

A Microtubule-associated Protein in *Drosophila melanogaster*: Identification, Characterization, and Isolation of Coding Sequences

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Abstract. Microtubules and microtubule-associated proteins (MAPs) have been isolated from cultured cells of *Drosophila melanogaster* by a taxol-dependent polymerization procedure. The principal MAPs are a group of four polypeptides with similar electrophoretic mobilities corresponding to $\sim M_r$ 205,000 (the 205K MAP). These proteins are resistant to precipitation by boiling. One mouse monoclonal antibody and one polyclonal rabbit antiserum specific for the M_r 205,000 MAP were produced and characterized by immunoblotting and indirect immunofluorescence. Both antibody preparations stain the M_r 205,000 molecules and an M_r 255,000 molecule in immunoblots of *Drosophila* cell homogenates; the rabbit antiserum also stains an M_r 150,000 triplet. Both preparations stain the microtubules of the mitotic spindle, and the

rabbit antiserum stains the cytoplasmic microtubules as well. Experiments using affinity-purified rabbit antiserum demonstrate that it is the M_r 205,000 species that is located in the mitotic apparatus and on cytoplasmic microtubules. A random shear genomic library was produced in the expressing vector lambda gt11 and screened with the rabbit antiserum to isolate the DNA sequences encoding these polypeptides. Several cross-hybridizing clones were recovered, shown to encode antigenic determinants in the M_r 205,000 MAP, and characterized by hybridization to Northern blots of mRNA and Southern blots of genomic DNA. Analysis by in situ hybridization reveals that the gene encoding the 205K MAP is located in polytene region 100EF.

A VARIETY of cellular motility processes such as ciliary beating and mitosis depend upon the proper organization and function of microtubules. While the predominant constituent of isolated microtubules is tubulin (Weisenberg et al., 1968), microtubules from some cell types contain additional polypeptides known as microtubule-associated proteins (MAPs)¹ (for review of early work see Sloboda et al., 1976; Vallee et al., 1984). MAPs differ widely in size and properties and the particular molecules found in a given preparation depend both upon the cell type from which they are isolated and the method used. For example, microtubule proteins isolated from mammalian neural tissue by cycles of assembly and disassembly contain two principal size classes of MAPs: the high molecular weight class and the tau class. The high molecular weight class contains at least two, and perhaps as many as six major polypeptides with apparent molecular weights of $\sim 300,000$ (Bloom et al., 1984a). The tau class probably contains at least six different molecules in the range M_r 47,000–80,000 (Drubin et al., 1984). The best studied MAPs from cultured cells of non-neural origin are the M_r 210,000 MAP from HeLa cells (Bulinski and Borisy, 1979, 1980a; Weatherbee et al., 1980, 1982), MAP 4 from

mouse (Parysek et al., 1984a, b), and the tau-like molecules found in cultured mammalian somatic cells (Duerr et al., 1981; Pallas and Solomon, 1982). While the neural and cultured somatic cell MAPs represent a diverse collection in terms of their sizes, they all share the ability to stimulate the assembly of dimeric tubulin into microtubules in vitro. Additional surveys of other cultured cell lines and organisms such as sea urchins suggest that an M_r 200,000 MAP may be common to most cell types and organisms, although there is considerable diversity in the MAPs isolated from these various sources (Vallee and Bloom, 1983; Duerr et al., 1981; Scholey et al., 1984; Parysek et al., 1984a, b).

While the in vitro behavior of MAPs has been intensively studied, their functions in vivo are unknown. Several hypotheses for the function of MAPs in vivo have been proposed based on their in vitro behavior and on immunocytochemical investigations. These hypotheses fall into two classes: (a) MAPs regulate the assembly state of tubulin (Murphy and Borisy, 1975; Weingarten et al., 1975), or (b) MAPs form bridges or connections either among microtubules or between microtubules and other structures or filaments (reviewed in Bloom and Vallee, 1983; Vallee et al., 1984). As yet, few experiments distinguishing these hypotheses have been reported.

1. Abbreviations used in this paper: MAP, microtubule-associated protein; TBS, Tris-buffered saline.

Antibodies directed against MAPs have been useful probes for studying MAP function. Immunofluorescence localization studies using antibodies that bind the tau or high molecular weight molecules indicate that these polypeptides are associated with cytoplasmic microtubules in neural tissue and with mitotic spindle and cytoplasmic microtubules in non-neural tissue (Connolly et al., 1977, 1978; Bloom and Vallee, 1983; Bloom et al., 1984a, 1984b). Studies using antibodies that recognize the HeLa *M*₁ 210,000 and 125,000 MAPs suggest that these molecules are associated with microtubules of the mitotic spindle and cytoplasmic microtubule system (Bulinski and Borisy, 1980b; Izant et al., 1982, 1983). In addition, injection of monoclonal antibodies that recognize the HeLa *M*₁ 210,000 MAP into dividing cells results in mitotic arrest (Izant, 1981; Izant et al., 1983). Finally, antibodies recognizing MAP 4 have been used to demonstrate its presence in cytoplasmic microtubules in numerous cell types and tissues in mouse (Olmsted et al., 1984; Parysek et al., 1984a, b). Taken together, these studies strongly suggest that several different MAPs are present on cytoplasmic and mitotic spindle microtubules and that at least one MAP has an essential function during mitosis.

We have begun an analysis of MAPs in *Drosophila melanogaster* as the first step in our project of bringing the tools of *Drosophila* genetics to bear on the problem of the function of MAPs in the mitotic spindle and cytoskeleton. In this paper, we present a description of microtubule proteins isolated from cultured cells of *Drosophila*, and detailed studies of the properties, distribution, and DNA sequences encoding one particular MAP.

Materials and Methods

Culture of Cells from *Drosophila melanogaster*

The *Drosophila* cells used were Schneider S-2 cells adapted to suspension culture (Lengyel et al., 1975). Cells were cultured in spinner flasks at 25°C under 5–10% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.5% lactalbumin hydrolysate, 0.1 mM non-essential amino acids (Gibco, Grand Island, NY), 100 U/ml penicillin, 100 µg/ml streptomycin. Cell densities were kept between 2×10^6 /ml and $1-2 \times 10^7$ /ml.

Isolation of Microtubules

Microtubules were isolated from cultured cells by a modification of the taxol-dependent polymerization procedure of Vallee (1982). Cells were grown to $\sim 10^7$ /ml, harvested by centrifugation at 4°C in a Sorvall GSA rotor spun at 6,900 *g*_{max}, washed once in phosphate-buffered-saline (PBS) at 4°C, washed again in microtubule assembly buffer (0.1 M Pipes, 1 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM GTP, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 2 µg/ml aprotinin, 2 µg/ml tosyl-arginyl-methyl ester) at 4°C, and then resuspended in 2 vol of 4°C assembly buffer. The cell suspension was sonicated at setting 7 for ~ 2 min on a Branson sonicator (Branson Sonic Power Co., Danbury, CT) fitted with a stepped microtip. To prevent heating, the sonication was done in a cold room and the cell suspension was cooled intermittently in a dry ice-ethanol bath. The cell suspension was monitored by phase contrast microscopy to ensure that >99% of the cells were disrupted. The suspension was homogenized with five passes of a motor driven Potter homogenizer fitted with a Teflon pestle in a cold room and allowed to incubate on ice for 15 min to depolymerize microtubules. Clarification of the cell extracts was accomplished in two steps. First, the extract was spun at 54,000 *g*_{max} in a Beckmann Type 65 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 30 min at 4°C. The resulting supernatant was carefully removed, avoiding material in and floating near the pellet. This supernatant was then spun at 218,000 *g*_{max} in a Type 65 rotor for 90 min at 4°C. Taxol and GTP were added to the clarified supernatant to final concentrations of 20 µM and 2 mM, respectively, and then incubated at room temperature ($\sim 22^\circ\text{C}$) for 30 min to allow microtubules to polymerize. This material was then layered

over a 15% sucrose cushion made up in assembly buffer supplemented with taxol and GTP and centrifuged. Generally 3 ml of microtubule-containing supernatant was layered over a 1-ml sucrose cushion and spun at 54,000 *g*_{max} for 35 min at 20°C in a Beckmann SW60 Ti rotor. The resulting pellet is a standard microtubule pellet; a typical yield is ~ 1 mg of microtubules from 1 liter of cells (~ 5 g of packed cells); $\sim 20\%$ of this is MAPs. This pellet can be washed in assembly buffer supplemented with taxol and GTP or used directly for extraction of MAPs; comparable results have been obtained both ways. Extraction of MAPs was accomplished by resuspending microtubules in assembly buffer supplemented with taxol, GTP, and 0.4 M NaCl, then subjecting the material to centrifugation at 42,000 *g*_{max} at 22°C for 30 min in a type 65 rotor to give a microtubule pellet depleted of the associated proteins, and a supernatant that contained the MAPs and was depleted of tubulin. For preparation of heat stable MAPs, such a supernatant was made up to 0.75 M NaCl, boiled for 5 min, cooled on ice, and subjected to centrifugation at 50,000 *g*_{max} at 4°C in a type 65 rotor. MAPs and microtubules were generally stored in liquid nitrogen.

Electrophoresis and Immunoblotting

The polypeptide compositions of our preparations were analyzed on 7.5% polyacrylamide gels run with a discontinuous Neville buffer system (Neville, 1971), or in later experiments, with a Laemmli buffer system (Laemmli, 1970). For immunoblotting (Towbin et al., 1979; Erickson et al., 1982), 4–11% polyacrylamide gradient gels were run and electrophoretically transferred to nitrocellulose sheets for 12–24 h at 8 V/cm in 25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS pH 8.3 in a Hoefer Transblot apparatus (Hoefer Scientific Instruments, San Francisco, CA) cooled with running tap water. In early experiments gels were stained with Coomassie Blue to ensure that no polypeptides remained after transfer. The nitrocellulose replicas were washed for 1 h to overnight with several changes of PBS/0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) (Batteiger et al., 1982) to remove residual SDS. Primary rabbit antibody was diluted 1:100 in PBS/0.05% Tween 20/10% heat-inactivated fetal bovine serum and incubated with the nitrocellulose replicas for 1 h at 37°C or diluted 1:1,000 in the same solution and incubated overnight at 4°C. Monoclonal antibody was applied to replicas by direct use of hybridoma-conditioned medium to which Tween 20 was added to a final concentration of 0.05%. After incubation in primary antibody, the replicas were washed five times for 5–10 min each in PBS/0.05% Tween 20, then incubated in peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Cappel Laboratories, Cochranville, PA) diluted 1:500 in PBS/0.05% Tween 20/10% fetal bovine serum for 1 h at 37°C. The replicas were again washed five times for 5–10 min each in PBS/0.05% Tween 20, then twice in Tris-buffered saline (TBS: 50 mM Tris-Cl pH 7.5, 150 mM NaCl) before developing in 4-chloro-1-naphthol (3 mg/ml of 4-chloro-1-naphthol in methanol diluted with 5 vol of TBS to which was added 0.01 vol of 30% hydrogen peroxide).

Preparation of cell homogenates for immunoblotting was conducted by harvesting 10 ml of healthy cells by centrifugation, washing the cells three times in 4°C PBS, then resuspending in 50 µl of PBS. This suspension was then sprayed into 250 µl of boiling sample buffer and boiled for 2 min. The DNA was sheared with a 26 gauge needle, and the sample was immediately loaded on a gel or stored at -20°C before gel electrophoresis; similar results have been obtained both ways. Alternatively and with equivalent results a cell suspension was sprayed into 10 vol of -20°C acetone, incubated 10 min at -20°C , washed once in -20°C acetone, dried, then resuspended in sample buffer and boiled.

Elution of Antibodies from Immunoblots

Portions of nitrocellulose replicas of polyacrylamide gels were used to affinity-purify antibodies by a modification of the method of Olmsted (1981). After electrophoretic transfer, nitrocellulose replicas were washed twice in TBS/0.05% Tween 20 for 5 min each, washed twice in TBS for 5 min each, and stained for 10 min in Ponceau S (20 ml of Sigma Ponceau S concentrate diluted to 200 ml with deionized water). After staining, the replicas were destained briefly in distilled water until the protein bands were clearly visible, and then the bands of interest were excised with a new razor blade. These nitrocellulose strips that contained the protein of interest were then washed twice in TBS/0.05% Tween 20, and incubated overnight at 4°C in antibody diluted 1:50 in TBS/0.05% Tween 20/3% bovine serum albumin (BSA)/0.8% Y1089 lysate (see screening below for details of preparation). The strips were then washed five times for 10 min each in TBS/0.05% Tween 20, loaded into a 5-ml syringe, and washed again by forcing two 5-ml aliquots of TBS/0.05% Tween 20 through the filters. 1 ml of 0.2 M glycine (pH 2.8)/0.05% Tween 20 was drawn up into the syringe through the filters, incubated for 3–5 min, expelled through the filters, drawn up again, then expelled into 1 ml of 100 mM Tris base. To this mixture 100 µl of 3 M NaCl, 100 µl of 1% Tween 20, 400 µl of newborn

calf serum, and 1.4 ml of TBS/0.05% Tween 20 were added. This antibody mixture was used directly in immunoblotting by incubating overnight at 4°C with nitrocellulose replicas or for immunofluorescence after diluting 1:1 with newborn calf serum. The strips from which antibody was eluted were saved, and on occasion reused.

Antibodies

A cell line (4D5.11.6) secreting an IgM isotype monoclonal antibody was recovered from a fusion of SP2/O cells with spleen cells from a mouse immunized with whole microtubules. The hybrids were selected in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum, 5% NCTC 109, 0.1 mM hypoxanthine, 0.01 mM aminopterin, and 0.03 mM thymidine, and propagated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. The line was cloned twice in soft agar before use. Large quantities of conditioned growth medium that contained antibody were obtained by growing the line in roller bottles.

Antigen for immunizing a rabbit with the *Drosophila* *M*, 205,000 MAP was obtained by preparing ~125 µg of the heat-stable MAPs, running this on a preparative 7.5% gel, staining with Coomassie Blue, and excising the *M*, 200,000 band. Preimmune serum from the rabbit was collected, saved, and checked to ensure that it gave a minimal background as assayed by both blots and immunofluorescence. For primary immunization the excised band was rinsed in distilled water and homogenized with an equal volume of 0.1% SDS, 0.05% 2-mercaptoethanol by passing through successively smaller gauge syringe needles. One-half of this material was emulsified with an equal volume of Freund's complete adjuvant by successive passes through a small gauge needle. The emulsion (4 ml) was then injected at eight sites subcutaneously on the back of a New Zealand white rabbit. 16 d later the rabbit was boosted with the remainder of the antigen in Freund's incomplete adjuvant by subcutaneous injection on the back. Serum was collected at weekly intervals for 2 mo without any noticeable drop in titer, at which time the rabbit was killed by cardiac puncture, and a large volume of serum collected.

Immunofluorescence

Schneider cells were seeded on sterile polylysine-coated coverslips at a density of 5×10^3 /ml in 30-mm petri dishes the day before fixation and staining. Coverslips were rinsed twice in room temperature PBS, rinsed once in anhydrous -20°C methanol, and fixed in anhydrous -20°C methanol for 4 min. The coverslips were then transferred to acetone at -20°C for 1 min, followed by two quick rinses in PBS and two 10-min washes in PBS. The coverslips were treated for 30 min at 37°C with 50% normal goat serum diluted in PBS, then washed twice for 5 min each in PBS. Incubation with primary antibody was for 1 h at 37°C followed by two quick PBS rinses and two 10-min washes in PBS. Secondary antibody incubation was for 1 h at 37°C followed by two PBS rinses, two 10-min PBS washes, and a distilled water rinse before mounting with Elvanol.

Bacterial Strains, Vectors, and Enzymes

Bacterial strains Y1088, Y1089, Y1090, and phage lambda gt11 are described in Young and Davis (1984). CSH 18 is described in Maniatis et al. (1982). JM103 and pUC 13 are described in Viera and Messing (1982). EcoRI and EcoRI methylase used during bank production were a gift of R. Young. Restriction enzymes for mapping and subcloning and DNA polymerase I were from New England Biolabs (Beverly, MA). EcoRI linkers and polynucleotide kinase were from Collaborative Research, Inc. (Waltham, MA). T4 DNA ligase was a gift of B. J. Meyer or from Collaborative Research, Inc.

Production of a Random Shear Genomic Library in Lambda gt11

A genomic library consisting of randomly sheared *Drosophila melanogaster* genomic DNA inserted into the unique EcoRI site of lambda gt11 was produced as follows. DNA to be inserted into the vector was prepared by sonicating genomic DNA from *Drosophila* embryos of strain Ore-R P2 in 2.5 M NaCl, 10 mM Tris-Cl pH 7.6, 1 mM EDTA (Maniatis et al., 1978) to a mean of 1 kb as estimated by fluorescence intensity on 1% agarose-ethidium bromide gels. The ends of the molecules were prepared for ligation by repairing the ends with S1 nuclease (10,000 U for ~100 µg of DNA in 30 mM sodium acetate pH 4.4, 250 mM NaCl, 1 mM zinc acetate at 10°C for 30 min). DNA in the size range of 0.5–10 kb was selected on 1% agarose gels by cutting a slit at 0.5 kb and inserting NA45 paper (Schleicher & Schuell, Inc., Keene, NH). The DNA was subjected to further electrophoresis at 150 V to run it onto the paper; the paper was then rinsed in 20 mM Tris-Cl pH 8.0, 1 mM EDTA, 100 mM NaCl, and then the DNA was eluted by the method of Burtis (unpublished work com-

municated by R. Young) in 1 M NaCl, 50 mM arginine (free base) for 1–2 h at 65°C. The eluted DNA was phenol extracted twice, ether extracted four times, and ethanol precipitated twice. The DNA was methylated with EcoRI methylase in 50 mM Tris-Cl pH 7.6, 5 mM dithiothreitol, 100 µM S-adenosyl methionine for 20–40 min, following which the methylase was heat-inactivated at 65°C for 10 min. EcoRI linkers were 5' phosphorylated with T4 polynucleotide kinase, after which 10 µg was added to ~50–100 µg of genomic DNA prepared as above and incubated with T4 DNA ligase in 1 mM ATP, 10 mM MgCl₂, 5 mM dithiothreitol for 18 h at 10°C. The resulting DNA was cut with EcoRI and size selected on a 1% agarose gel, using NA45 paper, to remove linker pieces. The *Drosophila* DNA was then inserted into the unique EcoRI site of lambda gt11, which had been cut with EcoRI and treated with calf-intestinal phosphatase to reduce self ligation of the arms, packaged in vitro (Promega Biotec, Madison, WI), and titered on Y1088. A total of 1.6×10^7 independent recombinant phage in three separate pools were produced and amplified once on Y1088 in plate lysates prepared at 42°C on LB/amp plates. Pool 1 was amplified by plating $\sim 4 \times 10^5$ phage per 150-mm plate with 1 ml of mid-log Y1088. Pools 2 and 3 were amplified by plating 2 and 2.5×10^6 phage per 150-mm plate, respectively.

In an experiment to assess the proportion of hypothetical recombinant phage (colorless plaques on X-gal) that actually contained DNA inserts, 32 colorless plaques were picked (20 from pool 1, 6 from pool 2, and 6 from pool 3), grown up in liquid culture, phage and phage DNA isolated, cut with EcoRI, and run on a 2% agarose gel. Only 27 of the original 32 gave enough phage for DNA isolation and of these, 24 were judged to yield enough DNA for accurate assessment of insert presence and size. 22 of these 24 had identifiable inserts in the size range of 0.5–2 kb leading to an estimate of 90% for the proportion of phage giving colorless plaques that were truly recombinant. The remaining 10% could be linker contamination though explanations such as instability of satellite DNA during phage growth, internal EcoRI sites in small inserts, or simply inserts too small to see above the background of degraded DNA and RNA running at the dye front of the gels seem more likely. Two of the phage examined contained two fragments; either two independent inserts or an internal EcoRI site in a single insert.

Screening of Plaques

Approximately 100,000 recombinant phage per 150-mm plate were plated on mid-log Y1090 (Young and Davis, 1984) in soft agar on LB/amp plates (pH 7.5) at 43°C for 3 h (until tiny plaques were just visible). The plates were immediately removed at 37°C and an isopropyl β-D-thiogalactopyranoside (IPTG) soaked and dried nitrocellulose filter was placed on the plates (filters were prepared by soaking in 10 mM IPTG for 1 h, blotting dry, and allowing to dry for 30–60 min). The plates were incubated for 2–3 h, the filters removed carefully, and processed.

Filters were processed by rinsing a few times in Tris-buffered saline (50 mM Tris-Cl, pH 7.5, 150 mM NaCl)/0.05% Tween 20 to remove bits of agar and cell debris, then incubated in primary antibody for the appropriate amount of time and temperature. Primary antibody was diluted in Tris-buffered saline/0.05% Tween 20/3% BSA. For monoclonal antibody incubations, undiluted cell culture supernatants made up to 0.05% in Tween 20 were used. Incubation conditions were overnight at room temperature, overnight at 4°C, 1 h at room temperature, or 1 h at 37°C. Incubations were in plastic petri dishes with rotary agitation (8 ml of antibody solution in a 100-mm petri dish with one filter plaque side up, 9 ml with two filters, one facing up, the other facing down). Antibodies that recognized *E. coli* antigens were removed from the rabbit antiserum by the boiled lysate method of Helfman et al. (1983) using an induced Y1089 (lambda gt11) lysogen. After incubation in primary antibody, filters were processed by the same methods as were used for immunoblots except that secondary antibodies were diluted 1:50 or 1:250 (depending on the particular secondary used). Filters were developed until signals were visible or background began to come up. Development was stopped by rinsing twice in distilled water. Filters were stored in the dark in acetate sheet protectors to prevent fading. Phage giving positive signals were picked and successively replated at lower densities to purify recombinants.

Production and Induction of Lysogens for Fusion Protein Analysis

Lysogens of lambda gt11 recombinants were made in the host Y1089 by infecting at a multiplicity-of-infection of 2–3 and plating at 30°C. Single colonies were picked and tested for growth at 30°C and 43°C to identify lysogens. To prepare gel samples of proteins produced in induced lysogens, a fresh overnight culture of a lysogen grown at 30°C was diluted 1:100 in 5 ml LB/ampicillin and grown to mid-log at 30°C. IPTG was added to a final concentration of 10 mM, the cells were induced at 43°C for 15 min, and then incubated

at 37°C for 2 h. 1.5 ml of cells were pelleted for 15 s in a microfuge, resuspended in 125 μ l of sample buffer, vortexed thoroughly, and heated to 100°C for 5 min. The suspension was thoroughly vortexed again, spun for 4 min in a microfuge, the supernatant recovered, and either run immediately on a gel (15–20 μ l/lane) or stored at –20°C (Carroll, S., personal communication).

Hybridization Conditions

For genomic DNA hybridizations, DNA was from Canton-S embryos or adults. Restriction enzyme-digested DNA was run on 0.6% agarose gels and transferred to Gene Screen Plus. Prehybridization was in 50% deionized formamide, 5 \times SSC (1 \times SSC is 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0), 50 mM sodium phosphate pH 6.5, 250 μ g/ml denatured salmon sperm DNA, 1 \times Denhardt's solution, 0.5% SDS for 2 h at 43°C. Hybridization was in the same plus 6.25% dextran sulfate for 18–24 h at 43°C. Washing of the filters was in 2 \times SSC for 30 min at 37°C, then twice in 1 \times SSC, 1% SDS at 65°C for 30–60 min each, followed by a final wash in 0.1 \times SSC at 37°C.

For hybridization to Northern blots of RNA, the source of the RNA was S2 cells grown in suspension culture in M3 medium. Cells were lysed in 2% SDS, 0.2 M NaCl, 10 mM Tris-Cl (pH 7.5), 10 mM EDTA, 250 μ g/ml proteinase K at 50°C for 30 min. The lysate was extracted twice with phenol/chloroform/isoamyl alcohol (24:24:1) then once in chloroform/isoamyl alcohol (24:1) followed by an ethanol precipitation (Kwan et al., 1977). DNA was removed by one lithium chloride precipitation (Cathala et al., 1983) followed by another ethanol precipitation. Poly A⁺ RNA was selected by oligo-dT cellulose chromatography (Maniatis et al., 1982). Gel electrophoresis of RNA was on 0.7% agarose gels run in 2.2 M formaldehyde, 20 mM 2-(*N*-morpholino)propane sulfonic acid, 5 mM sodium acetate, 0.5 mM EDTA followed by transfer to nitrocellulose. Denatured HindIII cut lambda DNA was used as molecular weight standard. Hybridization to RNA was as for DNA but washing was four times in 2 \times SSC/0.5% SDS at 37°C for 15 min each, followed by two washes in 0.1 \times SSC/0.5% SDS at 50°C for 30 min each.

Hybridization to polytene chromosomes was by the procedure of Bonner and Pardue (1976) using ³H-labeled plasmid subclones as probe. Hybridization was in 0.3 M NaCl, 20 mM Tris-Cl pH 7.5 at 65°C for 15 h followed by washing in 2 \times SSC at 60°C.

Analysis of Lambda gt11 Clones

During the analysis of subclones of the lambda gt11 clones containing the 205K MAP coding sequences, it was found that two clones (lambda gt11.205.16 and lambda gt11.205.23) gave two or three bands, respectively, in hybridization to Southern blots of genomic DNA, while one clone (lambda gt11.205.19) gave only one band. The latter result suggested that the gene was actually single copy. The multiple bands seen with lambda gt11.205.16 and lambda gt11.205.23 were traced to these phage containing DNA not related to the 205K MAP coding sequences in addition to their containing 205K MAP coding DNA. This conclusion was arrived at because (a) the 0.8-kb EcoRI/XbaI fragment, the 1.4-kb HindIII/XbaI fragment, and the 1.0-kb EcoRI/HindIII fragment isolated from pUC13.205.16 (see Fig. 10) each hybridizes to a different, single band in hybridizations to Southern blots of genomic DNA while the entire EcoRI subclone hybridizes to all three bands and (b) fragments from a genomic walk in the region of the 205K MAP coding sequences are all single copy and do not hybridize to DNA from the other regions found in lambda gt11.205.16.² It can be concluded that the area around the EcoRI site in lambda gt11.205.16 is the location of the 205K MAP coding sequence since (a) all of the cross-hybridization among the clones is localized in this region, and (b) this is the region containing protein coding sequence as indicated by a comparison of the sizes of the fusion protein and the DNA in lambda gt11.205.16. Preliminary data on cDNA clones also support this conclusion.²

Results

Isolation of Microtubules and MAPs from Cultured *Drosophila* Cells

Microtubules and associated proteins (MAPs) were isolated from a cultured *Drosophila* cell line (Schneider S2) as described in Materials and Methods. An electron micrograph of the microtubules from such a preparation is shown in Fig. 1; the numerous microtubules and the small amounts of other filamentous material are readily apparent in this negatively

2. Goldstein, L. S. B., and R. A. Laymon, unpublished observations.

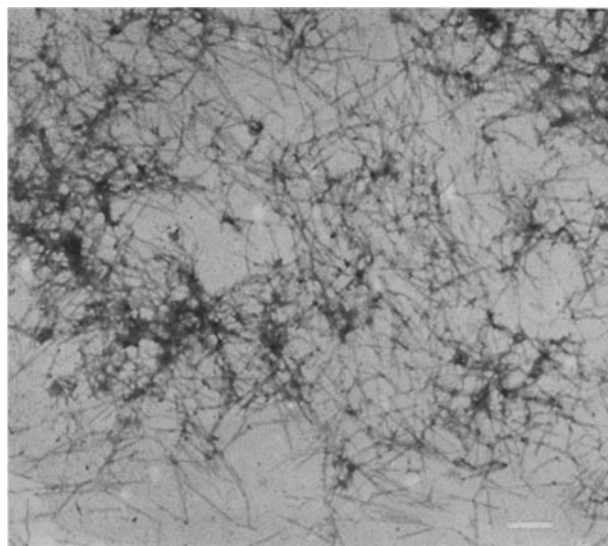


Figure 1. Electron micrograph of isolated microtubules negatively stained with uranyl acetate. Material shown is a first, unwashed, microtubule pellet. Bar, 150 nm.

stained preparation. The polypeptide compositions of the stages in a typical preparation of MAPs is shown in Fig. 2. The majority of the protein present in the isolated microtubules (Fig. 2, lane *MTP*) is tubulin, but, there are several associated species including a polypeptide with an electrophoretic mobility similar to actin and a few others which are highly enriched relative to the starting cell extracts, thus signifying their co-enrichment with tubulin. One highly enriched species has a mobility corresponding to M_r 205,000 (205K). Other species which may be enriched have mobilities corresponding to M_r 150,000 (150K), M_r 80,000–100,000, and M_r 60,000–70,000. In some, but not all preparations, we see enrichment of polypeptides with M_r >205,000.

Treatment of the *Drosophila* microtubules with 0.4 M NaCl results in the almost complete release of the associated proteins (Fig. 2, lanes *MTP*, *T*, and *MAP*). After centrifugation of such salt-treated material, the pellet (Fig. 2, lane *T*) is primarily composed of tubulin, a small amount of an M_r 43,000 polypeptide which may be actin, and variable amounts of other species. The supernatant (Fig. 2, lane *MAP*) contains the remaining associated proteins including the M_r 205,000 polypeptide. If *Drosophila* MAPs are heated to 100°C in 0.75 M NaCl for 5 min, most of the polypeptides precipitate and can be removed by centrifugation. The 205K and 150K polypeptides, however, remain soluble at 100°C and can be recovered in the supernatant (Fig. 2, lane *B*).

During the course of these studies it was noted that the *Drosophila* 205K polypeptide may be sensitive to degradation, as indicated by the apparent multiplicity of bands in the region of M_r 205,000 and by faint ladders of lower molecular weight bands often seen in immunoblots (see below). Examination of the gel in Fig. 2 reveals two or three subsidiary bands within the wide, heavy band at M_r 205,000 (also see immunoblots below).

Several experiments were done to investigate qualitatively the specificity with which these polypeptides co-sediment with tubulin. In the first experiment, a microtubule-containing pellet was resuspended and washed in assembly buffer con-

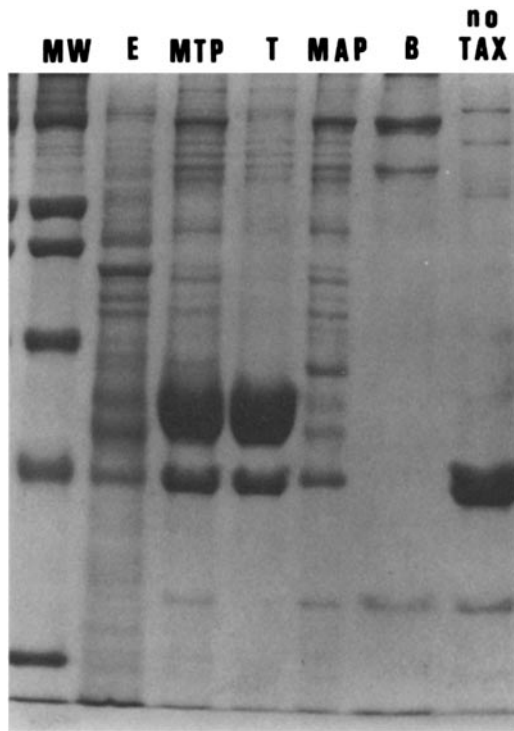


Figure 2. Polypeptide composition of various steps during MAP preparation. Various polypeptide preparations were run on 7.5% polyacrylamide gels and stained with Coomassie Blue. *MW*, molecular weight markers: carbonic anhydrase (29,000), ovalbumin (45,000), bovine plasma albumin (66,000), phosphorylase b (97,400), beta-galactosidase (116,000), myosin (205,000). *E*, 10 μ g of cell extract after sonication and clarification. *MTP*, 10 μ g of isolated microtubules. *T*, 10 μ g of salt-extracted microtubule pellet. *MAP*, 2.5 μ g of supernatant from salt extraction of microtubule pellet. *B*, 2 μ g of supernatant from boiling and centrifuging a MAP preparation. *no TAX*, 4 μ g of pellet from no taxol, no GTP control centrifugation.

taining taxol and GTP, then recentrifuged. The resulting pellet contained essentially the same proteins, although examination of the supernatant revealed that some components, but not the M_r 205,000 polypeptide, failed to resediment completely. None of the components, however, was removed completely by the wash. This suggests that some of the polypeptides cosediment with microtubules nonspecifically, bind to microtubules with low affinity, or lose activity during the wash. In another control experiment, microtubules were assembled in 0.4 M NaCl. The resulting pellet contained microtubules composed almost entirely of tubulin; few associated proteins were observed. In a third control experiment, cell extracts were incubated without taxol, then subjected to centrifugation. These conditions result in no pelleting of microtubules and little pelleting of other polypeptides. However, a considerable quantity of the M_r 43,000 species does pellet under these conditions, as do a few other polypeptides (see Fig. 2, lane *no TAX*). Taken together, these observations suggest that most of the associated proteins we observe do not pellet fortuitously, but, instead are dependent upon intact microtubules for sedimentation. Thus, these polypeptides are probably binding to the microtubules themselves and are strong candidates to be MAPs.

Production of Antibodies Recognizing an M_r 205,000 MAP

To study the intracellular distribution of *Drosophila* MAPs and to obtain tools for the cloning and genetic analysis of DNA sequences encoding MAPs, we have begun to produce monoclonal and polyclonal antibodies that recognize the polypeptides found in our preparations. In this paper, we report on both a polyclonal antiserum and a monoclonal antibody that recognize the 205K MAP.

The monoclonal antibody 4D5.11.6 is an IgM isotype antibody derived from fusion of SP2/0 myeloma cells with spleen cells from a BALB/c mouse immunized with whole microtubules. In immunoblots of purified microtubules or boiled MAPs, this antibody binds to four polypeptides in the M_r 205,000 region (see Figs. 3*a* and 4*h*; four bands are visible only when the gels are underloaded, usually only a doublet is

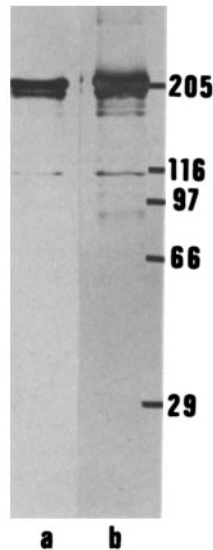


Figure 3. Immunoblot of boiled MAP preparation using monoclonal and polyclonal anti-205K MAP. Lane *a*, monoclonal anti-205K MAP; *b*, polyclonal anti-205K MAP.

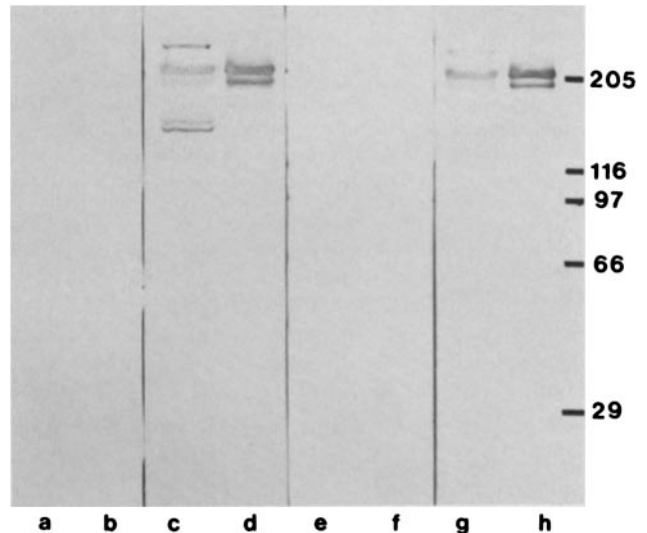


Figure 4. Immunoblots of cell homogenates and isolated microtubules using monoclonal and polyclonal anti-205K MAP. Lanes *a*, *c*, *e*, and *g*, cell homogenates; *b*, *d*, *f*, and *h*, isolated microtubules. Lanes *a* and *b*, preimmune serum (1:100); *c* and *d*, polyclonal anti-205K MAP (1:100); *e* and *f*, nonspecific monoclonal; *g* and *h*, monoclonal anti-205K MAP.

observed). Depending on the microtubule preparation, it sometimes stains a faint "ladder" of bands ranging from M_r 205,000 to $\sim M_r$ 130,000 (not visible in figures). In blots against Schneider S2 cell homogenates (Fig. 4g), this antibody recognizes two closely spaced doublets of M_r 205,000 and 200,000, respectively, and also an M_r 255,000 species. Because these cell homogenates were prepared so as to minimize proteolysis (see Materials and Methods), it is possible that the bands seen in immunoblots of whole cell homogenates are a better reflection of the molecular weights of the antigens than are those obtained with isolated microtubules. No cross-reactivity with HeLa MAPs has been observed in immunoblot experiments.

It was possible to take advantage of the heat stability and electrophoretic mobility of the 205K MAP to purify the polypeptide and use it to raise a polyclonal antiserum. This antiserum recognizes a polypeptide or polypeptides with distributions and characteristics similar to those recognized by the monoclonal antibody (Figs. 3b, 4c, and 4d; compare to 3a, 4g, and 4h). In immunoblots of microtubules or boiled MAPs, it recognizes four bands of M_r 205,000, and sometimes binds to a faint ladder of bands running from M_r 205,000 to M_r 80,000 (only a doublet is visible in Fig. 3b, see above for explanation). In immunoblots against whole cell homogenates, it recognizes a band of M_r 255,000, four bands at M_r 205,000, and a triplet at M_r 150,000 (Fig. 4c). In experiments to assess cross-reactivity of this antiserum, no cross-reactivity was seen in immunoblots of HeLa or bovine brain MAPs. Because of the complexity of the immunoblot pattern observed with the polyclonal antiserum, antibodies specific for the 205K polypeptide were affinity-purified using filter-bound 205K MAP as antigen (Olmsted, 1981; see Materials and Methods for details). The resulting antibodies recognize only the 205K MAP in immunoblots of cell homogenates or isolated microtubules (data not shown).

The cellular distribution of the 205K MAP recognized by the antibodies was assessed by indirect immunofluorescence staining of fixed Schneider cells. Both monoclonal and polyclonal antibodies stain microtubules in methanol-fixed but not aldehyde-fixed cells. The monoclonal antibody stains prophase asters and spindle microtubules (data not shown), and it may also stain cytoplasmic microtubules faintly. The anti-205K MAP antiserum gives intense staining of cytoplasmic microtubules, spindle microtubules, prophase asters, midbodies, and cell centers (see Fig. 5). Indeed, it gives a pattern that is indistinguishable in double-label experiments from that seen with a monoclonal antibody recognizing tubulin (Fig. 6). This same pattern is seen with the affinity-purified antiserum. Since both monoclonal and polyclonal antibodies that recognize the 205K MAP stain microtubules in fixed cells, and since the 205K polypeptide binds to microtubules *in vitro*, it can be concluded that this polypeptide is a MAP.

Isolation of DNA Sequences Coding for the 205K MAP

The strategy for isolating the DNA encoding the 205K MAP was as follows. A library was constructed of randomly sheared genomic DNA from *Drosophila melanogaster* inserted at the EcoR1 site in the bacteriophage vector lambda gt11 (Young and Davis, 1983). Because the unique EcoR1 site in this

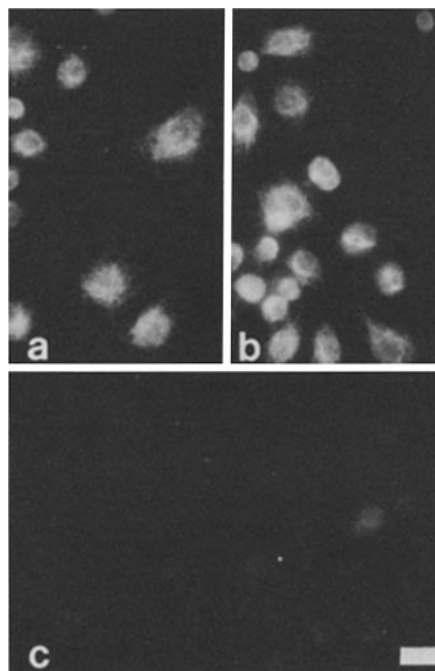


Figure 5. Immunofluorescence of Schneider cells with preimmune and immune sera. (a and b) Polyclonal anti-205K MAP (1:100); (c) preimmune serum (1:100). Bar, 10 μ m.

bacteriophage is located in the carboxyl-terminus of the beta-galactosidase gene, inserted DNA (in the appropriate frame and orientation) is expressed as protein fused with beta-galactosidase under the control of the lactose operon repressor. Thus, this library of recombinant phages could be screened with antibodies that recognize the 205K MAP in order to identify phage that carry DNA segments encoding the antigenic determinants of this protein.

Approximately 2×10^6 recombinant phage were screened using total rabbit 205K antiserum. In this primary screen, seven strong and 40–50 weaker signals were detected. Of the seven phage giving strong signals, four were plaque purified by repeated rescreenings at successively lower densities; three of these were analyzed in detail. All three phages, lambda gt11.205.16, lambda gt11.205.19, and lambda gt11.205.23, produced IPTG-inducible hybrid protein-containing 205K MAP antigenic determinants as judged by plaque assays. As described below, the two criteria that establish that these phage contain DNA sequences encoding portions of the 205K MAP are: (a) the fusion proteins encoded by the phage are recognized by antibodies specific for the 205K MAP and not by other possible contaminating antibodies present in the 205K antiserum; (b) the putative coding sequences for the 205K MAP encode more than a single antigenic determinant, eliminating the possibility that fortuitous sharing of a single determinant with an unrelated polypeptide is responsible for the immunological recognition (see for example Nigg et al., 1982).

To examine the fusion proteins produced by the recombinant phage, lysogens of the phage in the host Y1089 were produced. Upon induction of the lysogens and gel electrophoresis of the resulting proteins, each lysogen was observed to produce a new protein, not found in the other lysogens, visible by Ponceau S staining of nitrocellulose replicas. Each of these proteins had a molecular weight greater than that of intact

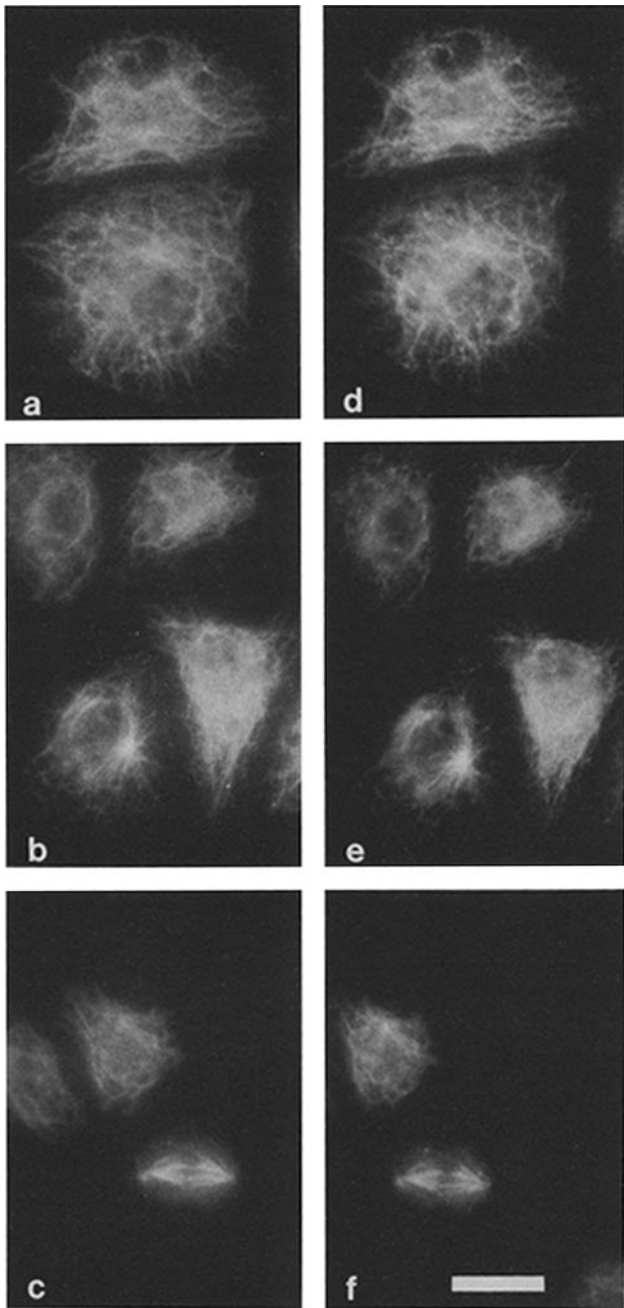


Figure 6. Double-label immunofluorescence experiment with monoclonal anti- α -tubulin and polyclonal anti-205K MAP. (a-c) Monoclonal anti- α -tubulin indirectly labeled with fluorescein-conjugated goat anti-mouse; (d-f) polyclonal anti-205K MAP indirectly labeled with rhodamine-conjugated goat anti-rabbit. Bar, 10 μ m.

beta-galactosidase and each was stained by anti-beta-galactosidase antiserum in immunoblots (data not shown), thus confirming the conclusion based upon IPTG-inducibility, i.e., they are fusions of eukaryotic protein with beta-galactosidase. The estimated molecular weight of each of the proteins, based on electrophoretic mobility, is 180,000 for lambda gt11.205.16, 150,000 for lambda gt11.205.19, and 140,000 for lambda gt11.205.23 (beta-galactosidase alone is 116,000).

Immunoblotting of lysates of each of the lysogens with

either polyclonal or monoclonal antibody to the *Drosophila* 205K MAP was then conducted. The fusion proteins produced by all three recombinants are recognized by the rabbit anti-205K antiserum but not by preimmune serum (Fig. 7, f-i). Only the fusion protein produced by lambda gt11.205.16 is recognized by the monoclonal antibody (Fig. 7d); it is not recognized by nonspecific monoclonal antibody. This result strongly suggests that lambda gt11.205.16 is not recognized by contaminating antibodies in the rabbit 205K antiserum. To better test lambda gt11.205.19 and lambda gt11.205.23, immunoblots of lysates of the recombinant phage lysogens were reacted with affinity-purified anti-205K antibody (described in the previous section and in Materials and Methods). The fusion proteins produced by all three recombinant phage are recognized by the affinity-purified anti-205K antibodies. In a second affinity-purification experiment, lysates of each of the three recombinant lysogens and a lysogen of non-recombinant gt11 were run on a gel, electrophoretically transferred to nitrocellulose, and stained with Ponceau S to locate the fusion proteins and beta-galactosidase. The regions of the nitrocellulose-containing fusion protein and beta-galactosidase were excised, as were control regions from other parts of the nitrocellulose containing only bacterial proteins. These nitrocellulose-bound proteins were then used to affinity-purify antibodies from the total rabbit 205K antiserum. These anti-

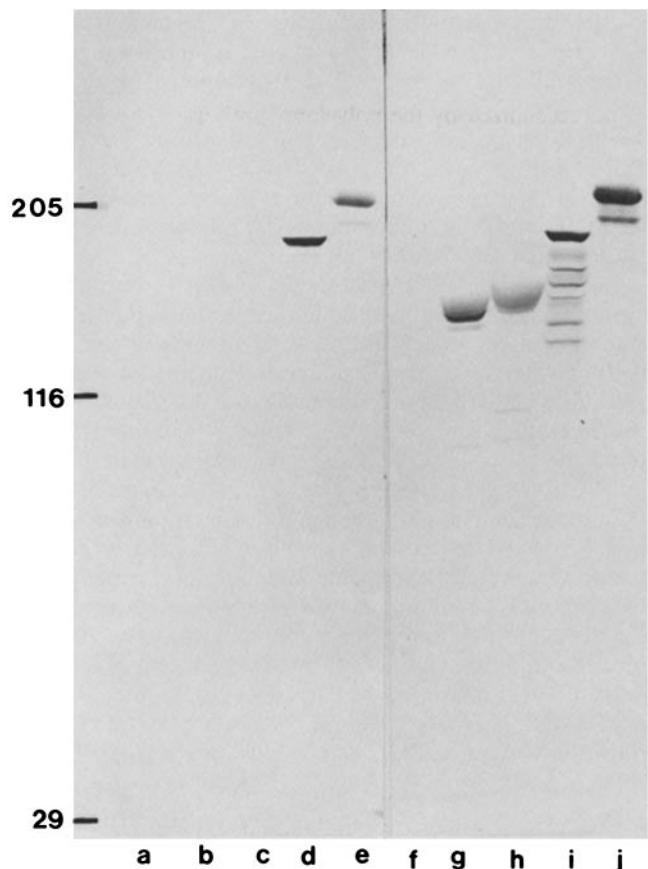


Figure 7. Immunoblots of proteins produced by recombinant and control lysogens using monoclonal and polyclonal anti-205K MAP. Lanes a-e, monoclonal anti-205K MAP; f-j, polyclonal anti-205K MAP. Lanes a and f, control lambda gt11 lysogen; b and g, lambda gt11.205.23 lysogen; c and h, lambda gt11.205.19 lysogen; d and i, lambda gt11.205.16 lysogen; e and j, isolated microtubules.

bodies were then used in immunoblots of purified *Drosophila* microtubules, total Schneider cell homogenates, and proteins produced in recombinant lysogens. It was expected that if antibodies in the 205K antiserum other than those that recognize the 205K MAP were responsible for recognizing the fusion proteins produced by the recombinant phage, then antibodies affinity-purified using the fusion proteins should not recognize the 205K MAP, and could possibly identify some other band in immunoblots of *Drosophila* microtubules or *Drosophila* cell homogenates. The result from such an experiment is that antibodies affinity-purified with the fusion proteins recognize only the 205K MAP (and not the 255K or 150K species) in purified microtubules and whole cell homogenates: no observable antibodies of any specificity were affinity-purified by the control proteins (either beta-galactosidase or other random bacterial proteins; see Fig. 8). Therefore, these experiments demonstrate that the antibodies that recognize these fusion proteins are the same as those that recognize the 205K MAP.

The above immunoblotting data also rule out the possibility that the phage clones encode a polypeptide unrelated to the 205K MAP, but which fortuitously shares an antigenic determinant. For example, lambda gt11.205.16, while sharing some homology with lambda gt11.205.19 (see below), encodes the monoclonal determinant, whereas lambda gt11.205.19 encodes determinants recognized only by the polyclonal antibody. Similarly, lambda gt11.205.23 and lambda gt11.205.19 have homology to contiguous stretches of DNA (see below) but not to each other, yet both encode determinants recognized by the polyclonal antiserum. Additionally, in some experiments where high yields of the lambda

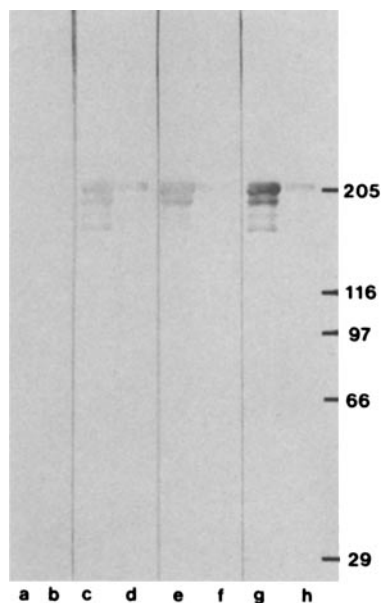


Figure 8. Immunoblots of antibodies affinity-purified using fusion proteins and challenged against *Drosophila* cell homogenates and microtubules. Lanes a, c, e, and g, isolated microtubules; b, d, f, and h, cell homogenates. Lanes a and b, antibody affinity-purified on low molecular weight *E. coli* proteins; c and d, antibody affinity-purified on fusion protein produced by lambda gt11.205.16; e and f, antibody affinity-purified on fusion protein produced by lambda gt11.205.19; g and h, antibody affinity-purified on fusion protein produced by lambda gt11.205.23.

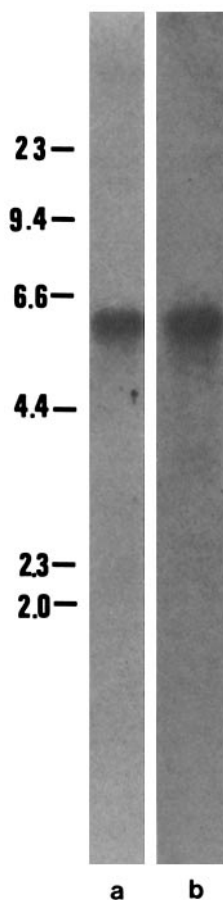


Figure 9. Hybridization of labeled DNA to Northern blots of 5 µg of poly A⁺ RNA from S2 cells. Lane a, hybridization to labeled pUC13.205.16; b, hybridization to labeled pUC13.205.19.

gt11.205.16 fusion protein have been obtained, it has been observed that there are proteolytic fragments of the fusion protein that stain with the polyclonal but not the monoclonal antibody. Therefore, at least two determinants recognized by the antibodies are encoded by lambda gt11.205.16. Finally, lambda gt11.205.19 and lambda gt11.205.23 encode distinct determinants as shown by experiments in which antibodies affinity-purified using either of the two fusion proteins recognize both the fusion protein on which they were affinity-purified and the fusion protein encoded by the overlapping clone in lambda gt11.205.16, but not the fusion protein produced by the other non-overlapping clone. Hence, these clones encode multiple determinants located on the 205K MAP. In addition, the last result demonstrates that these fusion proteins can be used to purify antigenically restricted subsets of antibodies from a polyclonal antiserum.

While these experiments provide strong immunological evidence that the lambda gt11 recombinant phage contain 205K MAP coding sequences, it was desirable to obtain independent structural evidence that supported this conclusion. Therefore, the size of the mRNA in Schneider S2 cells encoded by sequences in these phage was examined. Because the size of the polypeptide is quite large (~*M_r* 205,000 estimated from SDS gels), it was expected that the mRNA encoding it should also be quite large (estimate 5,000–7,000 nucleotides), larger than most cellular mRNAs. Examination of mRNA size using subclones (see next section) of lambda gt11.205.16 or lambda gt11.205.19 to probe Northern blots of polyA⁺ mRNA revealed this supposition to be correct (see

Fig. 9). The estimated size of the mRNA homologous to these clones is 6,000 nucleotides. Hence, these data and the immunological data taken together strongly support the identification of these clones as containing 205K MAP coding sequences.

Characterization of DNA Sequences Encoding the 205K MAP

To examine the relationships of the *Drosophila* DNA carried by the different recombinant phage and to elucidate the genomic organization of the sequences encoding the 205K MAP, DNA was isolated from each of the phage, restriction mapped at low resolution, and subcloned after EcoR1 digestion into the plasmid vector pUC13 (Viera and Messing, 1982). Hybridizations to Southern blots (1975) of restriction enzyme-digested plasmid subclones, using the plasmid subclones as probes, revealed that the inserts in lambda gt11.205.19 and lambda gt11.205.23 are homologous to the insert in lambda gt11.205.16 but not to each other (see map in Fig. 10). To examine the genomic DNA encoding the 205K MAP, labeled plasmid subclones or subfragments isolated from these subclones in the region of shared homology (see Materials and Methods for details) were hybridized to Southern blots of EcoR1 or HindIII digested genomic DNA. It was expected that if the 205K MAP coding sequences were single copy in the *Drosophila* genome, then probes homologous to DNA on opposite sides of the EcoR1 site in lambda gt11.205.16 should hybridize to different but single EcoR1 fragments of genomic DNA. In addition, because no HindIII site is present in the regions shared among lambda gt11.205.16, lambda gt11.205.19, and lambda gt11.205.23, probes from this region should hybridize to a single shared HindIII fragment. These expectations were met: labeled fragments from the lambda gt11 subclones around the EcoR1 site of lambda gt11.205.16 hybridize to a single shared band in HindIII digests (see example in Fig. 11) while the two sets of fragments homologous to DNA on opposite sides of the EcoR1 site in lambda gt11.205.16 hybridize to different, single EcoR1 fragments. Similar conclusions have been reached using subclones from a "genomic walk" in this region.² In situ hybridization to polytene chromosomes revealed that pUC 13.205.19 hybridizes to a single site in the genome located at 100EF (Fig. 12). Hence the *Drosophila* 205K MAP is likely to be encoded by a single copy gene.

Discussion

MAPs are operationally defined by their binding to tubulin polymers. There is considerable reason to think that MAPs play an essential role in microtubule function in vivo, but most studies of MAPs revolve around their behavior in vitro.

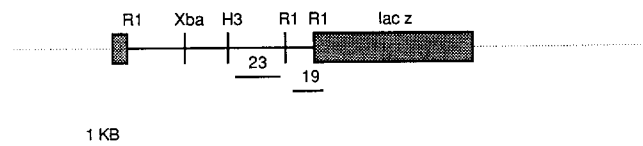


Figure 10. Low resolution restriction map of insert in lambda gt11.205.16 and positions of homology to inserts in lambda gt11.205.19 and lambda gt11.205.23.

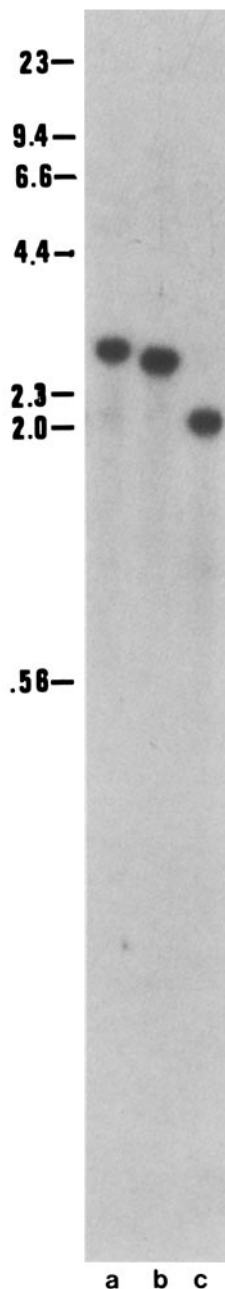


Figure 11. Hybridization of labeled 1-kb HindIII fragment from pUC13.205.16 to Southern blots of restriction enzyme digested genomic DNA. Lane a, 3 μ g of Oregon-R genomic DNA digested with EcoR1 and run on a 0.6% agarose gel; b, 3 μ g of Oregon-R genomic DNA digested with HindIII and run on a 0.6% agarose gel; c, 3 μ g of Oregon-R genomic DNA digested with EcoR1 and HindIII and run on a 0.6% agarose gel. Marker sizes are given in kilobases.

Thus, little is yet known about MAP function in vivo. Our goal is to use genetic analysis in concert with other methods to study the functions of those MAPs that are components of the mitotic apparatus in *Drosophila melanogaster*. In this paper, we describe the isolation of putative MAPs from a cultured *Drosophila* cell line, the production and characterization of specific antibodies directed against the most abundant group of these MAPs (205K), and present immunocytochemical evidence that at least one of the 205K MAPs is a component of the cellular mitotic spindle and cytoplasmic microtubule systems. The antibodies have been used to screen a genomic library in an expression vector and clones encoding 205K MAP epitopes recognized by these antibodies were identified and isolated. Hybridization analysis has revealed that the DNA sequences isolated are derived from a single gene located at 100EF. We are now poised to begin genetic

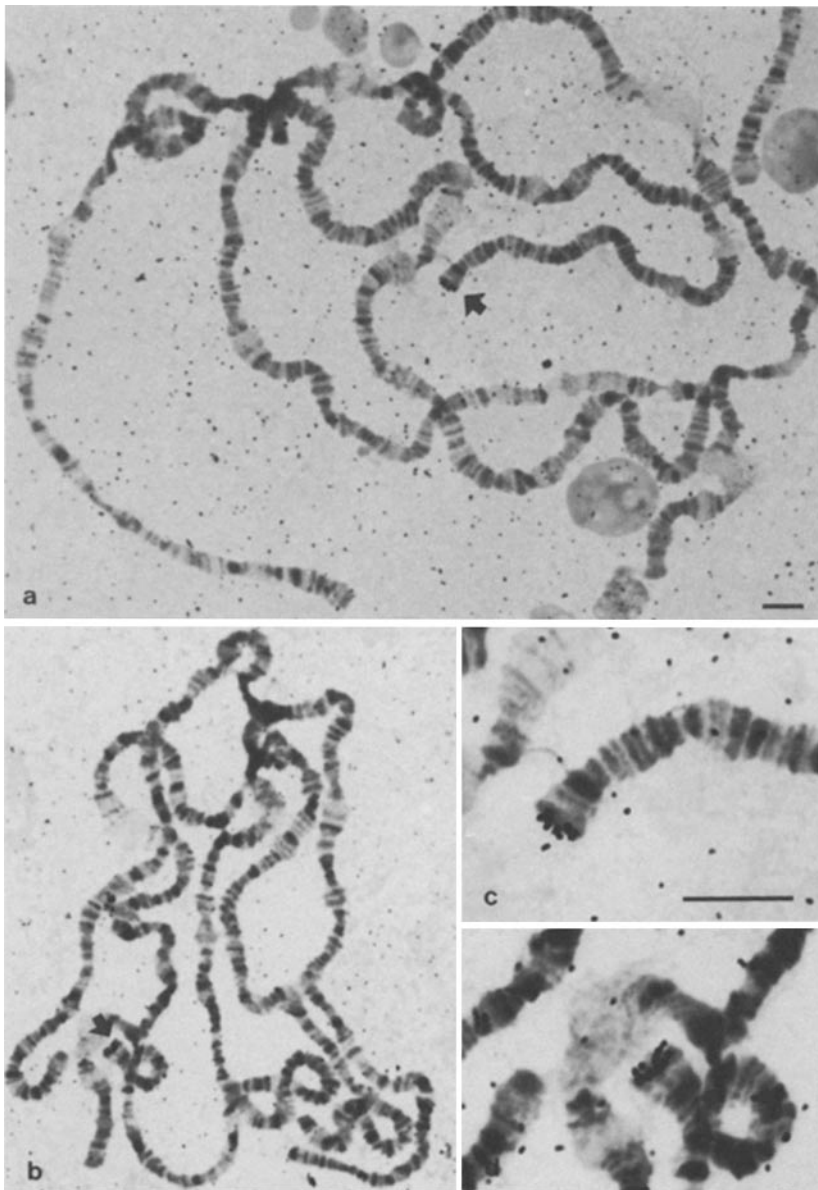


Figure 12. In situ hybridization of pUC13.205.19 to polytene chromosomes of Oregon-R. (a and b) Low magnification views of two nuclei from same slide; arrows indicate unique site of hybridization at 100EF. Bar in a, 10 μ m; a and b are at same magnification. (c and d) High magnification view of 3R tips in a and b (respectively) showing hybridization at 100EF. Bar in c, 10 μ m; c and d are at same magnification.

analysis of this gene so that we can elucidate the function of the 205K MAP in vivo.

MAPs have traditionally been identified as polypeptides that quantitatively co-purify with tubulin through cycles of temperature-dependent assembly and disassembly (see, for example, Berkowitz et al., 1977). Recent work has shown, however, that the taxol-dependent procedure reliably identifies polypeptides that are associated with microtubules inside cells (Vallee, 1982; Vallee and Bloom, 1983). In particular, work on HeLa cells, mammalian neural tissue, and sea urchins (Vallee, 1982; Vallee and Bloom, 1983; Scholey et al., 1984) demonstrates that the polypeptides identified in taxol-dependent isolations are an overlapping set of those identified by traditional means, and in some cases, are microtubule-associated in cells by the criterion of immunofluorescent localization studies using antibodies that recognize these polypeptides. In the experiments reported here, a taxol-dependent polymerization procedure (Vallee, 1982) was used to identify MAPs in *Drosophila melanogaster* Schneider line 2 cells. It

was observed that there was striking co-enrichment of polypeptide species of M_r 205,000 and tubulin during microtubule isolation and also, though not as enriched in all cases, a characteristic set of other polypeptide species is recovered during these experiments. The evidence that these associated proteins are specifically associated with microtubules rather than being fortuitously isolated comes from experiments in which the sedimentation of these polypeptides during centrifugation was tested for microtubule-dependency and also, in the case of the M_r 205,000 polypeptides, from immunofluorescent localization studies using specific antibodies (see below).

A monoclonal antibody and a rabbit antiserum directed against the 205K MAP were prepared and characterized. In immunofluorescence experiments, both probes stained mitotic spindle microtubules, asters, and in the case of the rabbit serum, cytoplasmic microtubules. In immunoblot experiments, both antibodies stained four polypeptides with similar mobilities of $\sim M_r$ 205,000 in isolated microtubules, and, both

antibody preparations stained four species of ~205K and one 255K species in whole cell homogenates. The rabbit serum additionally stains a 150K species in whole cell homogenates. Because we do not know whether the four bands of ~205K are four related molecules or are simply derived by proteolysis or modification of a single molecule, we refer to them for the purposes of discussion as the 205K MAP.

The above observations raise the question of whether it is the 255K, 205K, or 150K molecule that is located in the cellular microtubules. Two observations suggest that it is the 205K, and possibly the 255K molecules that are present in the mitotic spindle and cytoplasmic microtubules. First, the monoclonal antibody recognizes only the 205K and 255K molecules in immunoblots and stains the mitotic spindle and possibly cytoplasmic microtubules. Second, affinity-purification of the rabbit serum using filter-bound 205K MAP results in an antibody preparation that recognizes only the 205K species in immunoblots of whole cell homogenates but still stains mitotic spindle and cytoplasmic microtubules. The origin of the 150K staining in immunoblots of cell homogenates is unclear, but the affinity purification results and immunoblots against boiled MAPs (the source of gel-purified 205K MAP antigen) suggest that it is either a cross-reacting species or a proteolytic fragment.

An interesting outcome of these experiments is the identification of a *Drosophila* MAP species possessing properties very similar to the 210K MAPs found in HeLa and possibly all mammalian cells (Duerr et al., 1981; Olmsted et al., 1984; Parysek et al., 1984a, b). A similar protein may also be present in sea urchins (Vallee and Bloom, 1983). The HeLa 210K MAPs and MAP4 from mouse (also M_r 210,000–240,000) have been examined in detail, and it has been found that they co-purify with microtubules during taxol-dependent polymerization experiments, stimulate the assembly of dimeric tubulin into polymer, and do not precipitate from solution when boiled. The HeLa 210K MAPs may be related to a heavier, 255K, MAP species, and MAP4 is actually a group of proteins in the range of 210K–250K, the exact species present depending on the tissue. As evidenced by immunocytochemistry, the HeLa 210K MAPs are associated with spindle and cytoplasmic microtubules in vivo (Bulinski and Borisy, 1979, 1980a,b; Izant et al., 1982, 1983; Vallee, 1982; Weatherbee et al., 1980, 1982), and MAP4 is found on cytoplasmic microtubules (Parysek et al., 1984a,b). The 205K MAP identified in *Drosophila* cultured cells has a slightly faster mobility on denaturing polyacrylamide gels than the HeLa 210K MAP, but is otherwise very similar (heat-stability, binding to microtubules, and distribution in cells) to the mammalian protein. The staining of a polypeptide species of M_r 255,000 in immunoblots of whole cell homogenates suggests the possibility that in fly cells as in HeLa cells the 205K MAP is in some way related to a heavier polypeptide. Despite the similar properties of these molecules, antibodies that recognize the fly proteins do not cross-react with the HeLa proteins in immunoblots, and monoclonal antibodies directed against the HeLa protein do not recognize the fly protein (Goldstein, L. S. B., and M. Zavortink, unpublished observations). Nonetheless, the similar sizes and properties of these molecules suggest that a 205K/210K MAP may be found in all eukaryotes, and that, while the overall properties of the molecule may be conserved, its antigenic structure is not. This latter

conclusion is also supported by the observation that antibodies raised against the mammalian forms of this protein tend to be species or order specific (Bulinski and Borisy, 1980c; Izant et al., 1982, 1983).

The strategy chosen to isolate the 205K MAP gene was to create a large random shear genomic library of *Drosophila* DNA in the expressing vector lambda gt11, a vector that expresses inserted coding DNA as a fusion protein with beta-galactosidase. Phage that contained 205K MAP coding sequences were identified by screening this library with the antiserum that recognized the 205K MAP (Young and Davis, 1983, 1984). Three recombinant phage were isolated and characterized in detail. We conclude that these phage contain DNA sequences encoding the 205K MAP for three reasons. First, the proteins produced by the recombinant phage are recognized by the same antibody species that recognize the 205K MAP. This is supported by the observations that: (a) one recombinant phage, lambda gt11.205.16, produces a protein recognized by the monoclonal antibody directed against the 205K MAP; (b) antibodies affinity-purified using the 205K MAP recognize the fusion protein produced by each of these three clones; and (c) antibodies affinity-purified using fusion proteins produced by the phage recognize only the 205K MAP in immunoblots of cell homogenates and isolated microtubules. Second, our data show that a single contiguous stretch of DNA encodes multiple antigenic determinants. This rules out an argument suggested by observations such as those of Nigg et al. (1982), that is, the DNA we isolated encodes a single antigenic determinant fortuitously shared between the 205K MAP and an unrelated polypeptide. Third, determination of the size of the mRNA homologous to the DNA contained in these recombinant phage demonstrates an mRNA of sufficient and appropriate size to encode the 205K MAP. Thus, these data taken together strongly support the conclusion that these clones represent portions of the 205K MAP coding sequence. Preliminary data from hybrid-selected translation experiments also support this conclusion.²

Initial characterization of these DNA sequences suggests that they define a single gene in the *Drosophila* genome located in polytene region 100EF. To our knowledge no mutations likely to reside in the 205K MAP gene exist. Finally, we have identified no heterogeneity in mRNA present in S2 cells. The question thus remains as to the basis for the immunological complexity observed for the *Drosophila* proteins and perhaps for the mammalian proteins as well. We note, however, that Drubin et al. (1984) have described a clone encoding tau from rat brain, which while apparently single copy, generates several different size polypeptides from an apparently single mRNA species.

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