, 1992; Macdonald et al., 1993). The 3' untranslated region of actin mRNA is both necessary and sufficient for the localization of β -actin to the cell periphery and α -actin to the

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perinuclear region of cultured fibroblasts (Kislauski et al., 1993). Each localization signal may have a cytoskeletal receptor that mediates attachment to and/or motility along a cytoskeletal track. Although such receptors have not been identified, possible candidates include RNA-binding proteins (Schwartz et al., 1992). In *Drosophila*, three additional genes, *exuperantia*, *swallow*, and *staufen*, appear to be necessary for the association of *bicoid* RNA with the cytoskeleton (Berleth et al., 1988; Stephenson et al., 1988; St. Johnston et al., 1989; Pokrywka and Stephenson, 1991). The products of these genes may be mRNA chaperones or anchors to the cytoskeleton.

Messenger RNA may interact with the cytoskeleton directly, or through other components of the translational machinery, such as ribosomes or cytoplasmic ribonucleoprotein particles. In many cultured mammalian cells, clusters of ribosomes surround microtubules, and they are linked to the microtubule walls by short filaments (Wolosewick and Porter, 1976; Heuser and Kirschner, 1980; Ris, 1985). In hemipteran insects, ribosomes appear to move along microtubule tracks from the anterior ovarioles to the developing oocytes (reviewed in Stebbings, 1986). In the mitotic apparatus, ribosome-like particles are attached to adjacent microtubules by fine filamentous arms (Goldman and Rebhun, 1969; Salmon and Segall, 1980; Silver et al., 1980; Hirokawa et al., 1985; Suprenant et al., 1989).

We have developed a novel model system to study how and why components of the translational machinery interact with microtubules. Unfertilized sea urchin eggs are metabolically quiescent cells that contain large stores of unassembled microtubule protein as well as maternal mRNAs, of which only 1–5% are actively being translated. The ionic events at fertilization activate protein synthesis (reviewed by Rosenthal and Wilt, 1987), and they promote the polymerization of microtubules (reviewed by Schatten, 1984). Both processes appear to be driven by the alkalization of the cytoplasm that occurs within minutes of fertilization (Grainger et al., 1979; Johnson et al., 1976; Dube et al., 1985; Schatten et al., 1985; Suprenant and Marsh, 1987). In addition, the association of translationally active poly(A)⁺ RNA with the cytoskeleton correlates with the activation of protein synthesis (Moon et al., 1983), indicating that the cytoskeleton may play a direct role in translational regulation in sea urchins. We discovered recently that microtubule assembly in egg extracts *in vitro* is also promoted by alkaline pH (Suprenant and Marsh, 1987). These purified microtubules are decorated with densely stained particles that we characterized as monoribosome-like on the basis of their size, RNase sensitivity, and sedimentation in sucrose gradients (Suprenant et al., 1989). In selected sections prepared for electron microscopy, the ribosome-like particles are attached to the microtubule wall by a long, tapered stalk (Suprenant et al., 1989). This microtubule-ribosome complex is stable, and it can be repetitively assembled and disassembled.

In this report, we demonstrate that ribosomes associate with microtubules, and their association may be developmentally regulated, as polyribosomes rather than monoribosomes are associated with microtubules purified from two-cell embryos. We have developed methods to purify these polyribosome-microtubule complexes with intact messenger RNA molecules. Specific poly(A)⁺ RNAs are enriched in

these microtubule preparations, and they can be translated into polypeptides *in vitro*. This reconstituted system should prove valuable for dissecting how mRNAs and proteins are targeted to the cytoskeleton.

A brief account of these results has appeared in abstract form (Suprenant, K. A., and J. Drawbridge. 1991. *J. Cell Biol.* 115:341a).

Materials and Methods

Experimental Materials and Solutions

Sea urchins, *Strongylocentrotus purpuratus*, were purchased from Marinus, Inc. (Long Beach, CA). All nucleotides and analogues were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Ultrapure phenol was obtained from Gibco BRL (Gaithersburg, MD). Placental RNasin and nuclease-treated, rabbit reticulocyte lysate were purchased from Promega (Madison, WI) and Ambion, Inc. (Austin, TX). Primary antibodies were obtained from the following sources: anti-tubulin (DM1A) and anti-actin (C4) from ICN ImmunoBiologicals (Lisle, IL); anti-cytokeratin (BT-571) from Biomedical Technologies (Stoughton, MA); anti-kinesin (SUK4) from J. Scholey (University of California, Davis, CA); anti-dynein (71-4.2) from D. Asai (Purdue University, Lafayette, IN); anti-ribosome (40S) and anti-PABPs from M. Winkler (University of Texas, Austin, TX). These antibodies were used at the following dilutions: DM1A (1:1000); C4 (1:250); BT-571 (1:200); SUK4 (1:500); 71-4.2 (1:500); anti-ribosome (1:500); and anti-PABP (1:500). Secondary antibodies were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). All other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) and Research Organics (Rochester, NY). RNase-free glassware and plasticware were used throughout this study.

Gametes and Embryo Culture

Eggs and sperm were obtained by intracoelomic injection of 0.55 M KCl. Sperm were collected "dry" and stored on ice until needed. Eggs were shed into Millipore-filtered Instant Ocean (MF-IO) artificial sea water (Aquarium Systems, Mentor, OH), and were harvested by gentle centrifugation (100 g, 2 min). After three washes in artificial sea water, egg jelly coats were removed in "19:1" (500 mM NaCl, 27 mM KCl, 2 mM EDTA, pH 7.8).

Sea urchin eggs were fertilized in MF-IO, gently centrifuged (100 g, 2 min), and resuspended in Ca⁺⁺-free artificial sea water (Salmon, 1982), containing 10 µg/ml pronase, 1 mM DTT, and 17 mM MgCl₂ (Silver et al., 1980). After 15 min, the fertilization envelopes were removed by filtration through a 153-µm mesh nylon. The embryos were cultured in Ca⁺⁺-free sea water at 14°C with gentle stirring. Two-cell embryos (~2 h after fertilization) were concentrated by centrifugation (100 g, 2 min).

Ribosome Isolation

Ribosomes were isolated from unfertilized sea urchin eggs by preparative sucrose step gradients exactly as described (Hille and Danilchik, 1986).

Microtubule Protein Isolation

Microtubule protein was isolated from unfertilized sea urchin eggs by the pH and temperature-dependent assembly methods of Suprenant and Marsh (1987), as modified by Suprenant et al. (1989).

Microtubule protein was isolated from two-cell embryos as described above with the exception that 2 mM DTT, 100 µM emetine, and 500 U/ml RNasin were included in the lysis buffer. The microtubule reassembly buffer additionally contained 30 U/ml RNasin, 2 mM DTT, and 100 µM emetine. After three cycles of assembly and disassembly, purified microtubule protein was drop-frozen in liquid nitrogen and stored at -80°C.

Electron Microscopy

Microtubule pellets were fixed and embedded for thin sectioning as previously described (Suprenant and Marsh, 1987).

Microtubules were prepared for negative staining under three different solution conditions. In the first method, microtubule protein was diluted into PMEG (100 mM KPipes, 1 mM MgSO₄, 1 mM EGTA, and 1 mM GTP) and assembled into microtubules for 15–20 min at 30°C. In the sec-

ond method, microtubules were assembled in PMEG in the presence of 20 μ M taxol (Vallee and Collins, 1986). For the third preparation, microtubules were assembled in PMEG and fixed with 1% glutaraldehyde. For quantitation, two methods of negative staining for electron microscopy were used. In the first method, freshly cleaved mica sheets were lightly coated with carbon by vacuum evaporation (Valentine et al., 1968). The carbon film was floated on top of a 1-ml suspension of assembled microtubules. After 2 min, the carbon film was retrieved with the mica sheet and transferred to the negative stain (2% aqueous uranyl acetate) for 1 min. The carbon film was recovered on a copper electron microscope grid and air dried. In the second method, Formvar-coated grids were coated with a thin layer of vacuum-evaporated carbon. After ionizing the surface by glow discharging, the grids were inverted on top of a 50- μ l microtubule suspension for 2 min. The grids were gently rinsed three times with distilled water and negatively stained for 1 min on a drop of 2% aqueous uranyl acetate. Grids were air dried and viewed with an electron microscope (JEN-1200 EXII; JEOL U.S.A., Inc., Peabody, MA) at 80 kV.

The criteria used to quantitate the numbers of bound ribosomes to microtubules were modeled after previous studies of microtubule-bound adenovirus particles (Weatherbee et al., 1977). Electron micrographs were taken at a total magnification of 40,000 (approximate field of view = 4.5 μ m²), and microtubule length measurements were done on enlargements (\times 300) of randomly selected images. Ribosomes were presumed to be attached to a microtubule if they were \pm 4 nm from the microtubule edge. For each data set, measurements were made on \sim 75–100 randomly selected microtubules. Each experiment was done in triplicate. ANOVA and the Student's *t* test were used to analyze each data set.

Protein, RNA, Poly(A) Analysis, and In Vitro Translation

Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a protein standard.

RNA was isolated by proteinase K digestion in the presence of SDS, followed by phenol-chloroform extraction and ethanol precipitation (Winkler et al., 1985). Precipitated RNA was dissolved in a small volume of RNase-free water, and the total RNA concentration was determined by OD₂₆₀. Poly(A)⁺ RNA concentration was determined by a [³H]poly(U) hybridization assay. The fraction of poly(A) (percent of poly(A)) is expressed as the ratio of poly(A) (as determined by hybridization to [³H]poly(U)) to total RNA (as determined by OD₂₆₀ readings).

RNA was translated in vitro in a message-dependent rabbit reticulocyte lysate (Pelham and Jackson, 1976) (Ambion, Inc., Austin, TX). [³⁵S]-Methionine-labeled polypeptides were resolved on 4–16% acrylamide gels (below). Autoradiography of dried gels was done on XAR film (Eastman Kodak Co., Rochester, NY).

SDS-PAGE and Western Blotting

Proteins were analyzed on SDS-PAGE with the discontinuous buffer formulation of Laemmli (1970). For Western blot analysis, unstained gels were transferred to nitrocellulose (Towbin et al., 1979) and processed as previously described (Suprenant et al., 1993). Quantitation was by scanning densitometry using known concentrations of purified ribosomes as a standard.

Results

Ribosomes Are Attached to Egg Microtubules Assembled In Vitro

While developing a method for the purification of microtubule protein from unfertilized sea urchin eggs (Suprenant and Marsh, 1987), we discovered that purified egg microtubules are decorated with ribosome-like particles (Suprenant et al., 1989). These monoribosomes are attached directly to the microtubule wall by a long tapered stalk (Fig. 1; and Suprenant et al., 1989), and it is believed that this attachment is essential for their copurification with microtubules.

To determine what percentage of the egg's ribosomes copurify with these microtubules, we probed immunoblots with a polyclonal antiserum generated against the sea urchin 40S ribosomal subunit (Drawbridge et al., 1990). On West-

ern blots, this antiserum recognized predominantly a 40-kD polypeptide in both crude cytosolic extracts and purified microtubule preparations (Fig. 2). We estimated that 1–3% of the total ribosomes copurified with microtubules, which in turn comprise 1–3% of the soluble egg protein.

Quantitation of Microtubule-associated Ribosomes in Unfertilized Egg Preparations

A specific structural interaction between a ribosome and a microtubule would be reflected in a constant stoichiometry of binding. To accurately determine the number of ribosomes bound to microtubules under a variety of experimental conditions, a quantitative EM method was developed. Several methods of sample preparation, microtubule stabilization, and negative staining for electron microscopy were used to determine the most reproducible method for analysis. Initially, microtubules (2–4 mg/ml) were negatively stained with aqueous uranyl acetate on either a Formvar or carbon film support (see Materials and Methods). It was determined that 9.83 ± 1.45 and 9.36 ± 1.38 ribosomes were bound per micron of assembled microtubules that were negatively stained on carbon or Formvar films, respectively. Since there were no significant differences in these numbers, the Formvar method was chosen because it used much less protein and it produced high quality images.

At 2–4 mg/ml, microtubules were frequently tangled into large unquantifiable mats on the EM grid. To dilute the microtubule protein further (0.2–0.4 mg/ml), it was necessary to stabilize the microtubules against disassembly with either a fixative, glutaraldehyde, or the microtubule-stabilizing drug, taxol. Comparable numbers of ribosomes were associated with microtubules that were unfixed, glutaraldehyde fixed, or taxol stabilized, although there was a slightly larger statistical variance in the data obtained in the glutaraldehyde-fixed or taxol-stabilized microtubules (Table I). The number of ribosomes associated with egg microtubules under all the assay conditions ranged from 7 to 10 ribosomes per micrometer of microtubule.

The association of ribosomes with microtubules is not dependent on the presence of a motor protein such as dynein or kinesin. To examine the possible nucleotide requirements for ribosome association, taxol-stabilized microtubules were incubated in the presence and absence of several Mg nucleotides (ATP, ADP, AMP, ATP γ S, AMPPNP, GTP, UTP, CTP, and ITP), and sedimented through a 20% (wt/vol) sucrose cushion. In the presence of 20 μ M taxol and 2–10 mM Mg nucleotide, there were no significant differences in the number of microtubule-bound ribosomes, as assayed by immunoblotting (data not shown). In addition, we were unable to detect any cross-reactivity by immunoblotting of purified microtubule protein with either the SUK-4 or the 71-4.2 antibodies against kinesin and dynein, respectively (kindly provided by J. Scholey, University of California, Davis, CA, and D. Asai, Purdue University).

Polyribosomes Are Associated with Microtubules Purified from Two-cell Embryos

In addition to identifying the molecular components responsible for ribosome binding, we are also interested in what mRNAs may be associated with these microtubules assembled in vitro. The rate of protein synthesis in the unfertilized

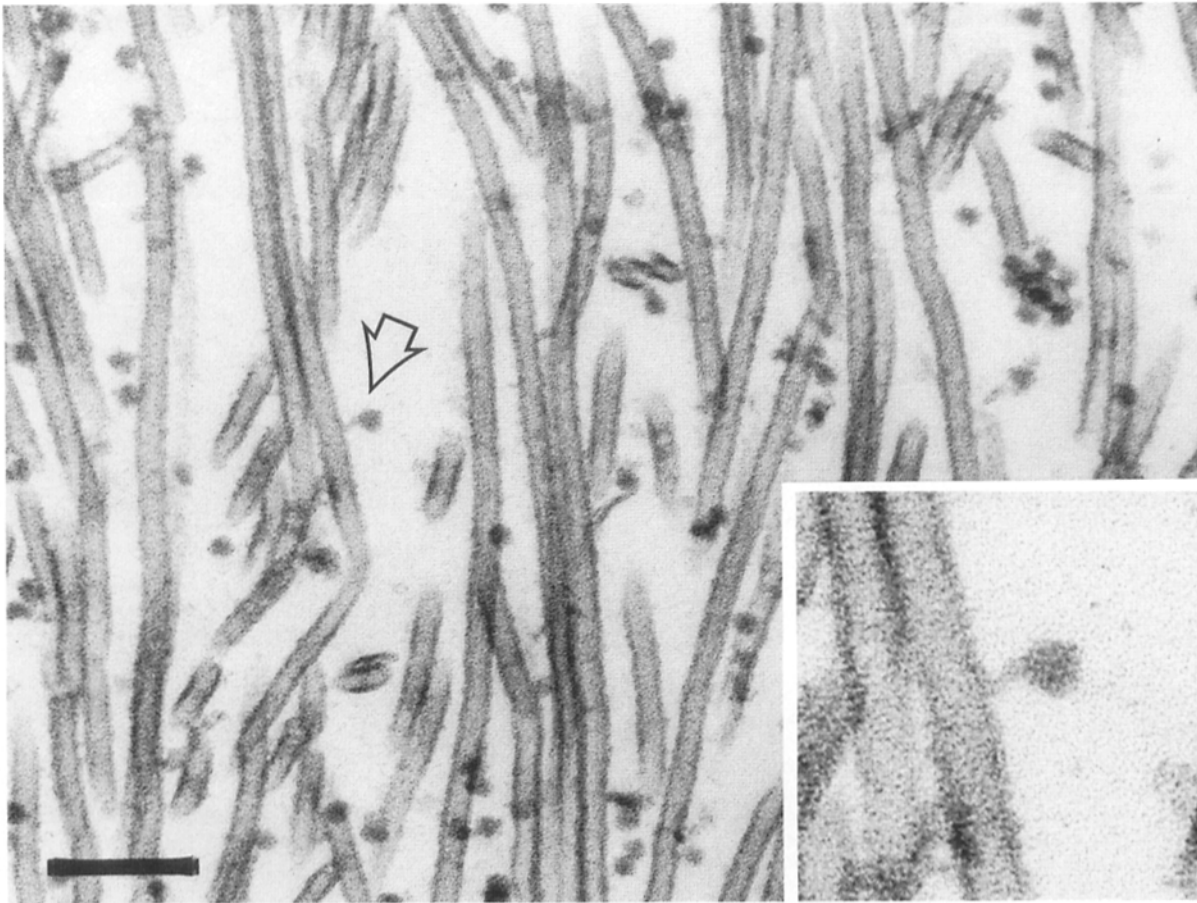


Figure 1. Electron micrograph of a third-cycle microtubule preparation from unfertilized sea urchin eggs. Arrow points to a single ribosome attached to the microtubule wall by a thin stalk. An enlargement of this region is shown in the lower right. Bar, 0.1 μm .

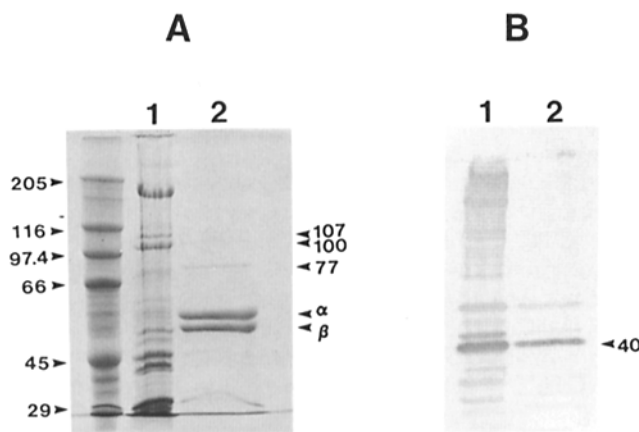


Figure 2. Ribosomal proteins copurify with microtubules isolated from unfertilized eggs. (A) Coomassie blue-stained gel of cytosolic extract proteins (lane 1) and purified microtubule proteins (lane 2). Molecular weight standards are shown to the left ($\times 10^{-3}$). The positions of α - and β -tubulins, as well as the abundant 107-, 100-, and 77-kD microtubule proteins are shown to the right. (B) Corresponding immunoblot probed with an anti-ribosomal antiserum generated against the 40S subunit (Drawbridge et al., 1990). The major polypeptide recognized by this antiserum is a 40-kD polypeptide.

egg is low, and after fertilization, the rate of protein synthesis increases 30-fold (Goustin and Wilt, 1981). Consequently, there are many more polyribosome complexes in the zygote than in the unfertilized egg. To identify mRNAs that may be associated with microtubules, we have developed a method for the purification of a polyribosome-microtubule complex from two-cell sea urchin embryos. The microtubule purifica-

Table I. Quantitation of Ribosome Binding to Microtubules

	Numbers of ribosomes/ μm MT	ANOVA
PMEG	9.83 ± 1.45	1.14
	9.36 ± 1.38	
PMEG-taxol	7.65 ± 1.94	4.37
	10.06 ± 2.35	
	5.46 ± 0.91	
PMEG-glutaraldehyde	8.52 ± 2.90	2.62
	7.07 ± 1.92	
	9.24 ± 2.83	

Microtubules were either assembled in PMEG or PMEG-taxol, or they were assembled in PMEG and then fixed with glutaraldehyde. Each sample was negatively stained for electron microscopy and quantitated as described in Materials and Methods. The number of ribosomes bound per length of microtubule (mean \pm SE for triplicate assays) and the ANOVA is shown for each experiment. *MT*, microtubule.

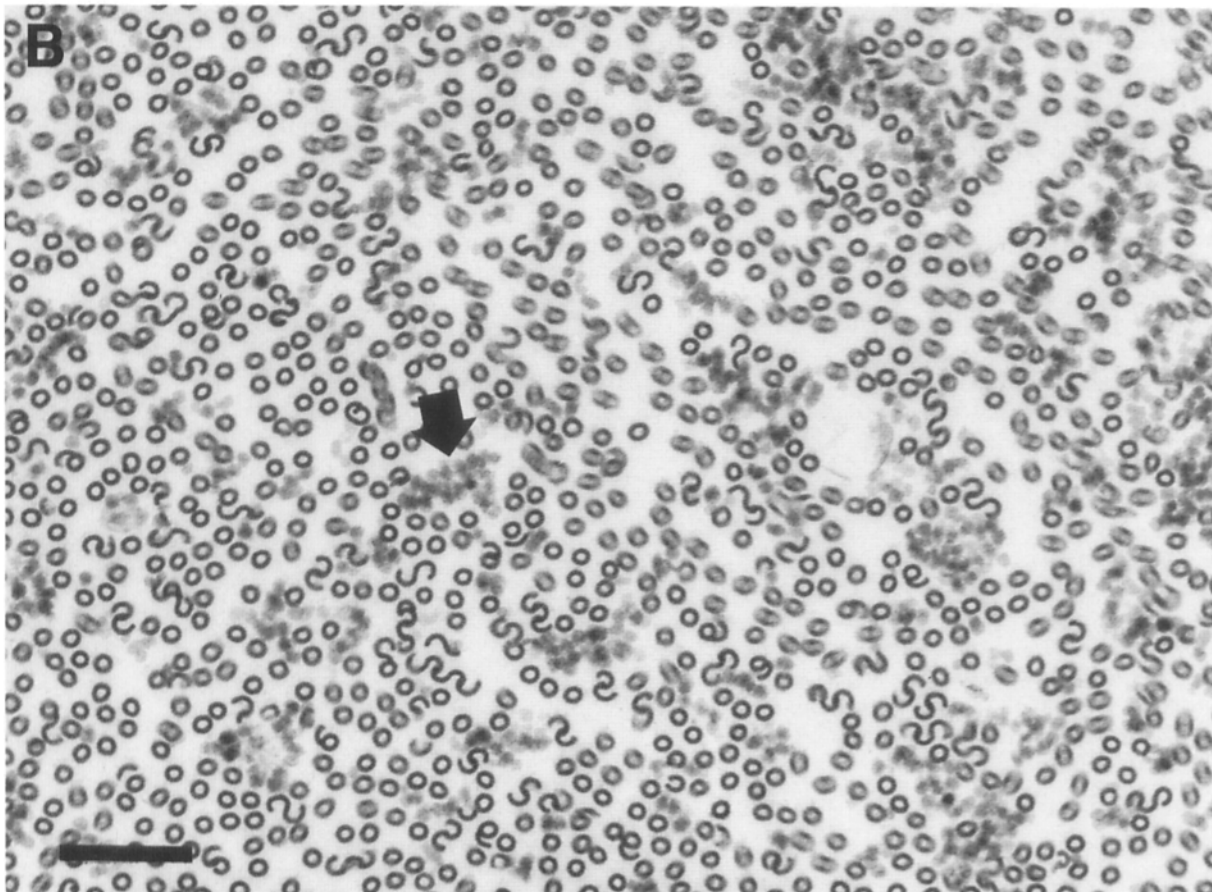
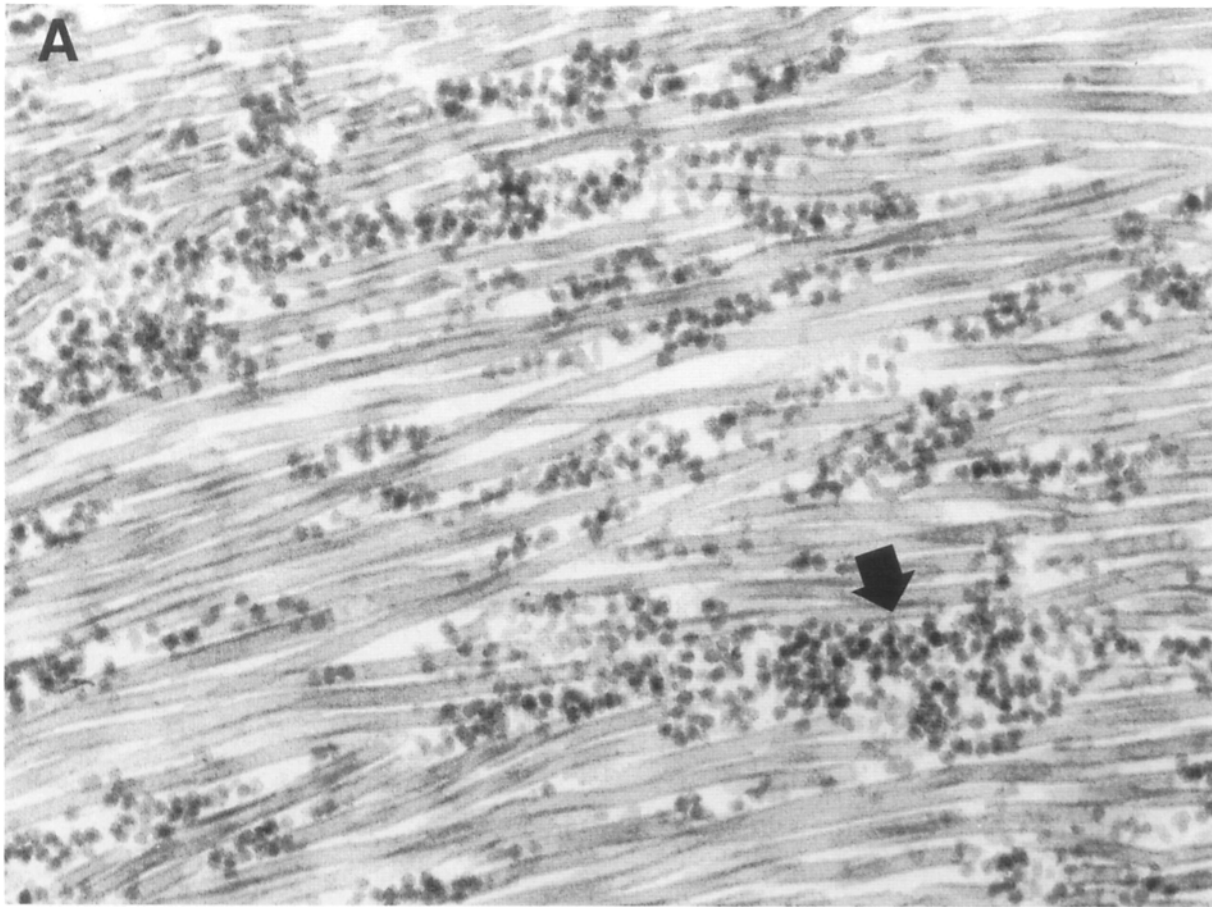


Figure 3. Electron micrograph of a third-cycle microtubule preparation from sea urchin embryos at the two-cell stage. In longitudinal section (*A*), clusters of polyribosomes (*arrow*) are interspersed with microtubules. In cross-section (*B*), a single polyribosome (*arrowhead*) contacts a single microtubule. Bar, 0.2 μm .

tion scheme developed for sea urchin eggs (Suprenant and Marsh, 1987; Suprenant et al., 1989) requires repeated cycles of temperature and pH changes over a 6–8-h period. This treatment frequently results in degradation of associated RNA. To stabilize mRNA in polyribosomes, emetine, an inhibitor of ribosome translocation, as well as placental RNasin, an RNase inhibitor, was included in the homogenization and assembly buffers (see Materials and Methods).

Fig. 3 illustrates that the microtubules assembled under these isolation conditions appeared normal in both longitudinal and cross-sections. There was, however, an increase in the number of incomplete microtubules or S-shaped protofilament sheets (see Fig. 3 B). This may be caused by the presence of RNasin, emetine, or the higher concentrations of DTT used to stabilize the placental RNasin. In addition to microtubules, polyribosome profiles were visible in both longitudinal and cross-sections (Fig. 3, A and B). In regions where the microtubules and ribosomes are tightly packed, it is difficult to see any attachments between the two organelles. In cross-section, the polyribosome complexes can occasionally be observed to contact a single microtubule at that level of section (see Fig. 3 B). In selected sections, ribosomes were aligned along the length of single microtubules. These ribosomes appear to be arranged periodically with a repeat distance of 28.2 ± 2.9 nm (Fig. 4). It is not known whether these ribosomes are in a polyribosome structure or whether they are bound singly to a repeated structural component of the microtubule. Either way, this striking alignment of ribosomes along the microtubule wall is indicative of a specific interaction.

The Copurification of Polyribosomes Depends on Microtubule Integrity

We considered the possibility that these polyribosomes were massive enough to pellet under the centrifugation conditions used to purify microtubules. To rule this out, third-cycle microtubules were assembled in the presence and absence of nocodazole, a microtubule assembly inhibitor, and cytochalasin D, an inhibitor of actin filament formation. Each microtubule preparation was centrifuged through a 30% sucrose cushion, and the pellets and supernatants were analyzed by SDS-PAGE and Western blotting (Fig. 5). Under conditions where microtubule assembly was prevented, polyribosomes did not sediment.

The major messenger RNA-binding proteins in sea urchin are two poly(A)-binding proteins of 80,000 and 66,000 *M*, (Drawbridge et al., 1990). Both the 80- and 66-kD poly(A)-binding proteins (PABPs)¹ are found in these third-cycle microtubule pellets, indicating that mRNA may be present in these complexes. Fig. 5 shows that, like ribosomes, sedimentation of PABPs is dependent on microtubule integrity. Cytochalasin D has little effect on the sedimentation of PABPs, ribosomes, or microtubule protein.

In addition to the inhibitor studies, Western blots were done to determine if there were any intermediate filament-like polypeptides in these microtubule preparations (Fig. 6). Low levels of a cytokeratin-like polypeptide were detected in these microtubule-ribosome complexes. No actin was de-

tectable in these preparations, which is consistent with the results of the cytochalasin experiments described above.

Limiting Factor for Ribosome Binding

There is a limit to the quantity of ribosomes that associate with microtubules assembled *in vitro* (Fig. 7). Increasing the amount of polymerized microtubules does not increase the quantity of sedimentable ribosomes. Aliquots of two-cell embryo extracts (39,000 g supernatants) were incubated in the presence of increasing amounts of phosphocellulose-purified brain tubulin. Polymerized microtubules were pelleted and analyzed by SDS-PAGE and immunoblotting. Increasing the amount of polymerized microtubules appeared to have no effect on the amount of cosedimenting ribosomes (Fig. 8). Identical results were obtained with the addition of purified sea urchin egg tubulin (data not shown).

Translational Status of Microtubule-associated Ribosomes

To examine the translational status of the purified polyribosomes, microtubules from two-cell embryos were purified by two cycles of pH- and temperature-dependent assembly and disassembly in the presence of emetine, cycloheximide, NaF, or puromycin. In all cases, the translational inhibitors did not affect the amount of ribosomes obtained (Table II). These results indicate that stable polyribosomes were preformed before their association with the assembled microtubules.

To determine whether the polyribosomes were actively translating any message, microtubule-bound polyribosome complexes were translated *in vitro* in a nuclease-treated reticulocyte lysate. Few detectable polypeptides were translated from the microtubule-associated polyribosomes (data not shown). These results indicate that the template activity of the microtubule-associated messages may be modulated by constituents or a general inhibitor of translation. The latter case is more likely since the microtubule preparation will dramatically reduce the translation of control Xef-1 RNA in a rabbit reticulocyte lysate (Fig. 9).

Specific Messenger RNA Molecules Are Enriched in Two-cell Embryo Microtubules

The presence of polyribosomes and PABPs indicate that mRNA may be a specific component of these microtubule complexes. To determine whether poly(A)⁺ RNAs are targeted to microtubules, total RNA was prepared from two-cell embryo extracts and from purified microtubules. Considering the amount of time that a microtubule preparation takes and the elevated temperatures required for the assembly of microtubules, it was difficult to retain intact mRNAs during purification. To achieve this goal, emetine, an inhibitor of ribosome translocation, and RNasin, an inhibitor of RNases, were included in all of the microtubule assembly buffers. In addition, an RNase-free environment, with baked glassware, sterile plasticware, and autoclaved solutions, was created.

Microtubules purified from two-cell embryos contain large amounts of RNA that can be purified by phenol-chloroform extraction. The bulk of this RNA is ribosomal; however, a [³H]poly(U) hybridization assay indicates that poly(A) tails comprise 0.02% of the total microtubule-associated RNA (by mass). On the basis of an average mes-

1. *Abbreviations used in this paper:* EMAP, echinoderm microtubule-associated protein; PABPs, poly(A)-binding proteins.

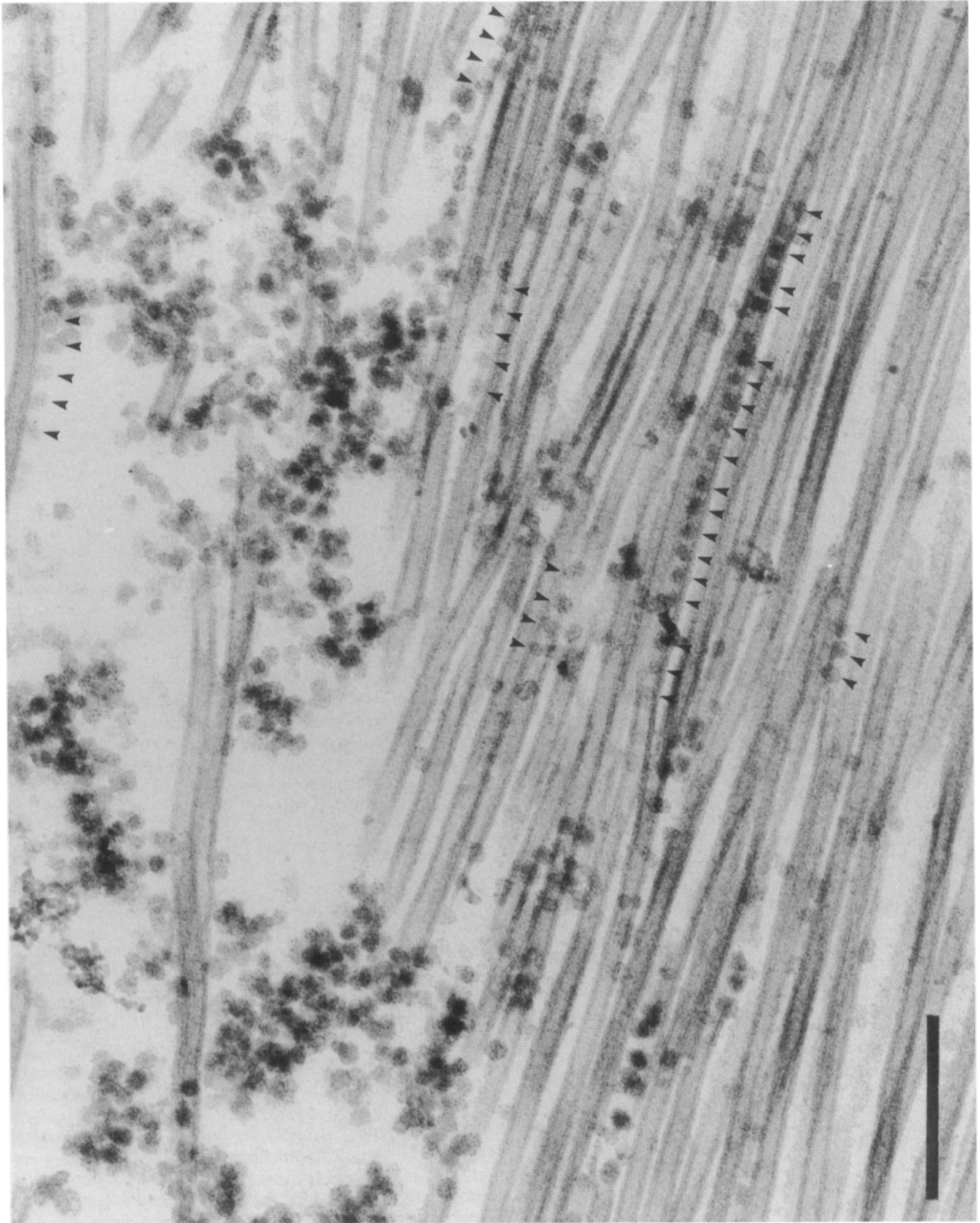


Figure 4. Higher magnification electron micrograph of a third-cycle microtubule preparation from two-cell embryos. In several places, ribosomes are lined up along the walls of a microtubule (*arrowheads*) with a distinct periodicity (28 ± 2.9 nm). Bar, $0.2 \mu\text{m}$.

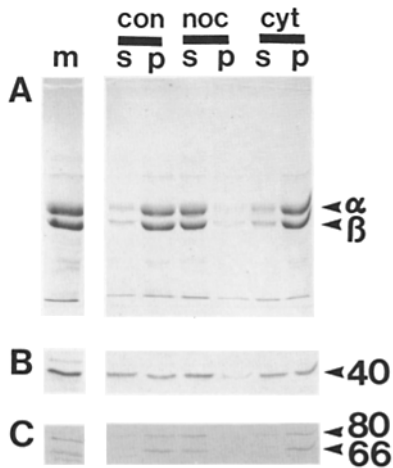


Figure 5. Sedimentation of polyribosomes requires microtubules. Microtubule protein (*m*) was diluted to a final concentration of 2 mg/ml in the presence of 1×10^{-4} M nocodazole (*noc*), 25 μ g/ml cytochalasin (*cyt*), or 1% (vol/vol) DMSO (*con*), the drug control vehicle. Samples were warmed to 30°C for 30 min to polymerize microtubules and were then centrifuged (39,000 *g* for 30 min at 30°C) through a 30% (wt/vol) sucrose cushion. Equal volumes of the supernatants (*s*) and resuspended pellets (*p*) were separated on an 8% polyacrylamide gel and were stained with Coomassie blue (*A*). (*B* and *C*) Corresponding Western blots probed with antisera against the 40-kD ribosomal protein and 66- and 80-kD PABPs, respectively.

sage size of 2,000–3,000 nucleotides with a 50-nucleotide poly(A) tract (Davidson, 1986), we estimate that 0.5–0.9% of the total microtubule-associated RNA is mRNA. Five times more poly(A)⁺ RNA is associated with microtubules purified from two-cell embryos than from unfertilized eggs (Table III).

The translation of unique polypeptides from microtubule-associated RNA would indicate that specific mRNAs are enriched in these microtubule complexes. Messenger RNAs were translated into [³⁵S]methionine-labeled polypeptides in a message-dependent, rabbit reticulocyte lysate (Fig. 10). Total RNA purified from two-cell embryo extracts translates into numerous polypeptides ranging from 20,000 to >200,000 *M_r*. An equal quantity of microtubule-associated

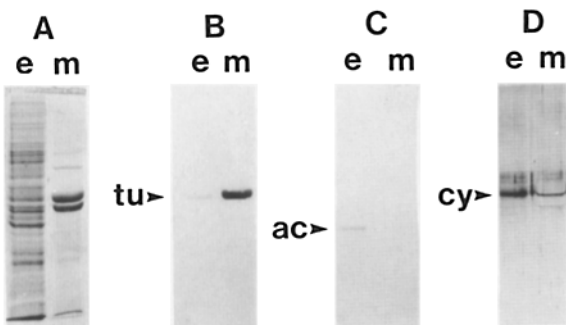


Figure 6. Microtubule preparations from two-cell embryos contain traces of a cyokeratin-like polypeptide and no detectable actin. Cytosolic extract (*e*) and microtubule protein (*m*) were separated by SDS-PAGE (*A*) and were immunoblotted with anti-tubulin (*B*), anti-actin (*C*), and anti-cyokeratin antibodies (*D*). The positions of α -tubulin (*tu*), actin (*ac*), and cyokeratin (*cy*) are marked.

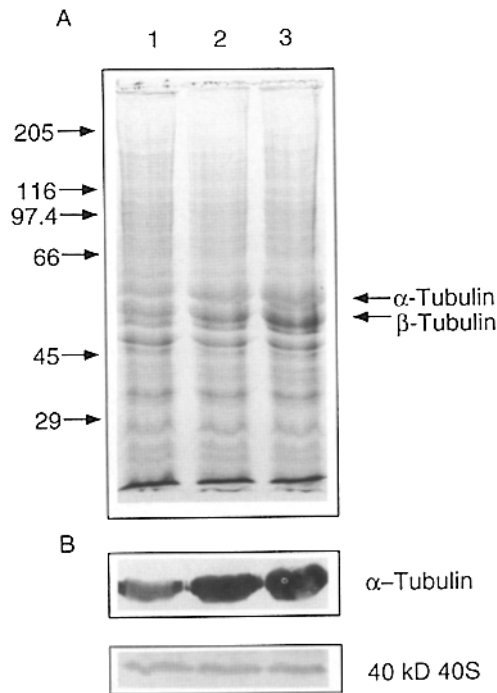


Figure 7. Limiting factor for ribosome binding. Sea urchin microtubules were coassembled in two-cell embryo extracts in the absence (lane 1) and presence of 0.5 mg (lane 2) and 1.0 mg (lane 3) phosphocellulose-purified bovine brain tubulin. The coassembling polypeptides were analyzed by SDS-PAGE (*A*) and by immunoblotting (*B*) with antibodies against α -tubulin and the 40-kD polypeptide from the 40S ribosomal subunit.

RNA also translated into numerous polypeptides spanning the same relative molecular mass range. Significantly, there are at least five labeled polypeptides that are highly enriched in the microtubule preparation. These polypeptides have relative molecular masses of 89, 77, 57, 51, and 39 kD. The identity of these polypeptides is not known at this time.

Discussion

There is a long history of observations of messenger RNA associations with cytoskeletal elements in various cell types (reviewed by Jeffery, 1989; Fulton, 1993; Singer, 1992; Steward and Banker, 1992; Suprenant, 1993; Wilhelm and Vale, 1993). Most of these observations have been based on the isolation of crude cytoskeletal preparations by subcellular fractionation of mechanically disrupted or detergent-extracted cells. The prevailing approach has been to prepare detergent extracts of whole cells, to call this a cytoskeleton, and to identify any polyribosomes remaining as "cytoskeletal associated." While these approaches were important and pioneering (Lenk et al., 1977), they are also very difficult to control for artifacts. In this report, we have demonstrated that we can purify and reconstitute a microtubule-poly(A)⁺ RNA-ribosome complex in sufficient quantity to permit biochemical analysis. This preparation should prove invaluable for dissecting which mRNA molecules interact with microtubules in vivo, and for the identification and characterization of molecular components involved in their localization.

Ribosomes copurify with microtubule preparations from unfertilized sea urchin eggs through repetitive cycles of

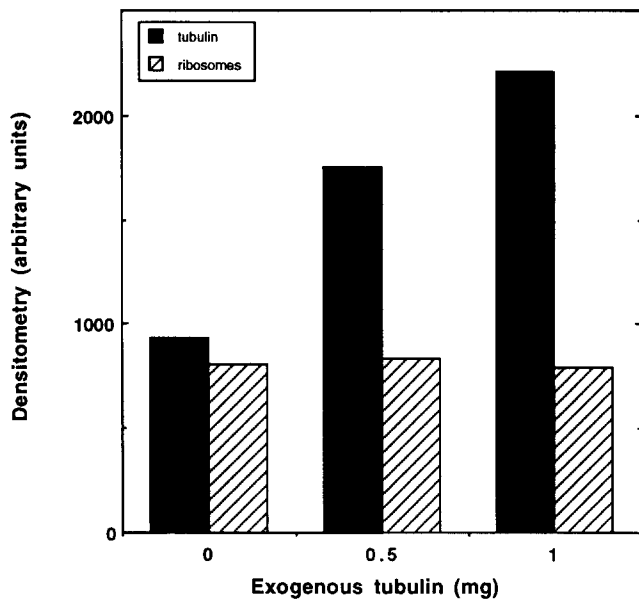


Figure 8. Quantitation of the amount of ribosomes that copurify with increasing amounts of microtubules. Histogram of the immunoblotting data presented in Fig. 7. With the addition of 0.5 and 1.0 mg brain tubulin, the amount of sedimentable tubulin in the form of microtubules doubles. In contrast, the amount of sedimentable ribosomal protein remains constant.

Table II. Percentage of Total Two-cell Ribosomes

Control	Emetine	Cycloheximide	NaF	Puromycin
1.0-3.0	1.2-3.5	1.5-4.3	1.5-4.4	1.4-4.0
n = 3	n = 3	n = 2	n = 3	n = 2

Quantitation of ribosome binding to microtubules in the presence of translational inhibitors. Immunoblotting was used to determine the fraction of the total ribosomes that copurified with microtubules in the absence and presence of 100 μ M emetine, 20 μ g/ml cycloheximide, 10 mM NaF, or 500 μ g/ml puromycin.

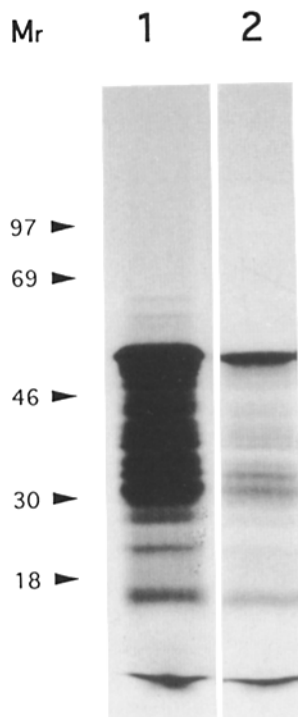


Figure 9. Translation of Xef-1 RNA is impaired in the presence of purified microtubule protein. (Lane 1) Xef-1 RNA alone. (Lane 2) Xef-1 RNA and two-cell microtubule protein.

Table III. Percentage of Poly(A) RNA/Total RNA

	Unfertilized egg	Two-cell embryo
Cytosolic extract	0.023	0.090
H ₁ P	0.013	0.056
H ₂ P	0.004	0.020

Quantitation of the percentage of poly(A) RNA in microtubule preparations from unfertilized eggs and two-cell embryos. The percent of poly(A)RNA/total RNA was calculated as described in Materials and Methods for the starting extracts and first cycle (H₁P) and second cycle (H₂P) microtubule pellets.

temperature- and pH-induced assembly and disassembly. Their microtubule association is mediated by ionic interactions, and in selected thin sections, a long tapered stalk can be observed to connect the two organelles (this report; and Suprenant et al., 1989). Because the stalk is trypsin sensitive and RNase resistant (Suprenant et al., 1989), it is believed to be composed principally of protein. Well-defined protrusions are visible on three-dimensional reconstructions of eukaryotic ribosomes, and it is conceivable that these ribosomes are attached to microtubules through a prominent stalk on the 60S subunit (Lake, 1985; Verschoor and Frank, 1990). Circumstantial evidence suggests that the abundant 77-kD echinoderm microtubule-associated protein (EMAP) may comprise part or all of the stalk (Suprenant et al., 1993). Ribosomes are associated with EMAP-containing microtubules, but not with EMAP-deficient microtubules. Moreover, microtubules extracted with moderate salt levels (0.35 M KCl) have neither ribosomes nor EMAPs associated with them (Suprenant et al., 1989, 1993).

Only a small percentage of the eggs' ribosomes (<3% by mass) copurify with the microtubules, suggesting that they may be a subset of distinct ribosomes or that there is a limit-

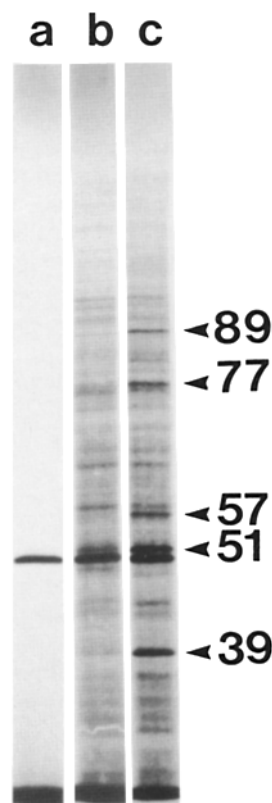


Figure 10. In vitro translation of microtubule-associated mRNA. Total RNA was purified by phenol-chloroform extraction from two-cell embryo extracts (lane b), as well as from microtubules purified from two-cell embryos (lane c). Equal amounts of RNA were translated in vitro in a message-dependent rabbit reticulocyte lysate in the presence of [³⁵S]methionine. The translated polypeptides were separated by SDS-PAGE and autoradiographed. A 50-kD product (arrow) is present in the zero-message control (lane a), as well as the experimental samples (lanes b and c), and should be disregarded. Five polypeptides (with relative molecular masses of 89, 77, 57, 51, and 39 kD) are enriched above background levels in the microtubule-derived RNA lane.

ing factor for their association. Although the conservation of ribosomes is often emphasized, the prototypical eukaryotic ribosome is composed of ~80 different proteins and four RNA species, and it is conceivable that transcriptional and translational mechanisms give rise to subpopulations of ribosomes during the life cycle of an organism. There are a few examples of organ-specific or stage-specific ribosomal proteins (Ramagopal, 1992; Etter et al., 1994), although it is not known how their function or regulation may differ. Perhaps the microtubule-associated ribosomes are specialized for protein and RNA targeting to the cytoskeleton.

There are several lines of evidence that lead us to believe that the ribosome-microtubule interaction is specific: a distinct morphological structure appears to mediate their association; they copurify with microtubules through several cycles of microtubule assembly and disassembly; they bind with constant stoichiometry; there is a limiting factor for binding; and their association appears to be developmentally regulated. It is unlikely that the ribosomes are nonspecifically trapped within a network of microtubules. Ribosomes remain attached to microtubules in nearly the same ratio, whether they are associated with cycle-purified microtubules or taxol-stabilized microtubules. In the latter case, the microtubules were diluted 10-fold. If ribosomes were trapped, these associations would be reduced upon dilution. An additional argument against trapping is that we have purified microtubules (using the same methods as for sea urchins) from a variety of cells that contain large quantities of ribosomes (clam oocytes, mouse B16 cultured cells, bovine brain), and yet these microtubule preparations do not contain ribosomes that we can detect in the electron microscope (Suprenant et al., 1993).

Little protein synthesis occurs in the unfertilized egg cytoplasm, and this fact is reflected in the predominance of monoribosomes in our microtubule preparations. Occasionally, polyribosomes were associated with unfertilized egg microtubules, but this was probably caused by the activation of protein synthesis by the alkaline pH of the microtubule assembly buffer (Grainger et al., 1979; Johnson et al., 1976). To obtain a preparation that consistently was composed of polyribosomes and microtubules, we developed methods to isolate microtubules from two-cell sea urchin embryos, a stage when protein synthesis has increased 30-fold over that in the unfertilized egg (Goustin and Wilt, 1981). At this stage of development, polyribosome-microtubule complexes can be routinely purified using multiple cycles of assembly and disassembly.

The copurification of polyribosomes with two-cell microtubules was dependent upon the presence of microtubules, and, in selected sections, a single polyribosome cluster can be seen to directly contact a single microtubule. The bridges between ribosomes and microtubules in unfertilized egg microtubule preparations are not observed in two-cell embryo preparations. This may mean that the stalks are not present at the later stage. It is more likely, however, that the stalks are difficult to resolve because of the crowded packing of polyribosomes surrounding the microtubule. In the two-cell embryo microtubule preparations, these polyribosomes frequently appeared to be stretched out lengthwise along the microtubule wall with an axial periodicity of 28 ± 3 nm. The periodicity of ribosomes in a fully-loaded sea urchin polyribosome is 16–17 nm (Martin and Miller, 1983) suggesting that

the longer axial periodicity along the microtubule wall may result from the interaction of ribosomes with a periodic structural component of the microtubules.

There are several polypeptides in these microtubule preparations that could be involved in ribosome binding. The most obvious candidate is the 77-kD EMAP (Suprenant et al., 1993). It was shown previously that bovine brain MAPs bind to isolated ribosomes in a phosphorylation-dependent manner (Jesus et al., 1984a, 1984b). In addition, tau-like immunoreactivity is detected on polyribosomes in both neuronal and glial cells (Papazosomenos and Binder, 1987), as well as in the nucleoli of human neuroblastoma cells (Loomis et al., 1990). These examples indicate that MAPs may interact directly with ribosomes. In addition to MAPs, we have detected a 50-kD cytokeratin-like polypeptide in these preparations. The cytokeratin-like polypeptide may be related to the 50–55-kD polypeptide found in isolated mitotic apparatuses and detergent-resistant cytoskeletons (Raymond et al., 1987; Rebhun and Palazzo, 1988; Steffen and Linck, 1992). In many studies, polyribosomes appear to be associated with a detergent-resistant, intermediate filament-rich cytoskeleton (Pachter, 1992; Jeffery, 1989).

In addition to cytoskeletal proteins, these microtubule-ribosome complexes contain both the 66- and 80-kD PABPs of sea urchin eggs (Drawbridge et al., 1990). PABPs are highly conserved mRNA-binding proteins that participate in the regulation of message stability (Sachs, 1993). In sea urchin eggs, the level of PABPs is much greater than needed to bind all of the poly(A) tails in the cell, and it is tempting to speculate that PABPs also are involved in the localization of ribosomes and poly(A)⁺ RNAs to the cytoskeleton.

Nearly 6% of the total RNA in the two-cell embryo can be phenol extracted from microtubules prepared at this stage. Although the bulk of the microtubule-associated RNA is ribosomal, significant amounts of mRNA were also detected. We identified five specific mRNAs that are highly enriched in two-cell microtubule preparations. Although the identity of the polypeptide products of these messages is unknown, it is worthwhile to note that the relative molecular masses of tubulin and the major proteins that copurify with it are identical to the relative molecular masses of the *in vitro* translation products of the five microtubule-enriched mRNAs. One possibility is that the messages that copurify with microtubules code for microtubule proteins, and that their association with microtubules is mediated through nascent chain targeting in the polyribosome complex (Singer, 1992; Suprenant, 1993). It has been suggested that titin and the myosin heavy chain, two large cytoskeletal proteins, may be cotranslationally assembled in this manner into nascent myofibrils (Isaacs and Fulton, 1987; Isaacs et al., 1989). The inability of puromycin to block polyribosome association does not rule out the possibility of nascent chain targeting because stable polyribosomes may be preformed in the two-cell embryo before isolation and microtubule association.

The poly(A)⁺ RNA itself may contain a localization signal that directs the polyribosomes to the microtubules. Localization signals have been identified in the 3' untranslated region of several localized messages (Macdonald and Struhl, 1988; Gottlieb, 1992; Gravis and Lehman, 1992; Mowry and Melton, 1992; Schwartz et al., 1992; Kislauski et al., 1993; Macdonald et al., 1993). The microtubule-associated RNA may contain similar localization signals. It is not

known whether the microtubule-associated RNA binds directly to the microtubules or to a receptor such as RNA- and/or microtubule-binding proteins.

Besides protein and/or mRNA targeting, microtubules may also be involved in translational regulation of the mRNAs with which they are associated (reviewed in Suprenant, 1993), perhaps in the autoregulation of β -tubulin mRNA levels (Theodorakis and Cleveland, 1992). In the future, it will be important to identify the specific mRNAs associated with these microtubules to distinguish among these possibilities. The ability to isolate and reconstitute a complex of specific mRNAs, ribosomes, and microtubules provides the first step toward this direction.

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