Monoclonal Antibodies to Dynein Subunits Reveal the Existence of Cytoplasmic Antigens in Sea Urchin Egg

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ABSTRACT Monoclonal antibodies directed against subunits of a sea urchin flagellar dynein were used to test for the presence of cytoplasmic antigens in preparations of fertilized eggs and mitotic apparati. A 9-10 S complex composed of 330,000-, 134,000-, and 126,000-mol-wt subunits was isolated from outer arms of *Strongylocentrotus purpuratus* sperm flagella and used to characterize the antibodies. Seven monospecific antibodies to the 330,000 subunit and two against the 134,000 subunit of the 9-10 S complex were identified by binding to nitrocellulose blots of electrophoretograms resolving polypeptides from different dynein preparations. The antibodies were applied also to blots of polypeptides from fertilized sea urchin egg at the first metaphase and a cellular fraction of mitotic apparati. Three of the antibodies to the 330,000 subunit bound to a cytoplasmic polypeptide of approximately the same molecular weight and the two antibodies to the smaller subunits recognized a polypeptide of 124,000 apparent molecular weight. Both antigens appeared to be enriched in the fraction containing mitotic apparati. These results indicate that polypeptides similar to two subunits of the 9-10 S complex are present in eggs at metaphase, and they are apparently associated with the mitotic apparatus.

The dynein arms of flagellar microtubules are structures involved in the generation of movement. The major constituents of the arms, the dyneins, in association with other proteins release the energy stored in ATP and perform the mechanical work that generates the sliding of adjacent doublet microtubules. A large body of experimental work has revealed different aspects of dynein arm structure, polypeptide composition, and function, and has been summarized in recent reviews (6, 2).

Early in the study of dynein arms, it was suggested that these structures could also be found associated with the other microtubule arrays where they could produce different types of movement such as intracellular transport and mitosis. However Weisenberg and Taylor (30) emphasized that it could be difficult to distinguish a cytoplasmic dynein from all other cellular molecules hydrolyzing ATP. Since then it has been suggested that dynein-like ATPases are present in sea urchin egg cytoplasm, cortical layer, and mitotic apparatus (30, 13, 20, 21). The identification of these activities as dyneins was based primarily on enzymatic properties of unpurified preparations. Recently an ATPase activity from unfertilized sea urchin eggs was purified to near homogeneity and identified as dynein by binding experiments of crude enzyme fractions to outer doublet microtubules (9). To date studies on the precise location and function of putative cytoplasmic dyneins have not been developed primarily because of the lack of probes that are specific for the structure of dynein molecules.

To explore the possibility that proteins similar to dyneins of flagellar axonemes are associated with the microtubular machinery of the mitotic apparatus, this study, which adopts the sea urchin, *Strongylocentrotus purpuratus* as a model system, has been undertaken. This organism is convenient because it provides flagella from spermatozoa and mitotic apparati from fertilized eggs at first metaphase in amounts that are suitable for biochemical studies. However in studying the microtubule-associated proteins of sea urchin embryos it is important to consider a potential problem. The precursors of dyneins that assemble in cilia at a later stage of embryogenesis may exist already in the egg at first metaphase (26) and may complicate the molecular analysis of the mitotic apparatus. This topic will be discussed in a following section in light of the results of our study.

This report describes the characterization of monoclonal antibodies to a specific flagellar dynein and their use to test for the presence of dynein-like molecules in the cytoplasm of fertilized sea urchin eggs and in the mitotic apparatus. A 9-10 S complex was isolated from a 15 S dynein of *S.*

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MAY 1984 1842–1850

purpuratus axonemes. Sets of monoclonal antibodies to high and intermediate molecular weight subunits of the 9-10 S complex were characterized and used to analyze egg and mitotic apparatus preparations. Polypeptides of molecular weight similar to the flagellar components were detected in homogenates of fertilized eggs at the first metaphase and were found to be enriched in cellular fractions containing mitotic apparati.

MATERIALS AND METHODS

Isolation of Sea Urchin Sperm Axonemes: Preliminary experiments of this study were performed with axonemes prepared according to the procedure of Gibbons and Fronk (7), previously applied to urchin *Tripneustes gratilla*. Having observed that during the isolation outer arms were partially extracted from the *S. purpuratus* axonemes, the preparation was modified in two details. (*a*) Flagella were detached from sperm heads by shearing in an isotonic solution of sucrose instead of being treated in a hypotonic solution in the presence of nonionic detergent. (*b*) The detergent was added to the flagella after all contaminating heads were removed by differential centrifugation. Axonemes prepared by the modified protocol and analyzed in thin section electron micrographs appeared well preserved (16). The extraction of outer arms from axonemes prepared by either of the two methods was correlated with the formation of a 15 S particle.

The modified method was applied at 0–4°C as follows: sperm in artificial sea water at 10–30 mg of proteins/ml was centrifuged at 3,000 *g* for 10 min and then suspended at the same concentration in 0.9 M sucrose, 0.1 M NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM Tris/Cl, pH 7. The suspension was homogenized with five strokes of a Dounce homogenizer and then centrifuged three times for 10 min at 12,000 *g* to remove sperm heads. 0.5% Nonidet P40 was added before sedimenting the axonemes by a 15-min centrifugation at 35,000 *g*. Axonemes were suspended at 5–10 mg/ml in 0.1 M NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM Tris/Cl, pH 7 and then extracted in the presence of 0.6 M NaCl as described (18).

Assays and Gel Electrophoresis: Procedures followed for ATPase and protein assays and gel electrophoresis were described before (18). All electrophoresis was performed on slab gels of reduced size (6 cm long, 20 cm wide, and 0.075 cm thick); volumes of the sample analyzed were 5–10 μl.

Sedimentation in Sucrose Gradients: In typical experiments 1-ml aliquots of 0.6 M NaCl extract from axonemes containing 1 mg/ml of proteins were applied on 5 to 20% sucrose gradients containing 0.5 M NaCl and 0.01 M HEPES, pH 7 and centrifugation was performed in a Beckman SW 41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm for 13 h at 5°C. 0.3-ml aliquots containing 0.1 mg/ml of the 15 S dynein complex were dialyzed against 0.5 mM EDTA, 14 mM 2-mercaptoethanol, 5 mM HEPES, pH 7 then applied on 5 to 20% sucrose gradients containing 0.5 mM EDTA, 14 mM 2-mercaptoethanol, and 5 mM HEPES, pH 7, and centrifugation was performed in a Beckman SW 55 rotor (Beckman Instruments, Inc.) at 55,000 rpm for 5 h at 5°C. ^{125}I-RU-1, 5-P_2carboxylase, and ^{125}I-catalase labeled with chloramine T method (11) were sedimented as standards in the gradients containing the samples.

Preparation of Hybridomas and Monoclonal Antibodies: Mice were immunized by two to five 0.4-ml injections of 50 μg of 15 S dynein complex in 0.13 M NaCl, 0.01 M Na phosphate pH 6.8 mixed 1:1 with complete Freunds' adjuvant (Difco Laboratories, Inc., Detroit, MI). Spleens were removed 3–4 d after the boost and fusion of spleen cells to myeloma cells was performed by polyethylene glycol Merck 4000 GK and standard procedures (4). A nonproducing myeloma line, P_3U_1, was given to us by Dr. Jay Unkeless of The Rockefeller University. This line was originally derived from the myeloma P3-X63 Ag 8 by Dr. Matthew D. Scharff (Albert Einstein College of Medicine, Yeshiva University, Bronx, NY).

Two tests were used for the selection of hybridomas producing antibodies of interest. In the first selection procedure, hybridoma culture media were screened against native axonemes bound to plastic of microtiter plates coated with 1% polylysine. In the second procedure, 3 × 0.2-cm strips of nitrocellulose (Millipore Corp., Bedford, MA, HAWP 304 FO) carrying axonemal polypeptides resolved by SDS PAGE and transferred to it by electrophoresis (29) were incubated in the media. Detection of antigen-antibody interaction was performed by ^{125}I or peroxidase labeled $F(ab')_2$ fragments of goat IgG anti-mouse IgG. Cloning of hybridomas on soft agar, ascitic fluid production, and purification of IgG were performed by standard procedures (4).

Protein Hydrolysis: Partial hydrolysis of 0.8 mg 0.6 M NaCl extract from *S. purpuratus* axoneme in 0.1% 2-mercaptoethanol, 0.05% SDS, 1 mM EDTA, and 20 mM Tris-borate, pH 8.64 was performed. We used 10 μg of *Staphylococcus* V8 protease (Miles Laboratories, Inc., Elkhart, IN; batch 2059, 500 U/mg) for 25 min at 25°C.

Preparation of Mitotic Apparati: This procedure was adapted by combination of different published methods (22, 24). *S. purpuratus* eggs in sea water were passed through four layers of cheesecloth to remove large debris and then washed three times by decantation in sea water at 14°C. Subsequent operations were performed at the same temperature. 25 μl of "dry" sperm was used to fertilize 10 ml of packed eggs in 100 ml of sea water; then the gametes were suspended by gentle shaking. After 1 min mercaptoethyl gluconamide was added to a final concentration of 2 mg/ml. From 2–5 min after fertilization, the fertilization membrane was removed by passing the eggs once through a 54-μm nylon mesh. The stripped eggs were washed twice in 300 ml of sea water by decantation. Incubation and gentle shaking were performed in 200 ml of sea water contained in a dish large enough to allow the formation of a monolayer of eggs. After 90 min from fertilization, eggs were washed twice by centrifugation at 160 *g* for 3 min in 200 ml 1 M dextrose warmed to 28°C. Lysis was performed at the same temperature in 10 ml 0.05% Triton X-100, 0.1 M NaCl, 1 mM EGTA, 5 mM morpholino-ethane sulfuric acid at pH 6.2 and the cell lysate was passed three times through a 54-μm nylon mesh. Sedimentation of mitotic apparati and formation of a loose pellet were obtained at 300 *g* for 5 min. The pellet was dispersed in 10 ml of lysis buffer in the presence of 500 KalliKrein Inactivator U/ml of Trasylol (FBA Pharmaceuticals, New York) and reformed by centrifugation as above. Cold soluble proteins were obtained from a suspension of mitotic apparati that were kept on ice for 1 h in lysis buffer at a concentration of 10 mg/ml. The extract was separated from the insoluble residue by a 30-min centrifugation at 30,000 rpm in a SW 65 Beckman rotor (Beckman Instruments, Inc.).

Preparation of Egg Homogenate: Fertilized sea urchin eggs at the first metaphase were suspended in one volume of lysis buffer and homogenized at 0°C by 10 strokes of a Dounce homogenizer. The homogenate was immediately diluted to a ratio 4:1 by a solution containing 5% SDS, 1% 2-mercaptoethanol, 0.01% EDTA, 0.3 M Tris/Cl, pH 8.8 and stored at −10°C.

RESULTS

Identification of Dynein Subunits

The purification of a dynein complex was obtained by sedimenting NaCl extracts of axonemes in sucrose gradients at high and low ionic strength, as was performed in previous isolations of dyneins from sea urchin (28). Extracts from axonemes of the sea urchin *S. purpuratus*, described in this study, differed in two respects from those prepared previously from the sea urchin *T. gratilla*. They were composed mainly of particles that had a sedimentation coefficient of 15 and not 21 S as in *T. gratilla* (7). The polypeptides with molecular weights close to 100,000 from the *S. purpuratus* 15 S fraction have different electrophoretic mobilities and antigenic properties than the constituents of *T. gratilla* 21 S.

Fig. 1*A* shows the sedimentation profile of Mg^{++}-activated ATPase activity present in the NaCl extract of *S. purpuratus* axonemes and the electrophoretograms of polypeptides found in the sucrose gradient fractions which also contain 0.5 M NaCl and 0.01 M HEPES, pH 7. The plot of the activity is superimposed on the photograph of a 4–11% polyacrylamide gradient gel that resolves polypeptides of molecular weight above 15,000. The maximum of the ATPase activity is found in fraction *9* and the sedimentation coefficient of the peak was estimated to be 15 S. The ATPase activity is found between fraction *1* and *11*, which contain polypeptides of molecular weight 330,000, 134,000, 126,000, 62,000, 54,000, and 50,000 as major components. These polypeptides are indicated by arrows. Trace amounts of other polypeptides are detected as well. The 54,000 and 50,000 constituents were identified as tubulin subunits by a radioimmune assay (17). To correlate the ATPase activity with a putative high molecular weight dynein subunit, the same fractions described in Fig. 1*A* were analyzed by an electrophoretic technique that resolves dynein heavy chains in the molecular weight range

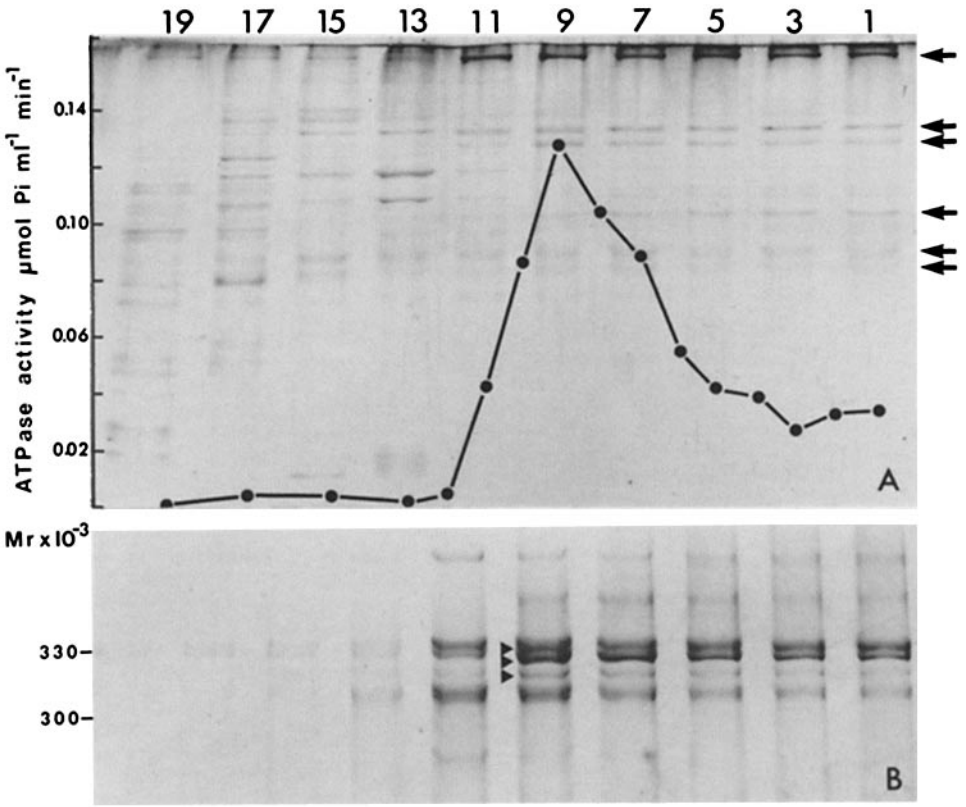

FIGURE 1 Sedimentation of a 15 S dynein. Polyacrylamide gel electrophoretogram of polypeptides extracted from axonemes and sucrose gradient sedimentation profile of ATPase activity. (*A*) 1 mg of proteins was applied to a 12 ml 5 to 20% sucrose gradient also containing 0.5 M NaCl and 0.01 M HEPES. The gradient was centrifuged as described and then divided in 20 fractions. ^{125}I-labeled RU-1,5-P_2 carboxylase and catalase were used as internal standards of sedimentation. 5 µl of each fraction were electrophoresed on separate tracks of a gel resolving polypeptides of molecular weight above 15,000. The gel was stained by silver. Arrows indicate major components present in fractions *1* to *11*. Other 5 µl of the fractions were used for the assay of Mg^{++}-activated ATPase activity. Plot of the activity is superimposed on the photograph of the gel. (*B*) Polyacrylamide gel electrophoretograms showing polypeptides of molecular weight above 300,000 that are present in fractions analyzed in *A*. 5-µl samples were applied to the gel that was stained by silver. Triangles indicate three bands that are present at the highest concentration in fraction 9.

300,000 to 330,000 (18). The result of the electrophoresis is shown in Fig. 1 *B*. Seven polypeptides are detected in fraction *9* which contains the highest Mg^{++}-activated ATPase activity. Three of the polypeptides that are indicated by triangles are present at the highest concentration in fraction *9*. In fractions *1* through *11* they appear to follow the distribution of the ATPase activity. Other polypeptides of high molecular weight show a different sedimentation behavior and are enriched in other fractions. On the basis of this evidence the polypeptides co-sedimenting with ATPase activity were identified as dynein subunits.

The isolation of a dynein heavy chain and evidence indicating that the 134,000 and 126,000 polypeptides are associated with it, was obtained by the sedimentation of the 15 S complex constituents in a sucrose gradient at low ionic strength. After a dialysis against 0.5 mM EDTA, 14 mM 2-mercaptoethanol and 5 mM HEPES pH 7.0 and a sedimentation in the same solution, the constituents of the 15 S fraction were dissociated into two groups. One sedimented as a 9-10 S complex and was composed of a dynein heavy chain of molecular weight close to 330,000 together with the 134,000 and 126,000 polypeptides (see Fig. 2, *A* and *B*). A second set sedimented with coefficients greater than 11 S and was formed by the other high molecular weight constituents (data not shown).

The electrophoretic analysis of components of *S. purpuratus* and *T. gratilla* dynein complexes is shown in Fig. 2. The electrophoretograms were obtained in 3.2–4% and 4–11% polyacrylamide gradient gels. Samples analyzed in Fig. 2*A* are *1*, *S. purpuratus* axonemes; *2*, NaCl extract of *T. gratilla* axonemes; *3*, the 15 S; and *4*, the 9-10 S complex from *S. purpuratus*. Only the last three samples are analyzed in Fig. 2*B*. Fig. 2, *A* and *B*, last two lanes, show that the sedimentation at low ionic strength resolved a dynein heavy chain away from other polypeptides of high molecular weight but not from 134,000 and 126,000 components. The single high molecular weight polypeptide in the 9-10 S complex corresponds to the faster component of molecular weight close to 330,000. Trace amounts of tubulin are also present in the 9-10 S fraction.

Fig. 2*A* also shows that heavy chains of the 15 S and 9-10 S complexes have the same electrophoretic mobility as the major high molecular weight component of the *T. gratilla* sample. In contrast Fig. 2*B* shows that none of the major intermediate molecular weight constituents of *T. gratilla* crude extract have the same mobility as the 134,000 and 126,000 polypeptides. It is evident that dynein heavy chains from different urchin species have the same apparent molecular weights. However they appear to be associated with different polypeptides of intermediate molecular weight (mo-

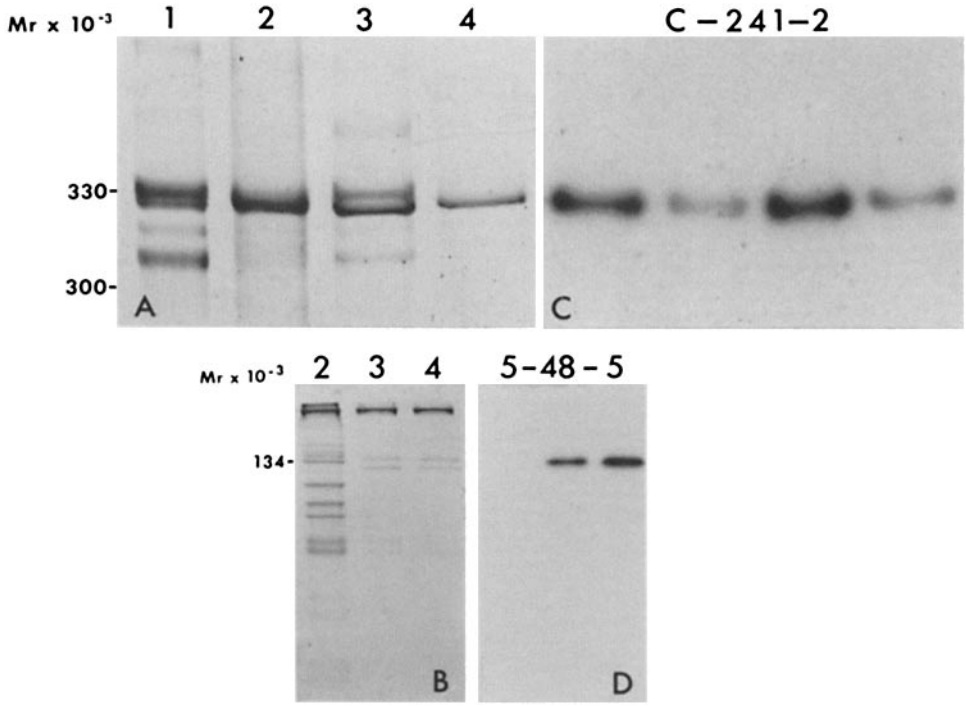

FIGURE 2 Antibody binding to dynein subunits. Polyacrylamide gel electrophoretograms of polypeptides and autoradiograms of nitrocellulose replicas. (*A*) Polyacrylamide gel electrophoretograms of polypeptides of molecular weight above 300,000. The samples applied to the gel are lane *1*, axonemes of *S. purpuratus*; lane *2*, NaCl extract of *T. gratilla* axonemes; lane *3*, 15 S; and lane *4*, 9-10 S complex prepared from *S. purpuratus*. (*B*) Polypeptides of molecular weight above 15,000 of the last three samples analyzed in *A*. (*C*) Replica of the gel shown in *A* incubated with C-241-2 antibody. (*D*) Replica of the gel shown in *B* incubated with 5-48-5 antibody.

lecular weight close to 100,000). Other investigators found that the 9-10 S dynein from *T. gratilla* is formed by two polypeptides of molecular weight 320,000 and 122,000 (1, 28, 2).

Isolation of Monoclonal Antibodies to Dynein Subunits

The 15 S complex containing the 330,000, 134,000, and 126,000 dynein subunits as well as other polypeptides was used to immunize mice. Nine monoclonal antibodies to the dynein subunits were prepared and characterized, as described in Materials and Methods. The designation of the hybridomas secreting the antibodies and some of the characteristics of the antibodies are listed in Table I. All antibodies bind specifically to one dynein subunit.

Evidence concerning the specificity of the antibodies was obtained by binding the antibodies to axonemal polypeptides that were resolved by polyacrylamide gel electrophoresis and then transferred to nitrocellulose sheets. Fig. 3*A* shows low resolution electrophoretograms of equal amounts of axonemes, NaCl extract, and postextraction axonemal residue. Tubulin subunits are major components of the axoneme and of the residue. The extract is enriched in polypeptides of molecular weights near 300,000 and in six to eight components of molecular weight between 60,000 and 134,000. Fig. 3, *B* and *C* show the autoradiograms of the nitrocellulose replicas incubated, respectively, with C-241-2 and 5-48-5 antibodies. Detection of antigen-antibody binding was achieved by using ^{125}I-labeled F(ab')$_2$ fragments of goat IgG anti-mouse IgG as secondary antibody. C-241-2 antibody binds to one component of molecular weight above 300,000 and 5-48-5 binds to a 134,000 molecular weight component.

TABLE I

Characteristics of Monoclonal Antibodies

Hybridomas*	Mol Wt of axonemal antigens	Binding to *Tripneustes* axonemal antigens‡	Binding to egg cytoplasmic antigens‡
[C-241-2	330,000	+	+
2-79-2]§	330,000	+	ND¶
[6-31-24	330,000	+	+
6-64-14]§	330,000	+	ND
4-69-14	330,000	−	+
132-7	330,000	−	−
C-26-2	330,000	+	−
146-28	134,000	−	+
5-48-5	134,000	−	+

* All hybridomas secrete IgG's.

‡ Antibody binding to axonemal polypeptides that were resolved by polyacrylamide gel electrophoresis and then transferred to nitrocellulose sheets as described in the text.

§ Designations of hybridomas secreting antibodies to similar antigenic determinants are between brackets. The two couples between brackets recognize different determinants.

¶ Not determined.

In all replicas the antigens appeared to be enriched in the sample of the NaCl extract and to be absent in the NaCl extracted residue. The antibodies 2-79-2, 6-31-24, 6-64-14, 4-69-14, 132-7, and C-26-2 behaved like C-241-2 and produced a binding pattern similar to that shown in Fig. 3*B*. The antibody 146-28 was indistinguishable from 5-48-5 by this test. There was no evidence of binding to other polypeptides, suggesting that the antibodies recognize one antigen and that the antigens are not proteolized. Moreover the extraction of the antigens by exposure of the axonemes to 0.6 M NaCl appears to be complete.

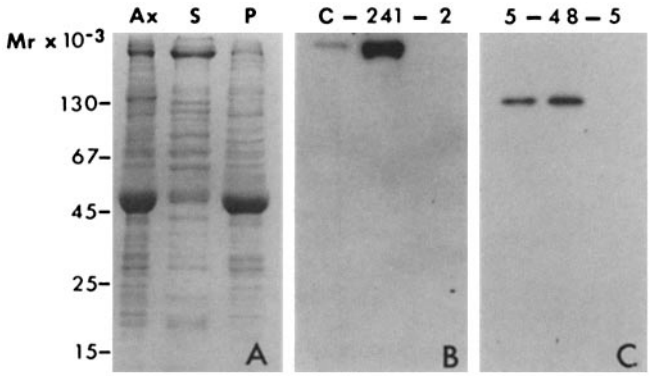

FIGURE 3 Monoclonal antibody binding to axonemal polypeptides. Polyacrylamide gel electrophoretograms of polypeptides and autoradiograms of nitrocellulose replicas. (*A*) Polyacrylamide gel electrophoretograms of polypeptides stained by Coomassie Blue. Position of molecular weight standards is indicated at *left*. 10 μg of axonemes, NaCl extract from the axonemes and postextraction axonemal residue were applied to the gel. Two sets of the same samples were electrophoresed in parallel and then transferred to nitrocellulose. (*B* and *C*) Autoradiograms of nitrocellulose replicas incubated with C-241-2 and 5-48-5, respectively.

More precise analysis of the specificity of the antibodies to the high molecular constituents was performed by binding the antibodies to axonemal proteins resolved in a low percentage polyacrylamide gel and then transferred to nitrocellulose. The major components present in the 15 S fraction were resolved under these conditions; therefore it was possible to observe that all seven antibodies bound to the same band that corresponds to the heavy subunit of 9-10 S complex (see Table I). These binding tests to the nitrocellulose replicas were performed with equal amounts of purified monoclonal immunoglobulins, which were in large excess over the quantity of the antigen, so that it was possible to estimate the relative affinity of each antibody for its antigenic determinant. Under these conditions it was evident that 132-7 and C-26-2 antibodies bound more weakly than the other antibodies (data not shown).

Evidence showing that the antibodies are specific for two subunits of 9-10 S complex was obtained by testing the binding to axonemal polypeptides and to sucrose gradient-purified fractions. The first and the last two lanes of Fig. 2, *A* and *C* show the binding of C-241-2 antibody to a subunit that has molecular weight close to 330,000 and is present in axonemes, in the 15 and 9-10 S complexes. This polypeptide was identified in the previous section as one of the 15 S dynein subunits. No other component of the three samples is recognized by the same antibody. Fig. 2, *B* and *D* show the binding of 5-48-5 to the 134,000 subunit present in the 15 and 9-10 S complexes. The antibody 146-28 was found to bind to the same 134,000 polypeptide (data not shown).

To test the antibodies listed in Table I for cross-reactivity to components of *T. gratilla* axonemes, *Tripneustes*, and *S. purpuratus*, samples were resolved in parallel by gel electrophoresis and analyzed. Fig. 2, *A* and *C*, second lane, show that C-241-2 antibody binds to the major component of high molecular weight present in the NaCl extract of *T. gratilla* axonemes. In contrast, Fig. 2, *B* and *D*, first lane, show that no reaction was found with 5-48-5 antibody to the 134,000 component. Cross-reaction with *T. gratilla* determinants was observed also with 2-79-2, 6-64-14, 6-31-24, and C-26-2 antibodies (data not shown).

At least five of the seven monoclonal antibodies to the 330,000 dynein subunit and both antibodies to the intermediate molecular weight chains recognize different antigenic determinants. Evidence of this diversity was obtained by binding the antibodies to nitrocellulose replicas carrying peptides prepared by partial digestion of the NaCl extract of axonemes with V8 protease. Fig. 4 shows the binding patterns obtained with antibodies to the dynein heavy chains when they were incubated with nitrocellulose strips replicated from the electrophoretogram of the partially digested antigen. Different patterns of multiple bands, corresponding to peptides containing the antigenic determinants were found by C-26-2, 4-69-14, 2-79-2, 6-64-14, and 132-7 antibodies that were applied to identical replicas. The antibodies C-241-2 and 6-31-24 appeared to have specificities similar to those of 2-79-2 and 6-64-14, respectively. The same procedure was followed to demonstrate that 146-28 and 5-48-5 antibodies to the 134,000 polypeptide are different.

In summary at least seven different antibodies to two subunits of the same dynein complex have been characterized by these experiments.

Separation of Mitotic Apparati from Fertilized Eggs at First Metaphase

Cellular fractions enriched in mitotic apparati were prepared as described in the Materials and Methods. The procedure involved washing the fertilized eggs twice with artificial sea water and twice with 1 M dextrose followed immediately by lysis of the eggs at metaphase. These treatments were performed with large volumes of solutions and were sufficient to eliminate any residual sperm from the preparation, as was assessed by light microscopic observation. After the lysis of the eggs the mitotic apparati were pelleted at 300 *g* for 5 min; this condition is not sufficient to sediment other cellular components. However, yolk granules and large debris remained trapped in the pellet. These contaminants could be partially removed by washing the preparation with the lysis

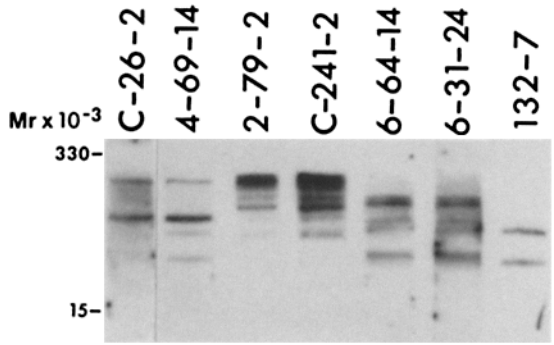

FIGURE 4 Diversity of the antibodies to the same dynein subunit. Autoradiograms of nitrocellulose replicas. Limited proteolysis of the polypeptides that are present in the NaCl extract of *S. purpuratus* axonemes was performed by V8 protease as described in Materials and Methods. The peptides were electrophoresed in a gel resolving molecules of molecular weight above 15,000 and then transferred to nitrocellulose sheets. Strips of nitrocellulose replicating parts of the same electrophoretogram were incubated with each of the antibodies indicated. Exposure time for autoradiography of the nitrocellulose strip treated with C-26-2 antibody was 10 times longer.

buffer and repeating the sedimentation step. However these treatments disrupted a small percentage of mitotic apparati and changed the morphology. Therefore the washing was performed only once.

Fig. 5 shows a photomicrograph of a typical preparation of mitotic apparati at metaphase, as they are recovered before the washing. Several apparati appear intact. Others have been divided in half. Asters, centrospheres, spindles, and chromosomes are easily recognized. Arrows indicate the chromosomes in the metaphase plate and asterisks indicate some chromosomes that remained attached to disrupted half spindles. Some debris floating in the medium is visible in the background.

The fraction formed by the mitotic apparati is enriched in microtubular structures and contains more polypeptides than are found in flagellar axonemes. These characteristics were made evident by the analysis of polypeptide components. Fig. 6 shows the electrophoretograms of polypeptides from an homogenate of eggs at their first metaphase, a mitotic apparatus preparation, a cold soluble extract of mitotic apparati, and axonemes. The amount of proteins analyzed from the first two samples was equal; the amount of protein in the third and fourth samples was adjusted so that the content of tubulin subunits was similar to that of the mitotic apparatus fraction. The composition of the mitotic apparatus preparation is very different from that of the egg homogenate, and tubulin subunits appeared to be major constituents of the samples. Evidence for the enrichment of microtubular structures in the mitotic apparatus preparation was derived from the analysis of an extract prepared by exposure of the mitotic

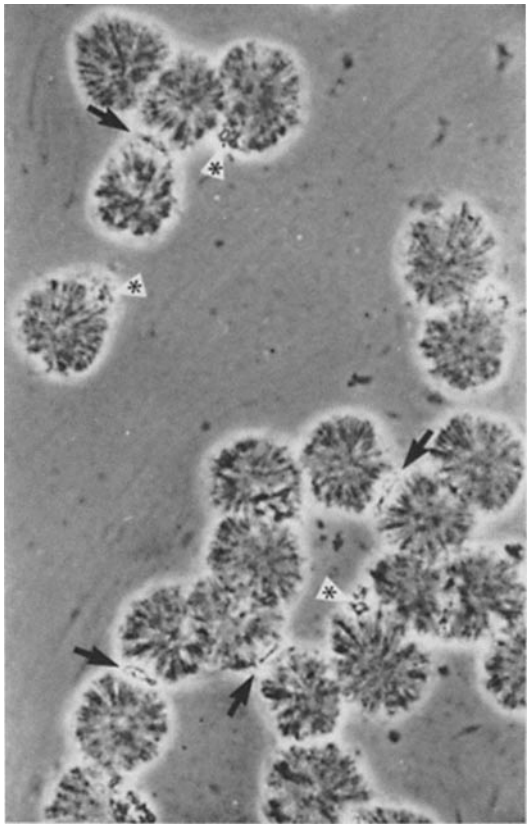

FIGURE 5 Photomicrograph of mitotic apparati: phase-contrast photomicrograph of mitotic apparati isolated from *S. purpuratus* eggs at the first metaphase. Arrows indicate metaphase plates and asterisks indicate half spindles. × 350.

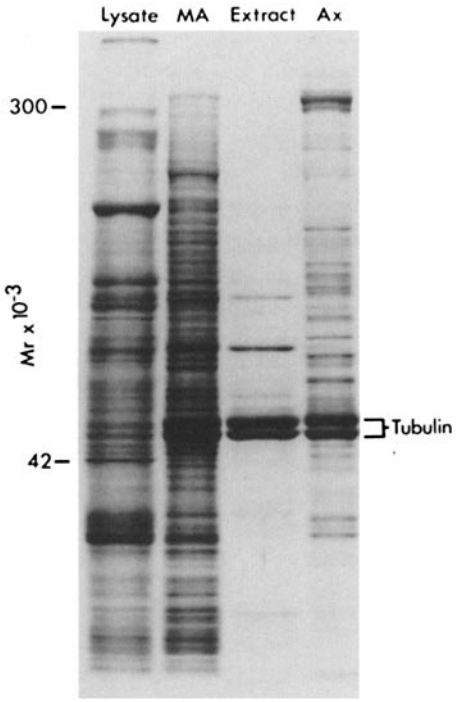

FIGURE 6 Polypeptide composition of sea urchin egg, egg subcellular fractions, and sea urchin sperm axoneme. Polyacrylamide gel stained by Coomassie Blue. Electrophoretograms of polypeptides of molecular weight above 15,000. The samples analyzed are sea urchin egg at the first metaphase, cellular fraction containing mitotic apparati, cold soluble proteins from the mitotic apparatus fraction, and axonemes from sea urchin sperm flagella.

apparati to 0°C. In fact, spindle and aster microtubules are labile at low temperature (22). 20% of the protein was solubilized under that condition and tubulin subunits represented the major constituents of the solubilized fraction. From the comparison of the second and fourth sample, it is evident that the ratio between the content of polypeptides other than tubulin and tubulin subunits is higher in the mitotic apparatus than in axonemes.

Identification of Cytoplasmic Antigens Similar to Dynein Subunits

The antibodies to the dynein subunits characterized so far were used to determine whether similar antigens could be detected in homogenates of eggs at metaphase and in preparations of mitotic apparati. It seemed likely that cytoplasmic antigens may be present in these samples at a very low concentration. Therefore the analysis was performed on the highest amounts of cellular proteins that could be processed by our methods (see Materials and Methods and legend of Fig. 7). The test was performed by binding the antibodies to nitrocellulose replicas of electrophoretograms obtained with 100 μg of proteins. As an internal standard, 1 μg of axonemal proteins was added to one sample of egg homogenate. Under these conditions C-241-2, 6-31-24, and 4-69-14 antibodies were found to bind a polypeptide of high molecular weight present in the preparations of the egg and of the mitotic apparatus. In the sample of egg homogenate, the band visualized by autoradiography was just detectable, whereas in the axoneme and mitotic apparatus samples, the bands visualized were both distinct and similarly intense. Low level of binding to other polypeptides was detected in the sample of the mitotic

apparatus (data not shown). 132-7 and C-26-2 antibodies were tested by similar experiments. No binding to cytoplasmic antigens was detected with these probes (data not shown). However, it is not yet clear whether they did not bind to the antigens or whether the binding was not detected because the affinity to the antibodies is low.

Because three antibodies to different antigenic determinants of the same axonemal polypeptide bound primarily but not exclusively to one cytoplasmic antigen in the sample of the mitotic apparatus, we tested whether the level of binding to other components was altered by simultaneous application of the three antibodies. Only the signal from the high molecular weight polypeptide was enhanced under these new conditions. Binding to other components appeared to be insignificant. Fig. 7 shows the binding of mixed C-241-2, 6-31-24, and 4-69-14 antibodies to a nitrocellulose replica of a gel resolving polypeptides from axonemes, egg, and a cellular fraction. Antigens present in the samples of axonemes, axonemes mixed with egg homogenate, egg homogenate, and mitotic apparatus preparation appear to have similar electrophoretic behavior. The cytoplasmic antigen is most concentrated in the fraction containing mitotic apparati.

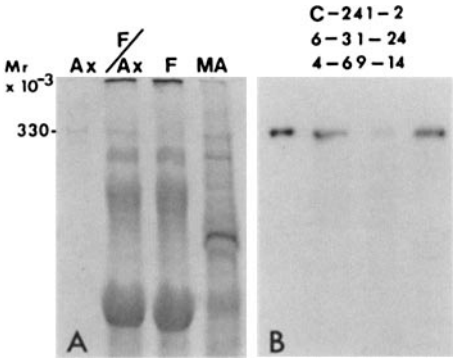

FIGURE 7 Antibody binding to polypeptides of mitotic apparatus preparations. Polyacrylamide gel electrophoretograms of polypeptides and autoradiograms of nitrocellulose replicas. (*A*) Coomassie Blue-stained polyacrylamide gel electrophoretograms of polypeptides of molecular weight above 150,000. From left to right the samples analyzed are 1 μg of axonemes; 100 μg of fertilized sea urchin egg at the first metaphase mixed with 1 μg of axonemes; 100 μg of fertilized sea urchin egg at the first metaphase; and 100 μg of a cellular fraction containing mitotic apparati. (*B*) Replica of the gel shown in *A* incubated with mixed C-241-2, 6-31-24, and 4-69-14 antibodies.

Further evidence of the existence of similarities among axonemal and cellular constituents was obtained by the application of 146-28 or 5-48-5 antibody. These antibodies bind to a polypeptide of 124,000 mol wt that is present in eggs at the first metaphase and is enriched in a fraction containing mitotic apparati. Fig. 8 shows the electrophoretograms of polypeptides from egg homogenate mixed with axoneme, egg homogenate, and mitotic apparati preparation resolved in a 4–11% polyacrylamide gel. Fig. 8, *B* and *C*, respectively, show the binding of mixed 146-28 and 5-48-5 antibodies and the binding of an anti-α tubulin antibody to the nitrocellulose replicas. The comparison of the binding activity detected in lane *2* and *3* of *B* indicates that the cytoplasmic antigen is enriched in the mitotic apparatus preparation. Compare lane *1* to lane *2* and *3* of the same panel: it is evident that axonemal and cellular components have slightly different mobilities. The analysis of homologous lanes in *B* and *C* clearly shows that in the fraction containing mitotic apparati the enrichment of the cytoplasmic antigen corresponds to the enrichment of α tubulin subunit. Moreover, assuming that the antibodies have the same affinity for the cytoplasmic antigen that they have for the axonemal constituents, it seems that the amount of the 134,000 subunit is present in the axoneme in a much higher ratio to tubulin than the 124,000-mol wt polypeptides in the mitotic apparatus sample. These results suggest that the cytoplasmic antigen is part of the mitotic

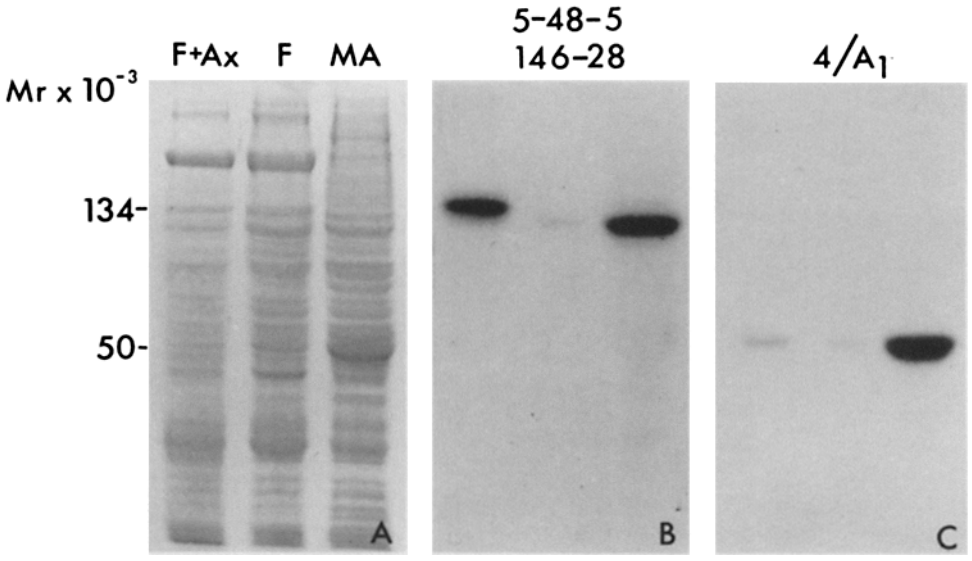

FIGURE 8 Antibody binding to polypeptides of mitotic apparatus preparations. Polyacrylamide gel electrophoretograms of polypeptides and autoradiograms of nitrocellulose replicas. (*A*) Coomassie Blue-stained polyacrylamide gel electrophoretograms of polypeptides of molecular weight above 15,000. From left to right the samples analyzed are 25 μg of fertilized sea urchin egg at the first metaphase mixed with 0.25 μg of axonemes; 25 μg of fertilized sea urchin egg at the first metaphase; and 25 μg of a cellular fraction containing mitotic apparati. Nitrocellulose replicas were prepared from gels similar to that shown but containing 100-μg aliquots of the samples. (*B*) Replica incubated with mixed 5-48-5 and 146-28 antibodies. (*C*) Replica incubated with anti-α tubulin antibody $4/A_1$.

apparatus and is assembled in a structure that is less abundant than the dynein arms in the axoneme.

In summary, five monoclonal antibodies raised to two subunits of a molecule, which is extracted from the axoneme, recognize five antigenic determinants in two cytoplasmic polypeptides of molecular weight similar to those of the immunogens. Both cytoplasmic polypeptides are enriched in the same fraction containing mitotic apparati. On the basis of these observations, it appears likely that the cytoplasmic antigens form a molecule similar to that of the 9-10 S axonemal complex.

DISCUSSION

A variety of experiments designed to test for the presence or the function of dynein-like activities in the cytoplasm have been reported earlier. McIntosh et al. (14), studying a cellular model permeabilized to proteins, showed that addition of flagellar dynein restored the movement of chromosomes previously arrested by the addition of salt. Using a polyclonal antibody to proteolytic fragments of a dynein fraction that contained more than one molecular entity, Mohri et al. (15) detected an antigen in the egg cortex and in the mitotic apparatus by immunofluorescence staining. Sakai et al. (23) found that chromosome motion in the isolated mitotic apparati was completely inhibited by the same antiserum. Chromosome movement in lysed Pt K_1 cells was found to be stopped by vanadate (3), an inhibitor of dyneins, as well as other ATPases (25). Experiments on a model system formed by *Tetrahymena* dynein and clam *Spisula* meiotic spindle supported the view that spindle microtubules are competent to bind dynein (27). In vitro reassembled microtubules formed by pure tubulin were observed to be cross-bridged by *Chlamydomonas* dynein (8).

Although several of these studies have considered that dyneins could interact with microtubules by cross-bridges similar to those formed by myosin in muscle systems, it is now evident that a dynein molecule is not as simple as myosin. Dyneins are associated with large axonemal structures, outer and inner arms, with molecular weights above one million. The degree of complexity of the molecules that are solubilized from the arms depends on the procedure used to extract them. This has made it difficult to distinguish between structural polypeptides forming the arms and the constituents that are essential for the hydrolytic activity of the dyneins. In addition, outer arms each contain two dyneins formed by different subunits (10, 28) and inner arms were found to be composed of two dyneins that were isolated in the form of complexes containing an actin-like polypeptide (19). The function of this actin-dynein association is not understood. Because of this complexity, it is evident that the search for a dynein in microtubular systems other than axonemes must rely on the use of specific probes.

Advances in immunology and in the molecular characterization of the dyneins enable us to adopt a new approach that tests for the presence of a dynein in egg homogenates and in cellular fractions. Monoclonal antibodies raised against axonemal polypeptides were used to recognize similarities between flagellar and cytoplasmic systems of microtubules at the level of polypeptide domains in a specific protein. The target chosen for the initial analysis was the simplest form of a dynein that was known to interact with flagellar microtubules in an ATP-dependent mode.

The choice of a 9-10 S complex was based on the results of binding experiments performed with the urchin *T. gratilla*. Outer arms of *T. gratilla* are dissociated from axonemes in the form of a 21 S particle composed of two dyneins, one sedimenting at 9-10 S and one in the range of 12-30 S under conditions of low ionic strength. The whole arm rebound to extracted flagella of the urchin *Colobocentrotus atratus* and restored the beat frequency of the structure (5). When the 9-10 S dynein was added to extracted axonemes, it appeared to block the restoration of beat frequency of subsequently added 21 S particle and this inhibition was reversed in the presence of ATP (28).

The 9-10 S complex isolated in this study differs from the 9-10 S form characterized in *T. gratilla* in molecular weight and in the antigenic properties of intermediate weight chains. However, the two complexes can be identified because both contain one heavy and one intermediate molecular weight chain that have similar electrophoretic properties. In addition, they both have similar sedimentation behaviors in high ionic strength (28) and in low ionic strength solution. This last condition causes the aggregation of the other constituents of the outer arms. Whether it will be possible to perform the same functional tests with *S. purpuratus* 9-10 S complex as were developed with *T. gratilla* and *C. atratus* flagella is not known. It appears that a further linkage between *T. gratilla* and *S. purpuratus* may be provided by antibodies like C-241-2, 2-79-2, 6-64-14, 6-31-24, and C-26-2 that bind to polypeptides from both urchins.

The experimental evidence presented in this study shows that at least two cytoplasmic antigens are similar to two subunits of 9-10 S axonemal complex. Both cellular polypeptides are enriched in a fraction composed of mitotic apparati that is free of major contaminants and contains tubulin as a principal constituent. These results suggest that a dynein-like molecule is associated with the mitotic apparatus. Further evidence supporting this model could derive from studies of localization of the antigens at the structural level and from further cellular fractionation followed by quantitative analysis of the cytoplasmic antigens. Future experiments of microscopy of the cytoplasmic antigens should be designed taking into account the fact that the antibodies, used as tracers, may generate a low signal over background noise. To obtain autoradiograms of comparable intensity from antigen-bound antibodies, mitotic apparatus and axoneme samples were analyzed by electrophoresis at a ratio 100:1 of protein mass. Assuming an equal affinity for antibody of the two antigens it would appear that the content of antigens in mitotic apparatus was one hundred times lower than in axonemes.

The apparent molecular weight of the larger cytoplasmic antigen is very close to that of the dynein that was isolated from unfertilized sea urchin eggs by direct interaction with Ca^{++}-calmodulin (9). If the two proteins are both recognized by the monoclonal antibodies described in this study, further studies on the precise location and function of this cytoplasmic dynein could be developed by combined use of antibodies, calmodulin, and drugs binding to calmodulin (12). The apparent molecular weight of the smaller cytoplasmic antigen is lower than that of the axonemal counterpart by about 10,000 daltons. Since precautions against proteolysis were taken during the preparations of egg homogenates and axonemes and because the same differences in electrophoretic mobilities were observed independently with samples of egg homogenates, mitotic apparati, and axonemes that were analyzed in pure forms or after mixing, it is evident that the

cellular polypeptide is not identical to the axonemal product. The difference in the polypeptide length could be generated by a number of different mechanisms. The cellular protein may be coded by its own gene or it may be processed from the transcription or translation products of the gene coding for the subunit of the 9-10 S complex.

It was mentioned in a previous section that precursors of dynein subunits that assemble in cilia at a later stage of embryogenesis may exist in fertilized eggs at first metaphase (26). These precursors may be detected in egg homogenates by the antibodies characterized in this study. To evaluate this possibility it was tested whether the antibodies bind to components of isolated embryonic cilia. It was found that each antibody recognizing a cytoplasmic antigen also bind to ciliary constituents (G. Piperno, unpublished data). Therefore, assuming that the ciliary precursors are present in sufficient amounts in the egg at first metaphase it is likely that they would be detected by the antibodies. The existence of precursor-product relationships between cytoplasmic antigens and ciliary constituents and the significance of the selective association of cytoplasmic antigens with fractions containing mitotic apparati are presently being investigated.

Other polypeptides or different complexes similar to axonemal constituents may be associated with cytoplasmic microtubules. The analysis developed in this study has been limited to two of the three subunits of the axonemal 9-10 S complex. However, if the antigens form a cellular molecule similar to the 9-10 S complex, a cytoplasmic counterpart of the third subunit may exist. The association of other cellular complexes with the 330,000 and 124,000 cellular polypeptides may be essential to form a structure that resembles the outer arms of flagellar axonemes and functions in a cytoplasmic system of microtubules.

I thank Dr. I. R. Gibbons (Pacific Biomedical Research Center, University of Hawaii, Honolulu) for the gift of a NaCl extract from axonemes of *Tripneustes gratilla*, Dr. Minx Fuller (Indiana University, Bloomington) for the gift of the monoclonal antibody $4/A_1$ to α-tubulin subunit, Anne Gibbons for technical assistance in the preparation and selection of hybridomas, and Sarah Daman for the preparation and analysis of sea urchin axonemes and mitotic apparati.

This work was supported by the grant GM28702 from the National Institutes of Health and in part by Biomedical Research Support Grant S07 RR0765.

Received for publication 29 September 1984, and in revised form 6 January 1984.

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