

# In Vivo Microtubules Are Copolymers of Available $\beta$ -Tubulin Isozymes: Localization of Each of Six Vertebrate $\beta$ -Tubulin Isozymes Using Polyclonal Antibodies Elicited by Synthetic Peptide Antigens

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**Abstract.**  $\beta$ -Tubulin is encoded in the genomes of higher animals by a small multigene family comprising approximately seven functional genes. These genes produce a family of closely related, but distinct polypeptide isozymes that are distinguished principally by sequences within the  $\sim 15$  carboxy-terminal amino acid residues. By immunizing rabbits with chemically synthesized peptides corresponding to these variable domain sequences, we have now prepared polyclonal antibodies specific for each of six distinct isozymes. Specificity of each antiserum has been demonstrated unambiguously by antibody binding to bacterially produced, cloned proteins representing each isozyme and

by the inhibition of such binding by preincubation of each antiserum only with the immunizing peptide and not with heterologous peptides. Protein blotting of known amounts of cloned, isotypically pure polypeptides has permitted accurate quantitative measurement of the amount of each  $\beta$ -tubulin isozyme present in the soluble and polymer forms in various cells, but has not revealed a bias for preferential assembly of any isozyme. Localization of each isozyme in three different cell types using indirect immunofluorescence has demonstrated that in vivo each class of microtubules distinguishable by light microscopy is assembled as copolymers of all isozymes expressed in a single cell.

**M**ICROTUBULES are ubiquitous structural components of eukaryotic cells. Composed principally of heterodimeric subunits of one  $\alpha$ - and one  $\beta$ -tubulin polypeptide, microtubules serve several divergent functional roles within cells. They are the primary components through which accurate chromosome segregation is achieved during meiosis and mitosis, and they serve as major structural components for ciliary- and flagellar-dependent cell motility, for establishment of the asymmetric morphology of neurons, and as a substrate for the transport of vesicles and organelles within the cytoplasm (Hayden and Allen, 1984; Vale et al., 1985). In addition, together with actin filaments and intermediate filaments, microtubules of the cytoskeleton play a major role in establishing and maintaining the dynamic and spatial organization of the cytoplasm (e.g., Heuser and Kirschner, 1980; Kirschner and Mitchison, 1986).

This heterogeneity of function is paralleled by a diversity in biochemical and molecular properties of microtubules. Microtubules within single cells show differences in their stability (Brinkley and Cartwright, 1975; Schulze and Kirschner, 1987), assembly (Schulze and Kirschner, 1987; Murphy and Wallis, 1985), and patterns of posttranslational modification (Gundersen et al., 1984; L'Hernault and Rosenbaum, 1985; Piperno et al., 1985). Furthermore, microtubules in different cells show dramatic differences in their

complement of microtubule associated proteins (Bulinski and Borisy, 1980; Binder et al., 1985; Bloom et al., 1984; Huber and Matus, 1984; Parysek et al., 1984). Nonetheless, the relationship of microtubule heterogeneity to microtubule function remains obscure, as do the mechanisms by which cells regulate the molecular properties of microtubules.

The possible role of the tubulin subunit itself as a modulator that can specify microtubule function remains unsettled (see Cleveland, 1987 for recent review). Our initial demonstration that eukaryotic genomes contain multigene families for  $\alpha$ - and  $\beta$ -tubulin (Cleveland et al., 1980) rekindled earlier speculations that such organisms possessed functionally differentiated tubulins that could be used to construct different kinds of microtubules (Fulton and Simpson, 1976; Stephens, 1975). It is now clear (principally from our efforts and those of Cowan and collaborators [Wang et al., 1986; Villasante et al., 1986]) that in vertebrates both  $\alpha$ - and  $\beta$ -tubulin are encoded by approximately six or seven functional genes. For the  $\beta$ -tubulins, our studies (Sullivan and Cleveland, 1984; Sullivan et al., 1986a, b; Sullivan and Cleveland, 1986) and those of Cowan's group (Hall et al., 1983; Lewis et al., 1985a, b; Wang et al., 1986) have established that these multiple genes encode polypeptides that differ in sequence and in patterns of expression. Within an otherwise conserved polypeptide framework, the distribution of amino

acid substitutions is not random but in large part restricted to domains at the extreme carboxy terminus, and to a lesser extent, near the amino terminus (Sullivan and Cleveland, 1986). Further, sequences of at least four of these carboxy-terminal region domains define evolutionarily conserved  $\beta$ -tubulin polypeptide isotypes (Sullivan and Cleveland, 1986).

To investigate further the functional properties of  $\beta$ -tubulin isotypes, we have now exploited the divergent carboxy-terminal domain sequences to chemically synthesize the peptides corresponding to each of the four evolutionarily conserved isotypic classes. Peptides for two additional chicken  $\beta$ -tubulin polypeptide sequences (not known to be found in other organisms) have also been prepared. We have used these peptides as immunogens to generate polyclonal antibodies to each  $\beta$ -tubulin isotype. The specificity of the antisera has been extensively characterized by using cloned DNA sequences to direct expression in bacteria of hybrid proteins containing the carboxy terminus of each of the six  $\beta$ -tubulin isotypes. The isotype-specific antibodies, in conjunction with the corresponding cloned proteins, have been used to develop an accurate and quantitative assay for the  $\beta$ -tubulin isotypic composition in the soluble and polymeric forms in various cell lines. Finally, indirect immunofluorescence analysis of isotypic composition *in vivo* has demonstrated that all microtubule classes distinguishable by light microscopy are assembled as copolymers of all available isotypes.

## Materials and Methods

### Peptide Synthesis and Coupling

Synthetic peptides were chemically synthesized using a peptide synthesizer (Applied Biosciences, Inc., Foster City, CA). After purification by high-performance liquid chromatography (on a C-18 column), sequences of each peptide were verified by analysis of amino acid composition and/or direct sequence analysis using an automated Applied Biosciences protein sequencer. Peptides ( $6 \times 10^{-7}$  mol in phosphate-buffered saline [PBS], pH 7.4) were coupled to the carrier protein keyhole limpet hemocyanin (KLH, Calbiochem-Behring Corp., San Diego, CA) ( $8 \times 10^{-9}$  mol in PBS) by cross-linking with glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA). The peptides and KLH were mixed on ice and  $10^{-4}$  mol of glutaraldehyde was added and then rotated for 20 h at room temperature. The complex was dialyzed against two changes of Tris-buffered saline (20 mM Tris, pH 7.4, 130 mM NaCl) for 16 h, aliquoted, and stored frozen ( $-20^{\circ}\text{C}$ ).

### Immunization of Rabbits with Peptides

Cross-linked peptide/KLH ( $6 \times 10^{-8}$  mol) and free peptide ( $4 \times 10^{-8}$  mol in PBS, pH 7.4) were sonicated into an emulsion with complete Freund's adjuvant. Each emulsion was injected subcutaneously into a New Zealand white rabbit (in four spots along the back). The inoculation was repeated after 20 d and again on day 35, this time with incomplete Freund's adjuvant. Rabbits were ear bled on day 48. The blood was allowed to clot, and the serum was collected and stored at  $-20^{\circ}\text{C}$ .

### Preparation of Affinity Columns Containing Peptide Covalently Linked to Sepharose

3.5 g of wet, cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) and 20 mg of peptide in 0.5 M NaCl, 0.1 M  $\text{NaHCO}_3$  (pH 8.8) were mixed gently overnight at  $4^{\circ}\text{C}$ . The Sepharose was pelleted by low-speed centrifugation and remaining unreacted sites were

quenched with 1 M triethanolamine, pH 8. The Sepharose was washed sequentially with acetate buffer (1 M NaCl, 0.1 M sodium acetate, pH 4), borate buffer (1 M NaCl, 0.1 M sodium borate, pH 8), and phosphate buffer (0.3 M NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , pH 6.9) prior to storage at  $4^{\circ}\text{C}$ .

### Affinity Purification of Antibodies

Immune serum and peptide-affinity columns were gently mixed overnight at  $20^{\circ}\text{C}$  to allow antibody binding to the immobilized peptide. Unbound antibodies were collected and the column was washed successively with P buffer (0.3 M NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , pH 6.9), with P buffer containing 0.2% Triton X-100, and with P buffer. The bound antibody was eluted with two column volumes of citrate buffer (0.3 M NaCl, 0.1 M sodium citrate, pH 2.2), and neutralized immediately with Tris-HCl, pH 9.1. The affinity-purified antibody was brought to 5 mg/ml bovine serum albumin (BSA) and stored at  $-80^{\circ}\text{C}$ .

### Construction of Expression Plasmids Containing the Carboxy-Terminal Sequences of Each of Six $\beta$ -Tubulin Isotypes Fused to the Bacterial Gene *trpE*

For each of six  $\beta$ -tubulin isotypes, a plasmid was constructed which carried a chimeric gene that directed the synthesis of a fusion protein in bacteria. In each instance the resultant fusion protein contained an amino-terminal 32 kD of the bacterial protein *trpE* linked to amino acid residues from  $\beta$ -tubulin beginning at codon 345 through to the carboxy terminus. To do this, we exploited the property that essentially all vertebrate  $\beta$ -tubulins contain a conserved Bam HI site within codon 344. The following  $\beta$ -tubulin fragments were isolated and inserted in the proper orientation into the unique Bam HI site of the *trpE* gene in pATH 1 (kindly provided by Dr. T. J. Koerner, Duke University):

**Type I.** A 0.5-kb Bam HI fragment from human gene  $\text{h}\beta 1$ . (This was obtained from a cDNA clone [Sullivan and Cleveland, unpublished results] for the human  $\text{h}\beta 1$  gene [originally named M40—Lee et al., 1983].)

**Type II.** Because the carboxy-terminal sequence of this isotype differed in two positions between the rat and mouse or chicken and porcine sequences, isotype plasmids were engineered to produce both the rat/mouse and chicken/porcine variants. For the rat/mouse variant: the 0.75-kb Bam HI fragment of mouse gene  $\text{m}\beta 2$  (Sullivan and Cleveland, 1986) was used. For the chicken/porcine variant, the 1.8-kb Bam HI fragment of gene  $\text{c}\beta 2$  (Lopata et al., 1983) was used.

**Type III.** Because the chicken and human type III carboxy-terminal sequences differ in two residue positions, two plasmids were constructed. For human type III, a 0.5-kb Bam HI fragment from a cDNA clone of  $\text{h}\beta 4$  (Sullivan and Cleveland, 1986) was used. For chicken type III, a 2.5-kb Bam HI fragment from gene  $\text{c}\beta 4$  (Sullivan and Cleveland, 1984) was used.

**Mammalian Type IV (m-IV).** A 0.5-kb Bam HI fragment from a cDNA clone (Sullivan and Cleveland, unpublished results) derived from the human  $\beta 2$  gene (Lewis et al., 1985a) was used.

**Chicken Type IV (c-IV).** The 4.4-kb Bam HI fragment from gene  $\text{c}\beta 3$  (Lopata et al., 1983) was used. (This gene encodes the dominant  $\beta$ -tubulin in chicken testis.)

**Chicken Type V (c-V).** The 4.8-kb Bam HI fragment from gene  $\text{c}\beta 5$  (Sullivan et al., 1986a) was used. (The  $\text{c}\beta 5$  gene encodes a chicken  $\beta$ -tubulin expressed in many tissues.)

### Induction of Cloned Fusion Proteins

Plasmids carrying each  $\beta$ -tubulin carboxy terminus linked in the correct orientation to the *trpE* gene were transformed into *Escherichia coli* strain CAG456 (Snyder et al., 1987). Expression of the fusion protein, which is controlled by the *trpE* promoter, was induced with 27 mM (5  $\mu\text{g}/\text{ml}$ ) indole acrylic acid (IAA, Sigma Chemical Co., St. Louis, MO). To do this, bacterial clones harboring each plasmid were grown overnight at  $30^{\circ}\text{C}$  in M9 minimal media containing 20  $\mu\text{g}/\text{ml}$  tryptophane (Maniatis et al., 1982). To induce the protein, the bacterial culture was diluted (1:10) into M9 media without tryptophane and grown 2 h at  $30^{\circ}\text{C}$  with vigorous aeration. The inducer (solubilized in ethanol) was added to 5  $\mu\text{g}/\text{ml}$  and the cultures were allowed to grow 2 h at  $30^{\circ}\text{C}$ . Extracts containing the fusion proteins were prepared by pelleting the bacteria by centrifugation, washing the cell pellets once with 10 mM Tris-HCl (pH 8), and resuspension in Laemmli gel sample buffer (Laemmli, 1970). After boiling for 5 min, bacterial DNA was pelleted by centrifugation (5 min at 12,000 g) and the supernatant containing solubilized proteins was recovered. Samples were electrophoresed immediately or stored at  $-20^{\circ}\text{C}$ .

1. *Abbreviations used in this paper:* KLH, keyhole limpet hemocyanin; CEF, chicken embryo fibroblasts.

## Gel Electrophoresis and Protein Blotting

Polyacrylamide gels for analysis of protein samples were run as described by Laemmli (1970). Proteins were transferred to nitrocellulose (BA83, Schleicher & Schuell, Inc., Keene, NH) in one-half strength Laemmli gel running buffer (Laemmli, 1970) plus 20% methanol. Typically, transfer was for 4 h at 50 V. Filters containing transferred samples were stained with Ponceau S (0.2% Ponceau S in 3% trichloroacetic acid) to identify positions of molecular weight standards. Nonspecific protein-binding sites were blocked by incubation in PTX-BSA (0.2% Triton X-100, 0.15 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 1 mM EGTA, 4% BSA) for 10 min. For immunologic detection of proteins, primary antibody in PTX-BSA was then added and allowed to react overnight at room temperature. In some cases, chemically synthesized peptides were preincubated with this primary antibody to test the specificity of the antibody binding. Unbound primary antibody was removed and the filters were washed five times (3 min each wash) in GB (0.5% Triton X-100, 50 mM triethanolamine, pH 7.4, 0.1 M NaCl, 0.1 mM EDTA, 0.1% SDS). <sup>125</sup>I-labeled protein A in PTX-BSA was added and incubated for 1 h at room temperature, followed by five washes (3 min each wash) in GB to remove unbound protein A. Binding was detected by autoradiography using Dupont Lightning Plus intensifying screens (DuPont Co., Wilmington, DE) and Kodak XAR film (Eastman Kodak Co., Rochester, NY).

## Preparation of Cell Extracts Containing Soluble, Polymeric, or Total Tubulin

To prepare extracts of total cell proteins, a 100-mm dish of ~50% confluent cells was washed four times with 5 ml of PBS before lysis with 200  $\mu$ l of 50 mM Tris, pH 6.8, 0.1% SDS. The lysate was scraped into a microfuge tube and boiled for 5 min, and the DNA was removed by centrifugation (3 min, 12,000 g). Protein concentration was determined by the bicinchoninic acid protocol (Smith et al., 1985).

For preparation of soluble and polymeric fractions of cell tubulin, cells were washed twice with PBS (at 37°C) and then twice with microtubule stabilizing buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, 4% polyethylene glycol 8000). Soluble proteins were extracted by lysis in 1 ml of stabilizing buffer containing 0.5% Triton X-100. Extraction was continued for 5 min at 37°C. The solution was removed, any insoluble material in the soluble fraction was pelleted by centrifugation (12,000 g for 1 min), and the soluble fraction was made 1X Laemmli gel sample buffer by addition of 280  $\mu$ l of a 5X stock and 120  $\mu$ l of 2-mercaptoethanol. The remaining cytoskeletal fraction (including the pellet from centrifugation of the soluble fraction) was solubilized in 1.4 ml of Laemmli gel sample buffer. After boiling for 3 min, DNA was removed from the polymeric fraction by centrifugation (12,000 g). Both fractions were concentrated by precipitation with 9 vol of cold methanol, after addition of tRNA carrier to 100  $\mu$ g/ml. After 1 hr at -20°C, precipitated protein was recovered by centrifugation at 9,000 g for 20 min at 4°C. Pellets were dried and resuspended in 200  $\mu$ l of Laemmli gel sample buffer. Samples were boiled and electrophoresed immediately or stored at -20°C.

## Quantitation of Amounts of Each $\beta$ -Tubulin Isotype

In order to use protein blotting to determine accurately the amount of each  $\beta$ -tubulin isotype present in various samples, the samples were electrophoresed on a series of parallel gels. On each gel, serial dilutions of a bacterial extract containing one of the cloned fusion protein isotypes were loaded adjacent to lanes of samples to be quantified. The gels were blotted and processed with the primary antibody corresponding to the fusion protein dilutions present on each blot. Autoradiography of the fusion protein dilutions provided internal standards for densitometric quantitation of the signals of each sample.

The amount of cloned fusion protein contained in a specific lysate dilution was determined by coelectrophoresis of a known volume of fusion protein extract with serial dilutions of known amounts of BSA. From densitometric comparison of the intensity of the Coomassie Blue staining of the fusion protein and the BSA standards, the amount of fusion protein was determined. By knowing the molecular weight of the fusion protein, the molar amount of  $\beta$ -tubulin carboxyterminus in a specified volume of lysate was then calculated.

## Immunofluorescence

Cells were grown overnight on glass coverslips. Coverslips were washed for

15 s at 37°C with stabilization buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, and 4M glycerol) and then incubated for 1 min at 37°C in stabilization buffer containing 0.5% Triton X-100. Coverslips were then rinsed in stabilization buffer (15 s at 37°C) and plunged into methanol at -20°C for 5 min. The coverslips were rehydrated in PBS and stained with the isotype-specific antibody (1:20 to 1:100 in PBS supplemented with 0.5% BSA) for 1 h at room temperature. They were then rinsed with PBS and a fluorescein-labeled anti-rabbit IgG antibody (Cooper Biomedical) was applied for 45 min at room temperature. The coverslips were rinsed with PBS and mounted in Aqua-mount (Lerner Laboratories, New Haven, CT). The cells were examined on an Olympus BH-2 microscope with epifluorescent optics and photographed on Kodak Tri-X film developed in Diafine.

## Results

### Synthesis of Peptides Corresponding to the Extreme Carboxy Termini of Six $\beta$ -Tubulin Isoforms

From comparison of known vertebrate  $\beta$ -tubulin sequences, we have previously identified four evolutionarily conserved classes of  $\beta$ -tubulin polypeptides (Sullivan and Cleveland, 1986). These classes are distinguished primarily through their divergent carboxy-terminal sequences and, so far as is known, each isotypic class is expressed in analogous developmental programs in each species investigated. To prepare polyclonal antibodies that would bind uniquely to individual isotypic classes, we chemically synthesized the carboxy-terminal peptides for each of the four classes. Fig. 1 displays the carboxy-terminal sequences of each of the various vertebrate  $\beta$ -tubulins and the boxed regions correspond to the actual peptides that were produced. Note that for both type II and type III isoforms, although the terminal regions are clearly homologous in each of the species for which the isotype has been identified, there are minor amino acid differences between the members of the class. For example, for type II the chicken and pig sequences have the dipeptide GE whereas the mouse and rat isoforms have EG. Similarly, the type III human and chicken variants differ in two of the final 20 positions. As shown in the figure, we chose to synthesize the rat/mouse type II and human type III peptide.

To prepare as complete a set of isotype-specific  $\beta$ -tubulin antibodies as possible, we also synthesized two additional peptides corresponding to chicken isoforms c $\beta$ 3 and c $\beta$ 5. Although we had tentatively proposed that c $\beta$ 3 represented the dominant testis  $\beta$ -tubulin isotype (Sullivan and Cleveland, 1986), in view of the high homology of c $\beta$ 3 to the type IV sequence, coupled with the demonstration from Cowan's laboratory (Wang et al., 1986) that the type IVB mouse gene (m $\beta$ 4' in our nomenclature [Sullivan and Cleveland, 1986] and m $\beta$ 3 in Cowan's) is the dominant mouse testis  $\beta$  tubulin, we now conclude that c $\beta$ 3 represents the chicken type IVB gene. For c $\beta$ 5, inspection of the available vertebrate  $\beta$ -tubulin sequences reveals that no cognate gene in other species has yet been reported for this isotype (even though c $\beta$ 5 is expressed in many chicken tissues [Sullivan et al., 1986]).

To raise polyclonal antibodies against these six carboxy-terminal peptides, each peptide was cross-linked with glutaraldehyde to the carrier protein KLH and, after emulsifying with adjuvant, injected into rabbits (see Materials and Methods for details).

### Determination of the Specificities of the Anti-peptide Antibodies

To assess unambiguously whether antibodies that recognized

**CLASS I ISOTYPE - CONSTITUTIVE**

human hβ1	E E E E D F	<b>G E E A E E E A</b>
rat rbt.3	E E E E D F	<b>G E E A E E E A</b>
mouse mβ5	E E E E D F	<b>G E E A E E E A</b>

**CLASS II ISOTYPE - MAJOR BRAIN ISOTYPE**

chick cβ1/cβ2	D E Q G E F	<b>E E E G E E D E A</b>
pig	D E Q G E F	<b>E E E G E E D E A</b>
rat rbt.1	D E Q G E F	<b>E E E E G E D E A</b>
mouse mβ2	D E Q G E F	<b>E E E E G E D E A</b>

**CLASS III ISOTYPE - MINOR BRAIN ISOTYPE**

chick cβ4	E E E G E M Y E D D E E E S E	<b>~ Q G A K</b>
human hβ4	E E E G E M Y E D D E E E S E S	<b>Q G P K</b>

**CLASS IV ISOTYPE**

**variant IV A - BRAIN SPECIFIC**

rat rbt.2	E E G E F	<b>E E E A E E E V A</b>
human 5β	E E G E F	<b>E E E A E E E V A</b>
mouse mβ4	E E G E F	<b>E E E A E E E V A</b>

**variant IV B - DOMINANT TESTIS**

human hβ2	E E E G E F	<b>E E E A E E E V A</b>
mouse mβ4' (mβ3)	E E E G E F	<b>E E E A E E E V A</b>
chick cβ3	E E E G E F	<b>E E E A E E E A E</b>

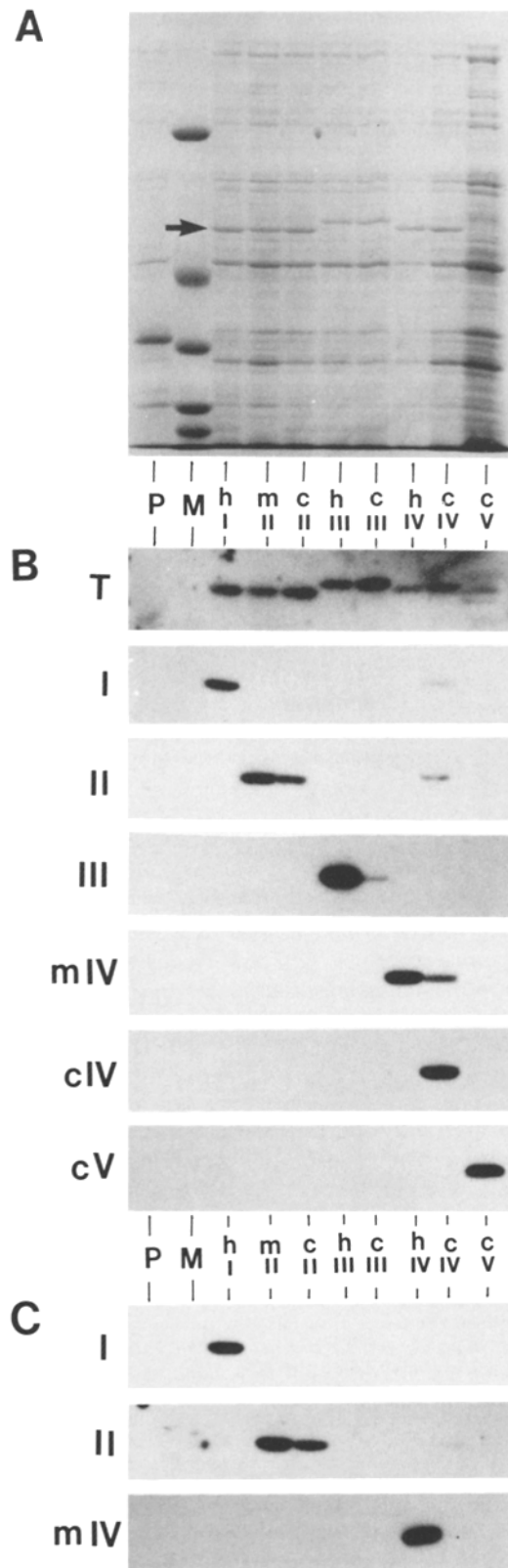
**CLASS cV ISOTYPE - UBIQUITOUS, EXCEPT BRAIN**

chick cβ5	N D G E E A F	<b>E D D E E E I N E</b>
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*Figure 1.* Carboxy termini of known vertebrate β-tubulin polypeptides. The one letter amino acid code has been utilized to show the extreme carboxyterminal sequences of known vertebrate β-tubulins. Except for cβ5 for which an homologous sequence has not been identified, sequences have been grouped into one of four evolutionarily conserved isotypic classes (adapted from Sullivan and Cleveland, 1986). Boxed regions delineate peptides chemically synthesized and used as immunogens for generating isotype-specific antibodies.

the appropriate isotype-defining carboxy terminus had been successfully generated using the peptide antigens, we isolated cloned DNA segments extending from amino acid codon 345 through to the translation termination codon for each β-tubulin isotype. These sequences were inserted into the bacterial *trpE* gene carried in the plasmid pATH 1 such that the plasmid encoded a hybrid protein consisting of an amino-terminal 32 kD of *trpE* linked to the carboxy-terminal ~100 β-tubulin residues. After transformation into an *E. coli* strain (CAG 456) carrying a mutation in the *lon* protease gene (Snyder et al., 1987), expression of the fusion proteins was induced with indole acrylic acid. SDS-PAGE of lysates of induced cultures (shown in Fig. 2 A) revealed that for fusion proteins carrying types I, II, III, mammalian IV (represented by a fusion containing the human isotype labeled *h-IV* in Fig. 2), or chicken IV (*c-IV*) carboxy termini, the fusion protein had accumulated to ~5% of bacterial proteins. For the last isotype, cβ5, the level of accumulation was consistently much lower (Fig. 2 A, lane *cV*), presumably the result of instability of this fusion protein in the bacterial host.

To test the specificities of antisera raised against each peptide, we electrophoresed our battery of fusion proteins (including both type II and type III variants) on eight parallel SDS-polyacrylamide gels. One gel, shown in Fig. 2 A, was



*Figure 2.* Specificity of binding of affinity-purified, peptide-derived antibodies to each β-tubulin isotype. (A) Coomassie Blue stain of an SDS-polyacrylamide gel of bacterial lysates containing induced, cloned fusion proteins, the amino-terminal region of which is the bacterial protein *trpE* and the carboxy-terminal domains are β-tubulin sequences beginning at β-tubulin residue 345. Lanes are loaded with: *P*, induced pATH vector alone; *M*, molecular mass

stained with Coomassie Blue. The remaining gels were blotted and each was probed with a different antibody. Part *T* of Fig. 2 *B* shows binding of a polyclonal antibody raised against chicken brain tubulin (Cleveland et al., 1981) and which was expected to react with all isoforms. As is evident, that expectation was, in fact, correct. Because whole sera gave higher backgrounds immunoblots (not shown), we first affinity-purified each antipeptide antibody using the appropriate peptide linked to Sepharose (see Materials and Methods). Immunoblots with these affinity-purified antibodies revealed:

**Type I.** Type I antibodies reacted strongly only with the type I-trpE fusion protein (Fig. 2 *B*, panel *I*). However, weak cross-reactivity was observed to the c-IV fusion protein. The only sequence common to type I and type c-IV that is not also common to other isotypes (which are not recognized by the antibody), is a single alanine that is the final residue in type I and the penultimate residue in type c-IV. Assuming an antibody binding site to consist of approximately five residues, we conclude that a major epitope recognized by the type I antisera contains AEEEA and that presence of the carboxy-terminal A is essential for binding (see Fig. 1).

**Type II.** Antibodies generated against the rat/mouse type II  $\beta$ -tubulin peptide strongly recognize both the rat/mouse fusion protein and the homologous (but not identical; see Fig. 1) chicken/pig type II fusion protein (Fig. 2 *B*, panel *II*). In addition, weaker binding is found to the chicken c-IV isoform, but not the m-IV isotype. No simple explanation for cross-reactivity unique to the c-IV isoform is apparent from inspection of the sequences (Fig. 1).

**Type III.** The type III antibody reacted uniquely with type III isoforms and did not recognize any of the other types (Fig. 2 *B*, panel *III*). Reaction with the human type III isotype was, however, much stronger than with the chicken type III. Because these two fusion polypeptides differ only in two residue positions (both within the terminal five amino acids), the principal epitope(s) recognized by the type III antibodies must lie within the extreme carboxy-terminal positions.

**Type IV.** The peptide-derived type m-IV antibodies reacted exclusively with type IV fusions, with strong staining of the mammalian type IV fusion protein (labeled *h-IV*) and weaker binding seen to the chicken c-IV isotope. This was in contrast to the antibody derived from injection of the c-IV peptide itself. That antibody reacted exclusively with the

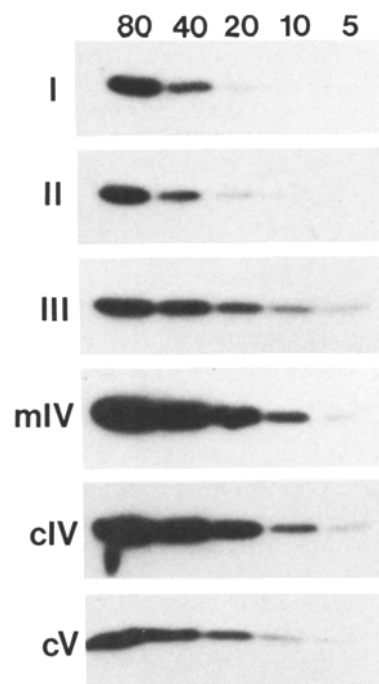
c-IV fusion protein (Fig. 2 *B*, panel *c-IV*). Because, like the type III example (above), the only sequence differences between the chicken IV and the mammalian IV fusion polypeptides are the final two carboxy-terminal residues, the c-IV antibody must exclusively recognize a determinant containing these residues.

**c-V.** Antibodies to the c $\beta$ 5 peptide (to be referred to henceforth as c-V) reacted exclusively with the c-V fusion protein (Fig. 2 *B*, panel *cV*). (The binding of this antibody to a fusion polypeptide of the appropriate size confirms the assignment of the minor polypeptide in Fig. 2 *A*, lane *cV*, as the c-V fusion protein.)

### Cross-reaction of Type I, II, and IV Antibodies Can Be Eliminated by Competition with the Cross-reacting Peptide

Because the goal of using peptide antigens was to prepare antibodies that bound solely to individual tubulin isoforms, the cross-reaction of types I and II antibodies to the chicken c-IV isotype was disappointing. Although this cross-reaction would be a significant problem only for analysis of isotype utilization in the chicken, we reasoned that such cross-reaction might be eliminated by competition of antibody binding in the presence of the excess c-IV peptide. To test this, we incubated parallel blots of cloned fusion proteins

markers (66, 45, 36, 29, and 22 kD); *hI*, human type I fusion; *mII*, mouse type II; *cII*, chicken type II; *hIII*, human type III; *cIII*, chicken type III; *hIV*, human type IV; *cIV*, chicken type IV; *cV*, chicken c-V. Arrow points to the position of the type hI fusion protein. (*B*) Autoradiograms of immunoblots of duplicate gels electrophoresed in parallel with that in *A*. *T*, general polyclonal antibody that recognizes all  $\beta$ -tubulin isotypes; *I*, type I antibody; *II*, type II antibody; *III*, type III antibody; *mIV*, type m-IV antibody; *cIV*, c-IV antibody; *cV*, c-V antibody. All peptide-derived antibodies were affinity purified except for anti-type III. (*C*) Immunoblots done in the presence of the c-IV peptide reduce c-IV cross-reaction of the type I, II, and IV antibodies. Autoradiograms of immunoblots of multiple gels electrophoresed in parallel with that in *A*. For each antibody used, antibody binding reaction was identical to that in *B* except that 3  $\mu$ M c-IV peptide was included to compete binding to the c-IV fusion protein. *I*, type I peptide antibody; *II*, type II antibody; *mIV*, m-IV antibody.



**Figure 3.** Quantitative immunoblot assay for detection of each of six  $\beta$ -tubulin isotypes. The concentrations of accumulated trpE/ $\beta$ -tubulin fusion proteins in lysates from induced bacteria were determined as in Materials and Methods. From those measurements, dilutions of each of the six fusion protein lysates were made to load 80, 40, 20, 10, and 5 ng of fusion protein on adjacent lanes of SDS-polyacrylamide gels and the resultant gels were immunoblotted with the peptide-derived antibodies. *I*, type I antibody; *II*, type II antibody; *III*, type III antibody; *mIV*, type m-IV antibody; *cIV*, type c-IV antibody; and *cV*, type c-V antibody. Panels represent overnight exposures except for *III* and *cV* which are 4-h exposures.

Table I. Utilization of Each  $\beta$ -Tubulin Isotype in CEF, 3T3, and CV1 Cells

Isotype	CEF cells				3T3 cells				CV1 cells			
	Amount of isotype in 40 $\mu$ g of cell protein ng	Portion of total $\beta$ -tubulin %	Portion of isotype in polymer %	Isotype	Amount of isotype in 40 $\mu$ g of cell protein ng	Portion of total $\beta$ -tubulin %	Portion of isotype in polymer %	Isotype	Amount of isotype in 40 $\mu$ g of cell protein ng	Portion of total $\beta$ -tubulin %	Portion of isotype in polymer %	Isotype
I	274	69	52	I	135	27	54	I	640	60	52	I
II	20*	5	ND	II	-†	-	-	II	-†	-	-	II
III	-§	-	-	III	-	-	-	III	-	-	-	III
IV	40	10	52	IV	270	53	52	IV	429	40	49	IV
c-V	62	16	48	c-V	100	20	50	c-V	-	-	-	c-V
Measured portion of total $\beta$ -tubulin in polymer (%)							53				52	
Calculated portion of total $\beta$ -tubulin in polymer (%)							51				52	

\* Measured with chicken type II fusion protein.

† Less than 10 ng (measured with human type II fusion protein).

§ Less than 30 ng (measured with chicken type III fusion protein).

|| Less than 3 ng (measured with human type III fusion protein).

with type I and type II affinity-purified antibodies, but this time in the presence of 3  $\mu$ M c-IV peptide. The outcome of that experiment is shown in Fig. 2 C. Clearly, the c-IV peptide had no discernible effect on binding of the specific type I or II antibodies, whereas it completely eliminated binding of the type I antibody to the c-IV fusion protein and severely diminished binding of the type II antibody to c-IV.

To test if peptide competition could eliminate cross-reaction between mammalian type IV and chicken type IV, a final blot of fusion proteins was probed with the m-IV antibody, again in the presence of 3  $\mu$ M c-IV peptide. The considerable cross-reactivity seen in the absence of competition (Fig. 2 B, panel *mIV*) was completely eliminated (Fig. 2 C, panel *mIV*).

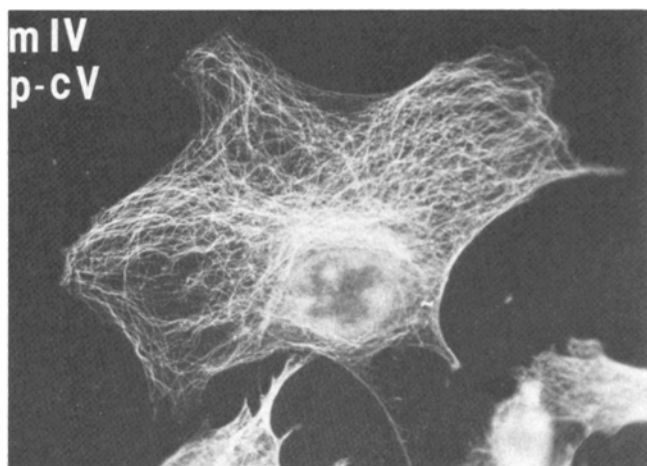
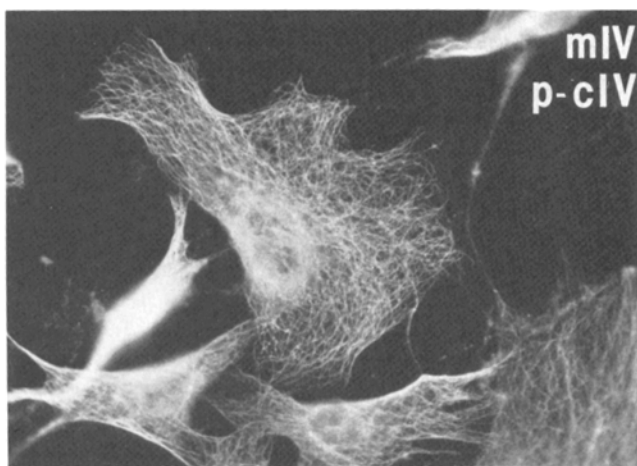
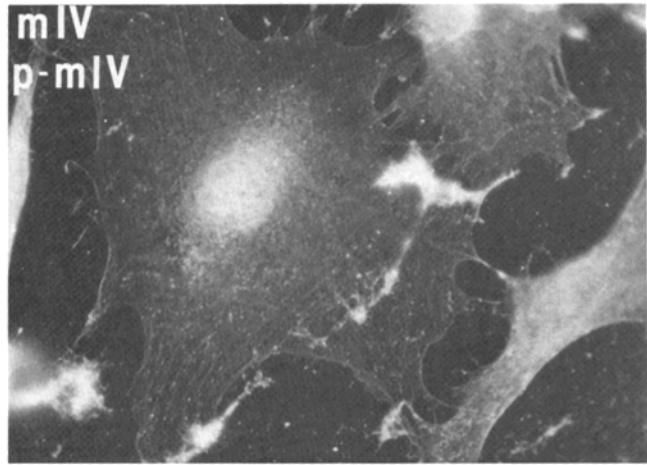
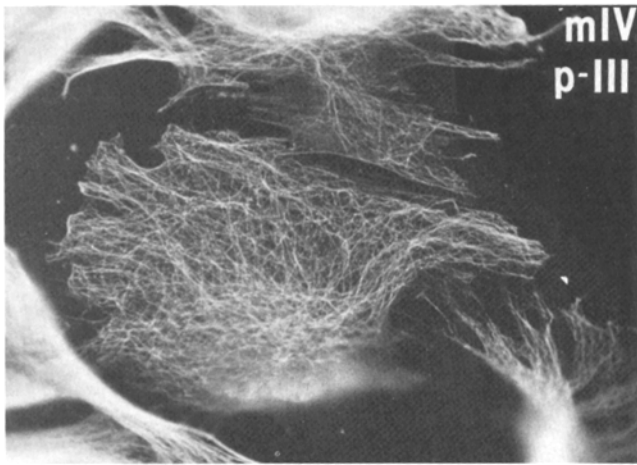
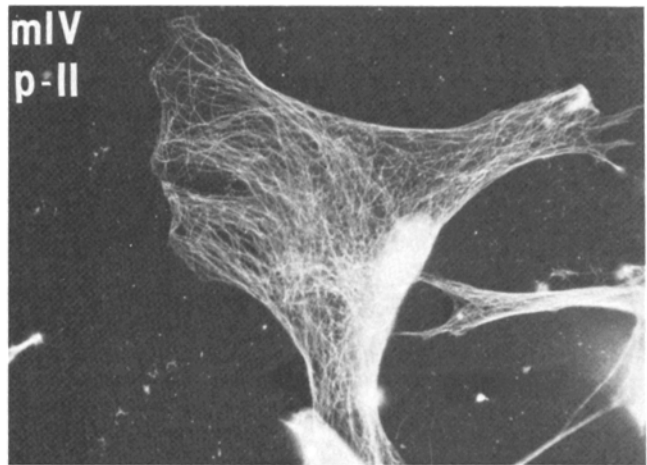
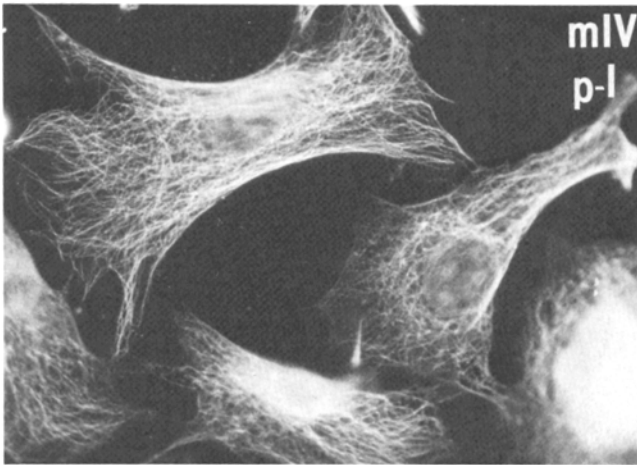
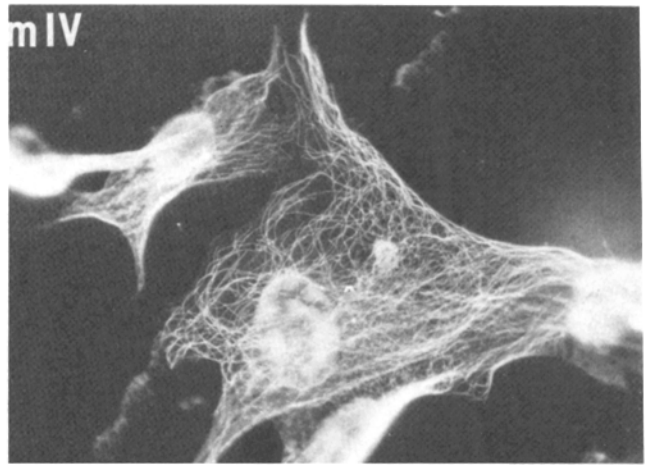
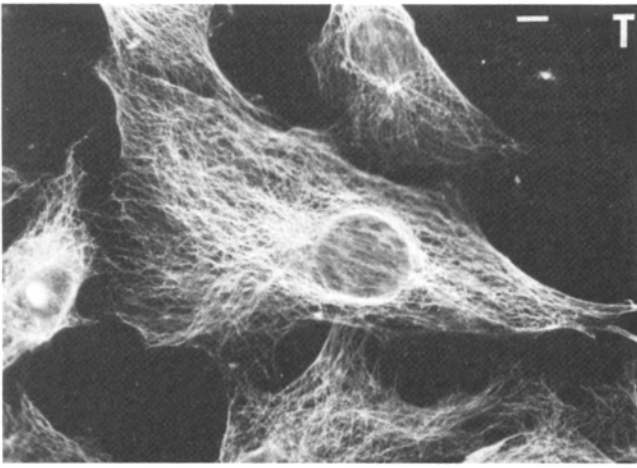
#### A Quantitative Blot Assay for Each of Six $\beta$ -Tubulin Isoforms

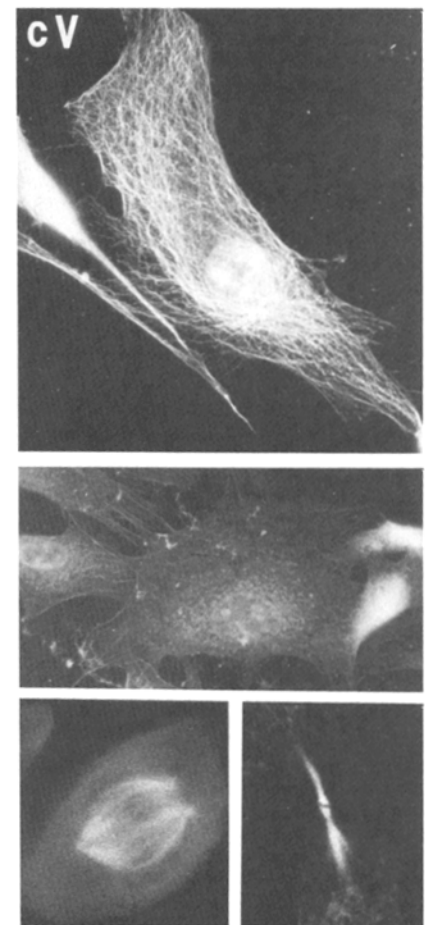
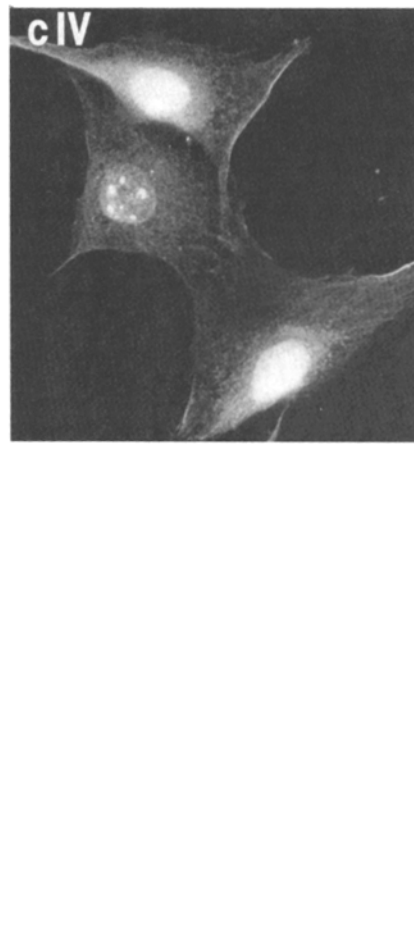
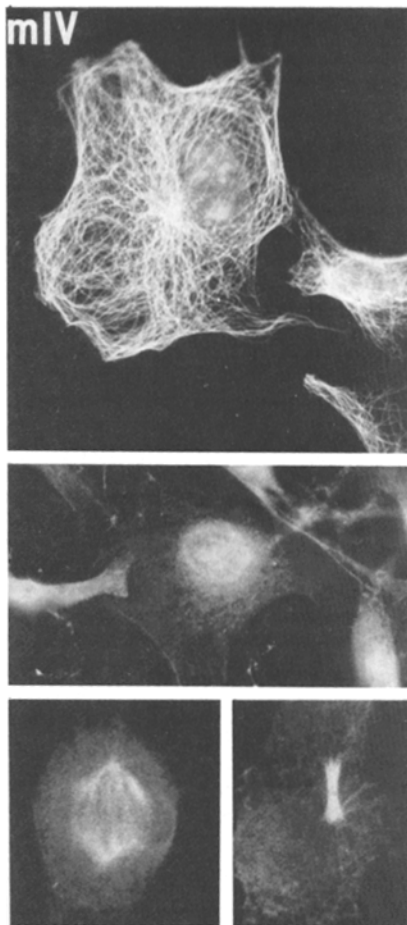
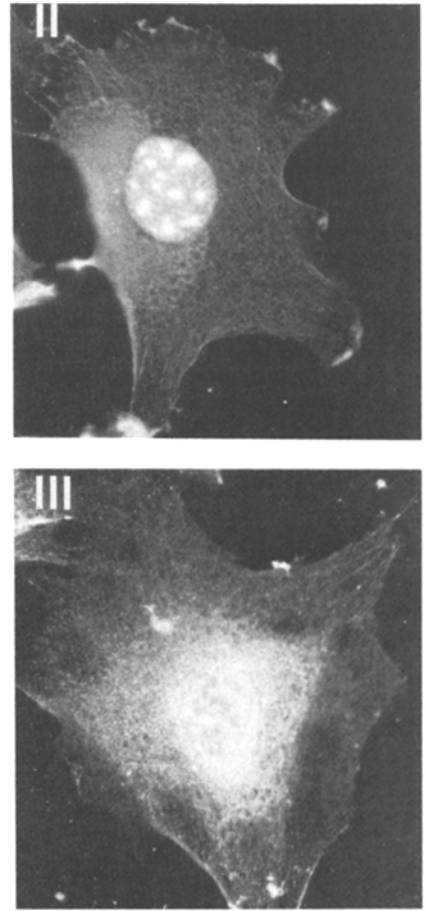
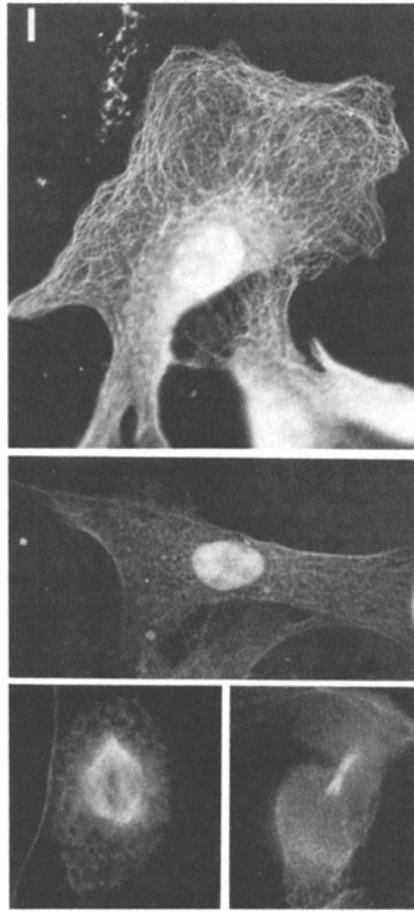
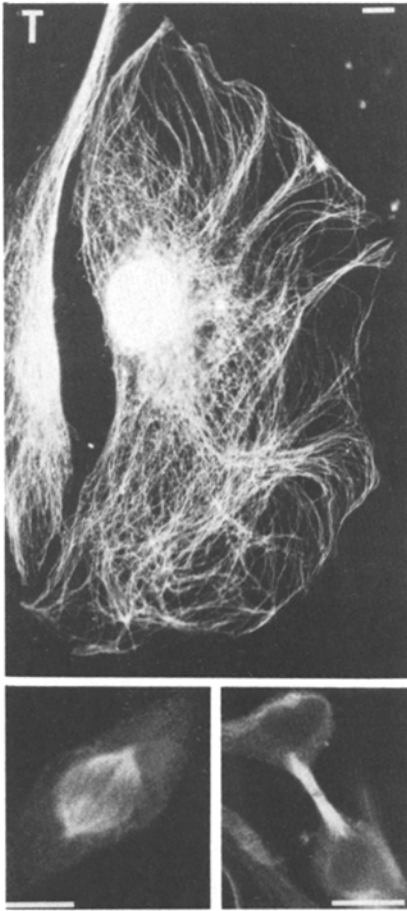
By electrophoresing serial dilutions of known amounts of cloned fusion protein lysates followed by immunoblotting (see Materials and Methods), we determined the sensitivity of each of our affinity-purified antibodies for recognition of the corresponding isotypes. Examples of those analyses are shown in Fig. 3. As might be anticipated, the six antibodies have different sensitivities of binding. For example, the type III, m-IV, c-IV, and cV antibodies could easily detect 5 ng of fusion protein, whereas the corresponding type I and II antibodies were approximately fourfold less sensitive. Nonetheless, for each antibody this protocol allowed a convenient method for quantitating levels of polypeptide type in a mixture of isoforms.

Using this immunoblot assay followed by densitometry, we analyzed the isotype composition of  $\beta$ -tubulins in 3T3 cells (an established line of mouse fibroblasts), secondary chicken embryo fibroblasts (CEF), and CV1 cells, a fibroblastic line of cells from monkey kidney. The quantitative results of that assay are presented in Table I. Types I and IV are the major isoforms in all of these fibroblastic cells, although the proportion of types I and IV are markedly different in each cell type. In addition, we found that the 3T3 cells contain an isotype that reacts with the anti-cV antibody (binding of which is blocked only by the c-V peptide). This was surprising because a gene that encodes such a polypeptide isotype has not yet been discovered outside of chicken. From these immunoblot results (in conjunction with immunofluorescent localization; see below) we conclude that mouse fibroblasts contain a c $\beta$ 5-like polypeptide isotype that comprises at least 20% of the  $\beta$  tubulins.

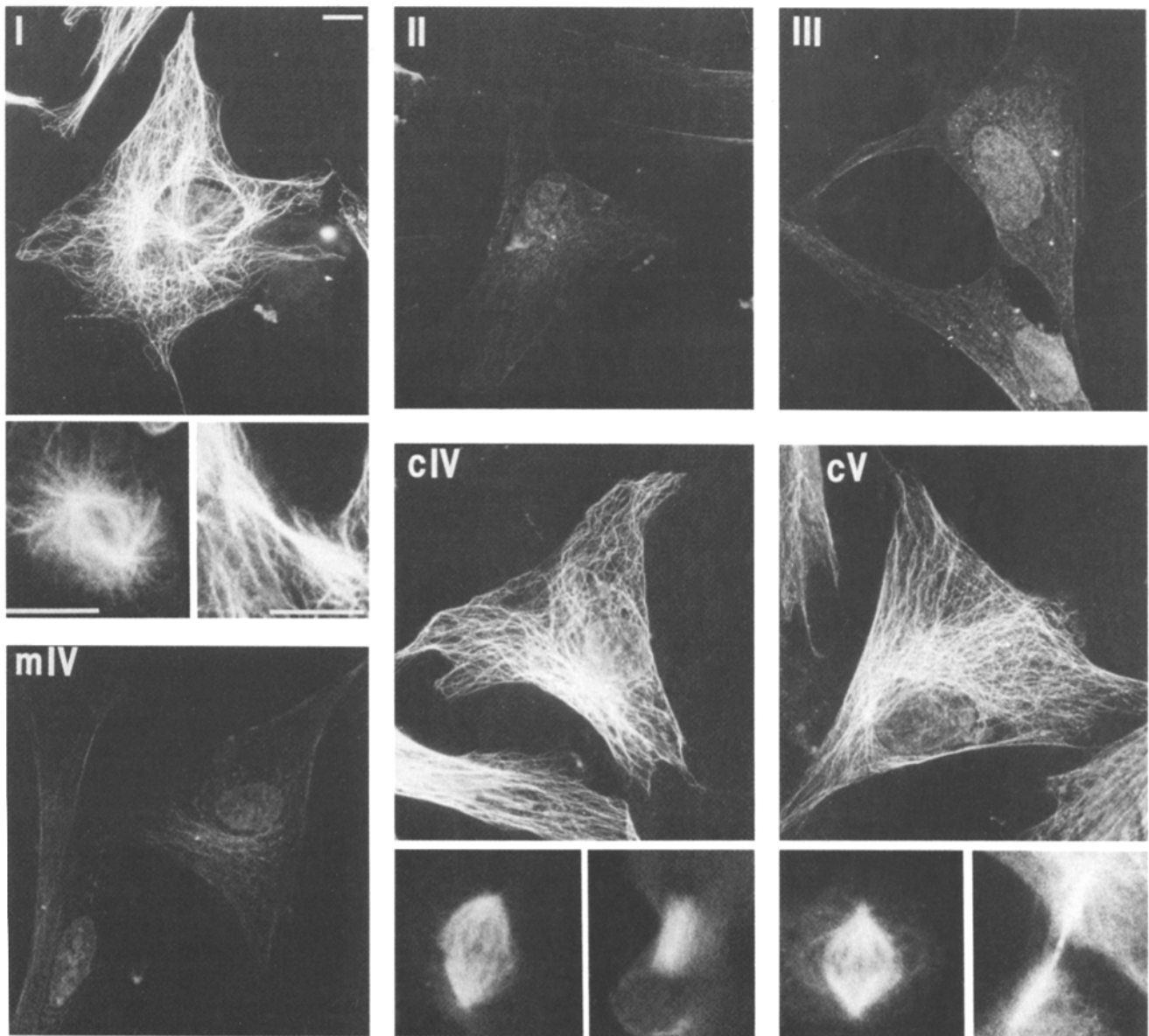
Figure 4. Specific binding of the anti-m-IV antibody to the m-IV isotype in 3T3 cell microtubules. Affinity purified, anti-m-IV antibody was used to stain microtubules in 3T3 cells using the technique of indirect immunofluorescence. Specificity of antibody detection of m-IV isotype in microtubules was determined by competing antibody binding by addition of peptides corresponding to each isotype. T, immunofluorescent staining of mouse 3T3 cells with an anti- $\beta$ -tubulin monoclonal antibody (Amersham Corp.); *mIV*, immunofluorescent staining of mouse 3T3 cells with the anti-isotype m-IV antibody. All other parts were anti-mIV staining but done in the presence of 3  $\mu$ M type I (*p-I*), type II (*p-II*), type III (*p-III*), type m-IV (*p-mIV*), type c-IV (*p-cIV*), or type cV (*p-cV*) peptides. Bars, 10  $\mu$ m.











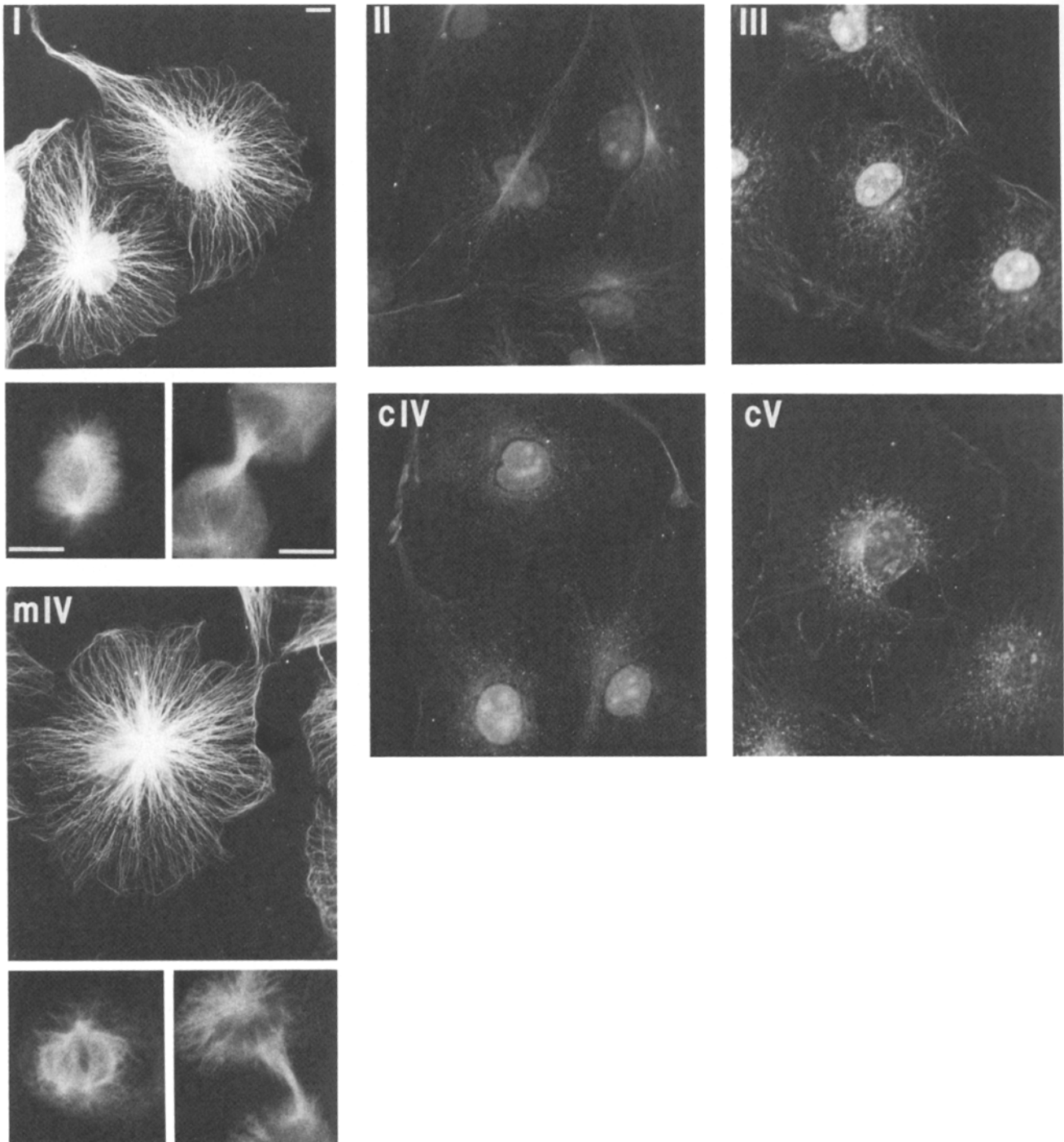
**Figure 6.** Detection of  $\beta$ -tubulin isotypes in CEF cell microtubules using indirect immunofluorescence. Isotypes present in CEF cells were detected by indirect immunofluorescence using the anti-peptide antibodies. *I*, antibody to type I  $\beta$ -tubulin; *II*, antibody to type II; *III*, antibody to type III; *mIV*, antibody to type m-IV; *cIV*, antibody to type c-IV; and *cV*, antibody to type c-V. Lower two sections of *I*, *cIV*, and *cV* display mitotic spindles and midbodies stained with the anti-type I, c-IV and c-V antibodies, respectively. All peptide antibodies except type III were affinity purified. Bars, 10  $\mu$ m.

### **The Peptide Antibodies Bind to Specific $\beta$ -Tubulin Isotypes in Microtubules**

Having demonstrated that the peptide-derived antibodies bind specifically to the corresponding isotypes on protein blots, we next determined whether the antibodies could also react with the appropriate isotypes when assembled into

microtubules. To do this, we first stained 3T3 cells with the anti-m-IV antibody corresponding to an isotype known to represent >50% of cell  $\beta$  tubulins (see Table I). Antibody binding was observed by indirect immunofluorescence. As shown in Fig. 4, panel *mIV*, interphase microtubule arrays showed extensive staining by the anti-m-IV antibodies. (In-

**Figure 5.** Detection of  $\beta$ -tubulin isotypes in mouse 3T3 cell microtubules using indirect immunofluorescence. Isotypes present in 3T3 cell microtubules were detected by indirect immunofluorescence using the anti-peptide antibodies. *T*, polyclonal antibody raised against chicken brain tubulin; *I*, antibody specific to type I; *II*, antibody to type II; *III*, antibody to type III; *mIV*, antibody to type m-IV; *cIV*, antibody to type c-IV; and *cV*, antibody to type c-V. Middle section of *I*, *mIV*, and *cV* display staining patterns when antibody binding is done in the presence of the 3  $\mu$ M type I, type m-IV or type c-V peptides, respectively. Bottom two sections of *T*, *I*, *mIV*, and *cV* display mitotic spindle and midbody staining, respectively. All peptide antibodies except type III were affinity purified. Bars, 10  $\mu$ m.

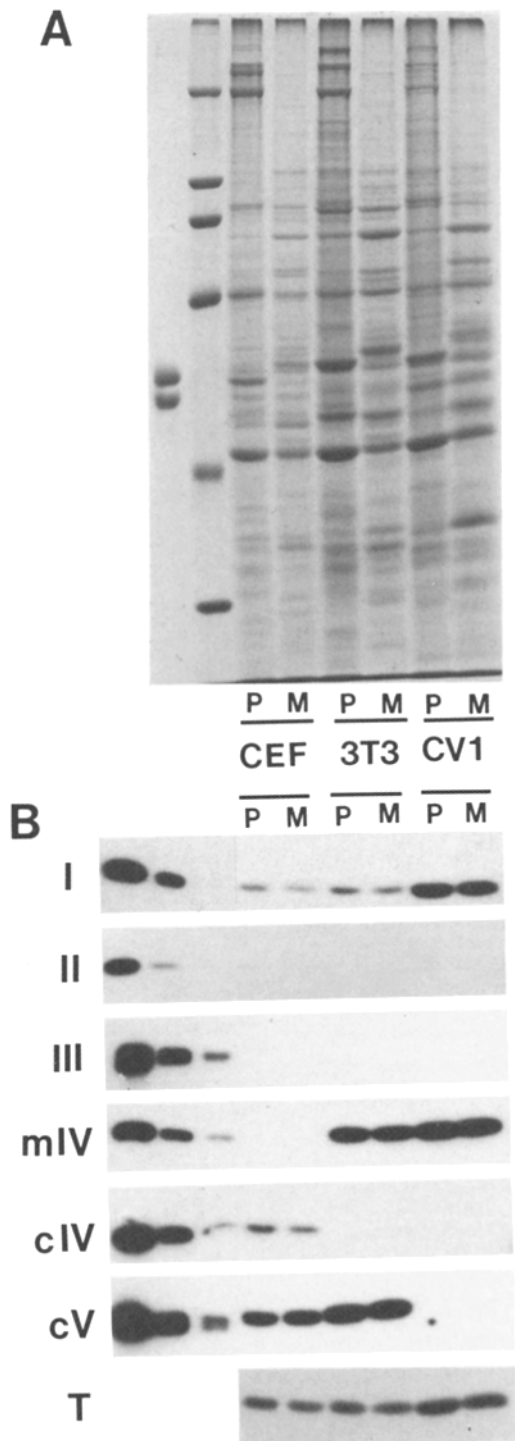


**Figure 7.** Detection of  $\beta$ -tubulin isotypes in CV1 cell microtubules using indirect immunofluorescence. Isotypes present in CV1 cells were detected by indirect immunofluorescence using the antipeptide antibodies. *I*, antibody to type I  $\beta$ -tubulin; *II*, antibody to type II; *III*, antibody to type III; *mIV*, antibody to type m-IV; *cIV*, antibody to type c-IV; and *cV*, antibody to type c-V. Lower two sections of *I* and *mIV* display staining of a mitotic spindle and a midbody with the anti-type I and type m-IV antibodies, respectively. All peptide antibodies except type III were affinity purified. Bars, 10  $\mu$ m.

deed, qualitatively the pattern of staining was indistinguishable from that seen with a  $\beta$ -tubulin monoclonal antibody [Fig. 4, panel *T*].) To test whether such binding represented specific binding to the appropriate isotype, we repeated the staining procedure on parallel aliquots of cells but this time added to each antibody binding mixture 3  $\mu$ M of one of the various peptides. As seen in Fig. 4, panel *m-IV/p-mIV*, addi-

tion of the type m-IV peptide to the type m-IV antibody completely blocked microtubule staining, whereas addition of any of the peptides for the other isotypes left staining undiminished (panels *p-I* through *p-cV*).

Analogous blocking experiments for each of the other anti-isotype antibodies revealed that staining was always blocked only by addition of the correct peptide (see below).



**Figure 8.** Quantitative analysis of soluble and polymer levels of each  $\beta$ -tubulin isotype in CEF, 3T3, and CV1 cells. Soluble tubulin and polymeric tubulin were prepared from dishes of CEF, 3T3, and CV1 cells (as detailed in Materials and Methods). Equivalent fractions of soluble and polymeric samples were electrophoresed on six gels (40  $\mu$ g of total cell protein was analyzed,  $\sim$ 50% of which was in the polymeric fraction). (A) Soluble and polymeric fractions as visualized by Coomassie Blue staining. The two lanes at the left display purified brain tubulin and molecular mass standards (205, 116, 97, 66, 45, and 29 kD), respectively. (B) To quantify the amounts of each isotype in each sample, serial dilutions of a bacterial lysate containing a specific trpE- $\beta$ -tubulin fusion protein were also loaded on parallel gels. The gels were blotted and incubated with

We conclude that the antibodies retain their isotype-specificity when binding to intact microtubules.

### *In Vivo Colocalization of Isotypes I, IV, and c-V in Interphase, Mitotic, and Midbody Microtubules*

We next sought to determine which  $\beta$ -tubulin isotypes were assembled in the various classes of microtubules distinguishable within an animal cell by light microscopy. For this, we stained microtubules in each of three different cultured cells (3T3, CEF, and CV1 cells).

The staining of 3T3 cells with each of the six isotype antibodies is shown in Fig. 5. As expected from the protein blotting experiment (Table I), antibodies to isotypes I, m-IV, and c-V stained microtubules (parts *I*, *mIV*, and *cV*), as did a tubulin polyclonal antibody (part *T*) that recognizes all isotypes (see Fig. 2A). No microtubule staining was observable for isotypes II, III, and c-IV. (For each peptide antibody, microtubule staining is specific to the appropriate isotype inasmuch as binding is completely blocked by addition of the corresponding peptide [see middle section of panels *I*, *mIV*, and *cV*].) Moreover, what is readily apparent is that each isotype is present in an apparently uniform distribution in all classes of microtubules (interphase, spindle, and midbody).

Analysis of microtubules in CEF and CV1 cells yielded results qualitatively similar to those from the 3T3s. All isotypes demonstrated to be present by immunoblot analysis (types I, c-IV, and c-V in CEFs and types I and IV in CV1s) were present in interphase, mitotic, and midbody microtubules (Fig. 6 and 7). Moreover, except for the possibility that in CEF cells astral microtubules may be enriched in the type I tubulin (see Fig. 6, panel *I*), no preferential utilization of any isotype was apparent for any microtubule class. (We believe that the stronger staining of astral fibers by anti-type I antibody reflects the large percentage of type I  $\beta$ -tubulin present [see Table I] and that the mitotic cell in Fig. 6, panel *I* represents a cell earlier in the cell cycle than those in Fig. 6, panels *cIV* and *cV*.)

That each antibody stains the entire repertoire of microtubules is further supported by double-immunofluorescence experiments. For such experiments, each peptide antibody was used simultaneously with a  $\beta$ -tubulin monoclonal antibody. No microtubules could be identified that were detected by the monoclonal but failed to stain with an isotype specific antibody (data not shown).

### *Quantitative Analysis of $\beta$ -Tubulin Isotypes in Cultured Animal Cells: Similar Interphase Monomer/Polymer Ratios for Each Isotype*

To determine whether an isotype (or isotypes) was preferentially utilized for microtubule assembly, the identities and

the appropriate antipeptide antibodies. Antibody binding was detected with  $^{125}$ I-protein A. *P*, polymeric tubulin; *M*, monomeric (soluble) tubulin; *I*, anti-type I-specific antibody; *II*, anti-type II-specific antibody; *III*, anti-type III-specific antibody; *mIV*, anti-type m-IV-specific antibody; *cIV*, anti-type c-IV antibody; *cV*, anti-type c-V antibody; *T*, monoclonal antibody that reacts with all isotypes. The first three lanes of *II*, *III*, *mIV*, *cIV*, and *cV* were loaded with 200, 60, and 20 ng of the corresponding cloned fusion protein. (Type III fusion protein was the human protein.) For *I*, fusion protein amounts were 600, 200, and 60 ng. All peptide antibodies except type III were affinity purified.

amounts of each  $\beta$ -tubulin isotype in the soluble and polymeric form in a variety of cultured animal cells were determined by quantitative immunoblotting. By gently lysing aliquots of cells under conditions that stabilize microtubules (Solomon et al., 1979; Osborn and Weber, 1982), we first fractionated cellular proteins into soluble and cytoskeletal fractions. Equivalent proportions of each fraction were electrophoresed along with appropriate levels of cloned fusion proteins corresponding to each  $\beta$  tubulin isotype. A gel of soluble and polymer fractions stained with Coomassie Blue is shown in Fig. 8 A. Immunoblots of gels run in parallel are shown in Fig. 8 B and the amount of each isotype in soluble and polymer forms is quantified in Table I. In all three cell types, just over 50% of cell tubulin is in the polymeric form (in agreement with measurements for fibroblasts by previous investigators [e.g., Hiller and Weber, 1978; Caron et al., 1985]). Moreover, a similar level of each isotype present is in the assembled form, although quantitatively a slightly lower (but reproducible) percentage of isotype c-V was assembled in both CEF and 3T3 cells.

## Discussion

The preparation of antibodies that recognize specific  $\beta$ -tubulin isotypes has permitted both quantitation of the amount of each isoform in the soluble and polymer forms and direct localization of each by indirect immunofluorescence microscopy. Several important observations emerge from such analyses.

First, the finding that each antibody appears to stain the entire repertoire of microtubules provides strong evidence that most, if not all, cellular microtubules are assembled as copolymers of available isotypes. This appears to be true for microtubules that comprise the interphase cytoplasmic array, for mitotic microtubules, and for microtubules within the midbody. This hypothesis is further supported by double-immunofluorescence experiments (not shown). Although it remains possible that a small fraction of microtubules may be composed exclusively or preferentially of a subset of available isotypes, it is nonetheless clear that in the examples examined thus far the majority of in vivo microtubules are copolymers of isotypes.

Secondly, not only are microtubules of divergent cellular functions composed largely (if not exclusively) of copolymers of various isotypes, we have been unable to detect any preferential utilization of any particular isoform in any subset of microtubules. Although the limited resolution of light microscopy may obscure subtle biases for incorporation of an isotype into some microtubule subset, no enrichment of any isotype is apparent in any microtubule class. Neither can any marked preferential utilization be detected by the proportion of each isoform that is assembled into polymer (although a slightly lower level of assembly of the c-V isotype was detected).

Thirdly, although we have previously noted the marked evolutionary conservation of the primary sequences and patterns of expression of each of four  $\beta$ -tubulin isotypic classes (Sullivan and Cleveland, 1986), our present quantitative analysis of isotypes present in chicken, mouse, and monkey fibroblasts show markedly different levels of isotypes (Table I). Although this may in part reflect differences between the established 3T3 line and the secondary CEFs, this observa-

tion is nonetheless difficult to reconcile fully with the notion that tubulin isotypes confer novel functional properties to the assembled microtubule. If tubulin isotypes contributed unique, albeit subtle, functional properties to microtubules, how is it that very different levels of each isoform are permissible in similar cell types from different species?

In conjunction with a similar analysis from Cowan's group that employed isotype-specific antisera and gene transfection (published while this manuscript was under review [Lewis et al., 1987]) and with the companion paper (Joshi et al., 1987) in which we demonstrate that the very divergent chicken erythrocyte  $\beta$ -tubulin (c $\beta$ 6) (which is normally assembled into marginal band microtubules in red blood cells) freely coassembles with other isotypes both in its normal erythrocyte environment and when expressed inappropriately in other cell types, these observations further support a variety of preceding genetic and biochemical evidence that multiple tubulin isotypes do not contribute to specifying essential functional distinctions among microtubule classes. The earliest (and in many ways most compelling) prior evidence came from Raff and co-workers (Kemphues et al., 1982), who demonstrated in *Drosophila* that all microtubules in developing spermatids (including microtubules involved in mitosis, meiosis, flagella assembly, and nuclear shaping) were all composed principally of a single multifunctional  $\beta$ -tubulin isotype. This work was followed by two divergent experiments from Solomon and co-workers that cast additional doubt as to the functional divergence of tubulin isotypes. These efforts included demonstration that an unusual hybrid  $\beta$ -tubulin (composed of a chicken amino terminus linked to a yeast carboxy terminus) coassembled into all classes of 3T3 cell microtubules that are distinguishable by light microscopy (Bond et al., 1986). In addition, in vitro reassembly experiments showed that the divergent erythrocyte  $\beta$ -tubulin (c $\beta$ 6) could be replaced by brain tubulin subunits in marginal band microtubules (Swan and Solomon, 1984).

On the other hand, we (Sullivan and Cleveland, 1986; Cleveland, 1987) and others (Villasante et al., 1986) have argued that the interspecies conservation of isotypic sequences and programs of expression of each isotype support functional differences among isotypes. Further, direct in vitro biochemical evidence has demonstrated marked differences in assembly properties of brain tubulins and the erythrocyte c $\beta$ 6 tubulin (Rothwell et al., 1986). Indeed, we have shown in a forthcoming article (Joshi et al., 1987) that the c $\beta$ 6 isotype is preferentially assembled into more stable erythrocyte microtubules. In addition, proteolytic removal of the isotype defining carboxy-terminal domain sequences yields subunits that are still competent to assemble in vitro, but with altered properties (Brattacharyya et al., 1985; Sackett et al., 1985; Serrano et al., 1984a, b). When coupled with evidence that the carboxy-terminal domain may be directly involved in binding some microtubule-associated proteins (Serrano et al., 1984b), these results suggest that tubulin assembly characteristics may be modulated in vivo by preferential binding of individual microtubule-associated proteins to particular isotypes.

How can these paradoxical findings be reconciled? Three possibilities seem most reasonable. First, the comingling of various isotypes may not be universal and in vivo examples of isotype segregation may yet emerge. Secondly, the coas-

sembly of various isotypes does not preclude the possibility that individual subunits may still provide preferential (or exclusive) binding sites for specialized microtubule-associated proteins. In this view, divergent functional characteristics of specialized microtubules would thus be established indirectly through associated protein binding to specific isotypes. Thirdly, despite some altered in vitro properties and evolutionary conservation of various isotypes, there may be no functional difference in vivo between some (or all) of the tubulins. The conservation in sequence may simply reflect slow ticking of the evolutionary clock since the time of gene duplication.

In any event, if significant differences among tubulin isotypes do exist, they must be subtle and manifest only in conjunction with other specific microtubule components.

We gratefully acknowledge the enthusiastic support and advice of Dr. Kevin F. Sullivan during the early stages of this work. We thank Margarete Heck for technical assistance in preparing CEF cells. This work has been supported by grants from the National Institutes of Health and March of Dimes to Dr. Cleveland, who is also the recipient of a National Institutes of Health Research Career Development Award.

Received for publication 26 May 1987, and in revised form 22 July 1987.

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