Generation of Antisera That Discriminate among Mammalian α-Tubulins: Introduction of Specialized Isotypes into Cultured Cells Results in Their Coassembly without Disruption of Normal Microtubule Function

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Abstract. To assay the functional significance of the multiple but closely related α-tubulin polypeptides that are expressed in mammalian cells, we generated three specific immune sera, each of which uniquely recognizes a distinct α-tubulin isotype. All three isotypes are expressed in a tissue-restricted manner: one (Mα3/7) only in mature testis, one (Mα4) mainly in muscle and brain, and the third (Mα6) in several tissues at a very low level. A fourth specific antiserum was also generated that distinguishes between the tyrosinated and nontyrosinated form of a single α-tubulin isotype. Because individual tubulin isotypes cannot be purified biochemically, these sera were raised using cloned fusion proteins purified from host *Escherichia coli* cells. To suppress the immune response to shared epitopes, animals were first rendered tolerant to fusion proteins encoding all but one of the known mammalian α-tubulin isotypes. Subsequent challenge with the remaining fusion protein then resulted in the elicitation of an immune response to unique epitopes. Three criteria were used to establish the specificity of the resulting sera: (*a*) their ability to discriminate among cloned fusion proteins representing all the known mammalian α-tubulin isotypes; (*b*) their ability to uniquely detect α-tubulin in whole extracts of tissues; and (*c*) their capacity to stain microtubules in fixed preparations of cells transfected with sequences encoding the corresponding isotype. The transfection experiments served to demonstrate (*a*) the coassembly of Mα3/7, Mα4, and Mα6 into both interphase and spindle microtubules in HeLa cells and NIH 3T3 cells, and (*b*) that the Mα4 isotype, which is unique among mammalian α-tubulins in that it lacks an encoded carboxy-terminal tyrosine residue, behaves like other α-tubulin isotypes with respect to the cycle of tyrosination/detyrosination that occurs in most cultured cells.

MICROTUBULES mediate a broad range of both essential and specialized biological functions in the cells of higher eukaryotes. These functions include, for example, the segregation of chromosomes during meiosis and mitosis; the maintenance of cell shape via dynamic modulation of cytoplasmic microtubules; cell motility, as an integral part of cilia and flagella; and intracellular transport, involving the bidirectional shunting of organelles along axons. Several possible factors could contribute to this functional diversity. First, the two major soluble microtubule proteins, the α- and β-tubulins, are each encoded by multigene families (Cleveland et al., 1980; Lee et al., 1983). In mammalian species, these multigene families encode about six α- and β-tubulin polypeptides, termed isotypes, that are distinct from one another by virtue of one or more amino acid substitutions in the polypeptide chain (Hall et al., 1983; Lewis et al., 1985*b*; Villasante et al., 1986; Wang et al., 1986; Sullivan and Cleveland, 1986). In principle, therefore, genetically encoded differences between tubulin isotypes could contribute to microtubule function. Second, in addition to the α- and β-tubulins, microtubules possess accessory proteins (microtubule-associated proteins) whose number and composition vary among functionally distinct kinds of microtubule (e.g., Huber and Matus, 1984; Bloom et al., 1984; Parysek et al., 1984; Binder et al., 1985). Third, there are populations of microtubules in which the tubulin proteins themselves undergo specific posttranslational modification (e.g., L'Hernault and Rosenbaum, 1983; Barra et al., 1974; Raybin and Flavin, 1977; Argarana et al., 1978). However, the extent to which these (and most likely other) determining factors contribute to the diversity of microtubule function remains essentially unknown.

An intriguing paradox exists with regard to the functional significance of the expression of multiple α- and β-tubulin isotypes. Our analysis of these isotypes in mouse (Lewis et al., 1985*b*; Villasante et al., 1986; Wang et al., 1986) and man (Hall et al., 1983; Cowan et al., 1983; Lee et al., 1983; Lee et al., 1984; Lewis et al., 1985*a*) taken together with data

from other mammalian species (Elliot et al., 1986; Farmer et al., 1984) shows that, in general, their characteristic sequences (in particular, a heterogeneous region spanning the 15 carboxy-terminal amino acids) as well as their patterns of expression have been rigidly conserved since the mammalian radiation. A single major exception to this interspecies conservation rule, namely the 10% sequence divergence of a hematopoietic-specific β-tubulin isotype since the mammalian radiation (Cowan et al., 1987), merely serves to underlie the extent of the selective pressure operating on the other isotypes. An obvious possible explanation for the interspecies conservation of isotype sequences would be that different tubulin isotypes themselves contribute to the functional distinctions among microtubules by a mechanism involving either segregation or selective enrichment. However, neither genetic experiments on lower eukaryotic species nor the development of antisera that discriminate among several vertebrate β-tubulin isotypes have provided evidence to support this idea. For example, in *Drosophila*, studies on mutations in a single β-tubulin gene have shown that this isotype contributes to all the many types of microtubule involved in spermatogenesis in that organism (Kemphues et al., 1982; Fuller et al., 1987). In yeast, although the two α-tubulin genes in *Saccharomyces cerevisiae* are 10% divergent (Schatz et al., 1986*a*), each isotype on its own can support all the microtubule functions involved in yeast cell growth and division (Schatz et al., 1986*b*) while one of the two α-tubulins in the yeast *Schizosaccharomyces pombe* is nonessential (Adachi et al., 1986). In *Aspergillus nidulans* the β-tubulin gene expressed in vegetative cells can substitute for that expressed in conidiation (May et al., 1985; Weatherbee et al., 1985). Finally, experiments with β-tubulin isotype-specific antisera have shown that at least in cultured mammalian cells, microtubules are mixed copolymers of all expressed β-tubulin isotypes (Bond et al., 1986; Lewis et al., 1987; Lopata and Cleveland, 1987).

The patterns of expression of β-tubulin isotypes are not paralleled by corresponding patterns among α-tubulin isotypes; that is, α- and β-tubulin isotypes are not expressed in pairs. This lack of correspondence, plus the fact that α-tubulin is capable of undergoing two unique kinds of post-translational modification (i.e., acetylation of lysine residues [e.g., L'Hernault and Rosenbaum, 1985*a*] and cycles of carboxy-terminal tyrosination and detyrosination [e.g., Gundersen et al., 1987]), prompted us to examine whether mammalian α-tubulin isotypes, like their β-tubulin counterparts, freely assemble into functionally distinct microtubules. Here we report the generation of antisera that discriminate among several distinct mammalian α-tubulin isotypes, and their use to analyze microtubules formed after their introduction into tissue culture cells by transfection.

Materials and Methods

Engineering of Constructs Designed to Express α-Tubulin Isotypes

To express α-tubulin isotypes as cloned fusion proteins in *Escherichia coli*, 3′ Eco RI fragments from cDNAs encoding Mα1, Mα7, Mα4, and Mα6 (Villasante et al., 1986) were cloned into the Eco RI site of the inducible expression vector pATH11 (generously provided by T. Koerner). The fusion proteins expressed by these constructs are diagrammed in Fig. 1 *A*. Two of these constructs were altered by site-directed mutagenesis and are shown in Fig. 1 *B*. A mismatched antisense oligonucleotide 5′AGGCTCCAAGTAGCCTAGTACTCTTCTTCTCCCTC3′ was synthesized and used by the procedure of Kunkel (1985) to add a tyrosine codon to the carboxy terminus of the trpE/Mα4 fusion protein, while the antisense oligonucleotide 5′CCCATGCGCTCACTACTCCTCCCCT3′ was used to change the encoded carboxy-terminal tyrosine residue of the trpE/Mα3/7 construct to a stop codon. (Mismatched residues are underlined in the oligonucleotide sequences.) The fusion protein sequences were altered through the cloning of a 250-bp Sph I–Bam HI fragment from each construct into M13, and checked by dideoxy sequencing (Sanger et al., 1980).

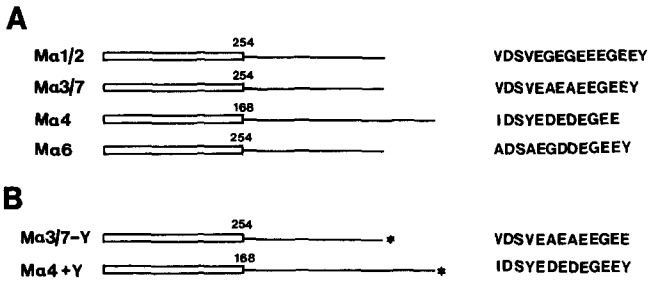


Figure 1. Fusion proteins used for the generation of mammalian α-tubulin isotype-specific sera. (*A*) Cloned cDNA sequences encoding mammalian α-tubulin isotypes extending 3′ to either amino acid 168 (Mα4) or amino acid 254 (Mα1/2, Mα3/7, Mα6) (*solid lines*) were fused to sequences encoding the *E. coli* trp E protein (*open boxes*) in vectors designed to express the fusion protein upon induction in host *E. coli* cells (see Materials and Methods). Mα1/2 represents both isotypes Mα1 and Mα2 (Villasante et al., 1986), since these isotypes differ only at amino acid 232, which is not included in the fusion protein. Mα3/7 represents a single isotype encoded by two testis-specific α-tubulin transcripts, Mα3 and Mα7 (Villasante et al., 1986). (*B*) Sequences encoding Mα3/7 and Mα4 were modified by site-directed mutagenesis to remove the encoded carboxy-terminal tyrosine residue (to generate Mα3/7–Y) or add an encoded carboxy-terminal tyrosine residue (to generate Mα4+Y). In each case, the expressed fusion proteins were purified from whole bacterial extracts and used as either tolerogens or immunogens for the elicitation of a specific immune response. The 13–15 carboxy-terminal amino acids that are characteristic of each isotype are shown at the right. Asterisks mark the location of the deleted (Mα3/7–Y) or added (Mα4+Y) encoded tyrosine residue in engineered constructs.

To express α-tubulin isotypes in cultured cells, full-length cDNAs encoding Mα3/7, Mα4, and Mα6 (Villasante et al., 1986) were inserted into the eukaryotic expression vector pSV-dhfr (Mulligan and Berg, 1981), replacing the dhfr-encoding sequences. Hybrid Mα3/7/Mα6 constructs were made by substituting either the 5′ or 3′ Eco RI fragment of the Mα6 cDNA in the pSV expression plasmid with the homologous fragment from the Mα7 cDNA. In all these constructs, tubulin cDNA sequences are flanked by SV-40 T-antigen promoter and terminator sequences. Transfection was by the method of Wigler et al. (1979).

Generation of α-Tubulin Isotype-Specific Sera

Five of the six cloned fusion proteins described above were used either as tolerogens or as immunogens to raise isotype-specific antisera in rabbits and guinea pigs. The method was as described in Lewis et al. (1987) with two modifications: (*a*) the cyclophosphamide dose used for guinea pigs was 75 mg/kg on 3 successive days, and (*b*) the animals received two (rather than one) injections of immunogen in Freund's complete adjuvant with a 2-wk interval before the final boost.

Affinity purification of the sera was accomplished by absorption onto and elution from nitrocellulose strips cut from Western blots of SDS gels heavily overloaded with whole extracts of *E. coli* expressing the appropriate trpE/α-tubulin isotype fusion protein. Briefly, the procedure was as follows. Strips were cut into small squares and blocked for 0.5 h in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 3% BSA and 0.04% Na azide. Approximately 1 ml of whole serum was incubated

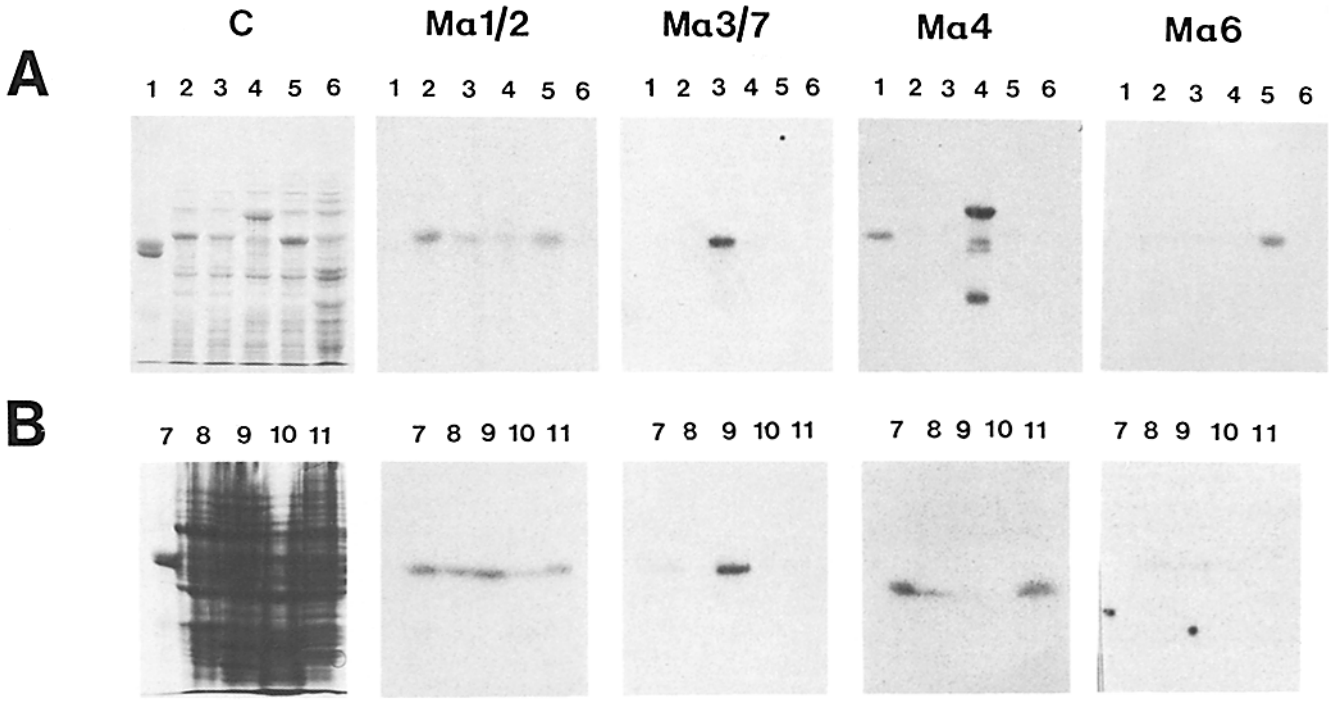


Figure 2. Specificity of antisera to mammalian α-tubulin isotypes. Cloned sequences encoding the carboxy-terminal portions of Mα1/2, Mα3/7, Mα4, or Mα6 were fused to sequences encoding *E. coli* trp E (see Fig. 1) and expressed in host *E. coli* cells. The resulting fusion proteins were purified and used to generate affinity-purified isotype-specific antisera (see Materials and Methods). The specificity of each of these antisera was tested against whole extracts of bacteria expressing all four fusion proteins (*A*) and against whole extracts of mouse tissues (*B*). *Column C*, Coomassie Blue stain of 8.5% SDS-polyacrylamide gels. Lanes *1* and *7*, marker bovine brain tubulin (5 μg). Lanes *2–6*, bacterial extracts expressing Mα1/2 (lane *2*), Mα3/7 (lane *3*), Mα4 (lane *4*), Mα6 (lane *5*), or *E. coli* trp E alone (lane *6*). Tracks *8–11*, whole extracts of mouse brain (lane *8*), testis (lane *9*), ovary (lane *10*), and spleen (lane *11*). Columns designated *Mα1/2*, *Mα3/7*, *Mα4*, and *Mα6* show autoradiographs of Western blots prepared from gels identical to those stained with Coomassie Blue (*C*) that had been reacted with the respective isotype-specific antisera. Detection of specifically bound antibody was with ^{125}I-labeled protein A. Note that the Mα4 fusion protein is larger than the other α-tubulin fusion proteins (see Fig. 1). The several smaller immunoreactive bands in the Mα4 track are degradation products of this fusion protein, as they do not appear in any of the control tracks.

with the nitrocellulose strip containing the appropriate bound antigen for 30 min. The strip was rinsed briefly twice with PBS, once with 0.1 M $NaHCO_3$, and once with 0.1 M $NaHCO_3$/0.5 M NaCl, and eluted for 5 min in 0.7 ml 0.1 M glycine-HCl, pH 2.6/150 mM NaCl. The pH was adjusted to 7.5 with 1 M Tris. All steps were carried out at room temperature. After affinity purification against the strip with bound α-tubulin isotype used as antigen, the sera were all further purified by negative absorption against nitrocellulose strips containing bound fusion proteins encoding the other α-tubulin isotypes. The nitrocellulose strips were eluted as above, reblocked, and stored at 4°C. Each strip could be reused several times. Affinity-purified sera were adjusted to 1% BSA, 0.04% azide, 40% glycerol, and stored at −20°C.

Western Blotting and Immunofluorescence

Western blots of bacterial and tissue lysates were prepared as described (Lewis et al., 1987). HeLa cells were grown in DME containing 5% FCS, and NIH 3T3 cells were grown in DME containing 10% FCS. Populations of interphase or mitotic cells were fixed and immunofluorescence was performed as described previously (Lewis et al., 1987). Primary antibodies used were the affinity-purified guinea pig anti-Mα3/7, rabbit anti-Mα4, rabbit anti-Mα6, rabbit anti-Mα4+Y, rabbit anti-Mα1/2, and guinea pig anti-Mα1/2 (the last two as general antitubulin sera) all described in this work, plus the rabbit anti-β-tubulin and guinea pig anti-β-tubulin sera described previously (Lewis et al., 1987). Second antibodies used were a rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) and an FITC-conjugated goat anti-guinea pig IgG antibody (Kirkegard Perry Laboratories Inc., Gaithersburg, MD). Cells were viewed and photographed under an immunofluorescence microscope using a Zeiss Planapochromatic 63× objective.

Results

Generation of Specific Antisera to Three Mammalian α-Tubulin Isotypes

Five mouse α-tubulin isotypes have been described, each distinguished by the presence of unique amino acid substitutions within the coding region (Villasante et al., 1986). Three of these isotypes (Mα1, Mα2, and Mα6) are widely expressed; Mα1 and Mα2 differ by only a single amino acid substitution. Mα3/7 (encoded by two mRNAs, Mα3 and Mα7) is expressed exclusively in testis, while Mα4 is expressed predominantly in muscle and brain and is unique among mammalian α-tubulins in that it lacks an encoded carboxy-terminal tyrosine residue.

To investigate the biological significance of the mammalian α-tubulin multigene family, we decided to raise isotype-specific sera. The specificity of the resulting sera was established in two ways. First, to investigate potential cross reactivity among different isotypes, affinity-purified sera were used on Western blots of whole extracts of bacterial cells expressing fusion proteins corresponding to all the α-tubulin isotypes. The data are shown in Fig. 2 *A*. The antibody raised against Mα1/2 is not specific, as it cross reacts with three out of four of the cloned fusion proteins. In con-

trast, the sera raised against Mα3/7, Mα4, and Mα6 are all specific for their respective isotypes. Second, the specificity of these sera for epitopes unique to α-tubulins was tested on Western blots of whole extracts of mouse tissues (Fig. 2 *B*). The only protein detected by the sera is α-tubulin. Because the fusion protein encoding Mα4 contains 86 amino acids (5′ to amino acid 254) that are not present in the other three fusion proteins (Fig. 1 *A*), the possibility existed that the apparent specificity of this antibody shown in Fig. 2 *A* might be due to epitopes in this unshared region. However, this possibility is ruled out by the observation that the Mα4 antibody fails to recognize any α-tubulin in whole extracts of testis, where Mα4 is not expressed (Fig. 2 *B*).

An Antiserum That Discriminates between the Tyrosinated and Nontyrosinated Form of a Single α-Tubulin Isotype (Mα4)

An interesting feature of the biochemistry of α-tubulins is a posttranslational modification in which a carboxy-terminal tyrosine residue is either added to the heterodimer, or removed from the assembled microtubule (Thompson et al., 1979; Kumar and Flavin, 1981; Gundersen et al., 1987). To establish the specificity of our isotype-specific sera with respect to the presence or absence of the carboxy-terminal tyrosine, we engineered two fusion proteins by site-directed mutagenesis (Fig. 1 *B*). First, we wanted to know whether the Mα4 antibody would recognize any detyrosinated α-tubulin isotype or whether it was truly specific for Mα4. This question arises because Mα4 is the only mammalian α-tubulin isotype (and therefore the only one of our cloned fusion proteins) that is translated without a carboxy-terminal tyrosine residue. Therefore, using Mα3/7 as representative of cloned fusion proteins ending in tyrosine, we expressed an altered Mα3/7 fusion protein in which the tyrosine codon (TAC) was changed to a termination codon (TAG). Second, we engineered a fusion protein in which a tyrosine codon was inserted between the penultimate codon and the termination codon of Mα4. Western blots of whole bacterial extracts expressing altered and unaltered fusion proteins are shown in Fig. 3. The data show that the Mα3/7-specific antibody recognizes both tyrosinated (Mα3/7) and nontyrosinated (Mα3/7–Y) forms of this isotype, but neither form of Mα4 (Mα4 or Mα4+Y). On the other hand, the Mα4-specific antibody recognizes preponderantly the nontyrosinated form of this isotype on Western blots (Fig. 3 *A*) and exclusively this form in fixed microtubules (see below). However, it does not detect either form of Mα3/7 and is therefore truly specific. Because of the bias of the Mα4 antibody, we raised an additional antiserum against the engineered fusion protein Mα4+Y (Fig. 1 *B*). The specificity of this serum with respect to all six cloned fusion proteins is demonstrated in Fig. 3 *B*; there is dominant recognition of Mα4+Y. Although the serum raised against Mα4 detects tubulin in extracts from brain and spleen, the antibody raised against Mα4+Y is negative on Western blots from these tissues (data not shown) suggesting that in these tissues the Mα4 isotype is predominantly present in its nontyrosinated form. This implies one of two things: either the α-tubulin tyrosine ligase activity is not present in the particular cells in which Mα4 is expressed or, more likely, Mα4 exists mainly in stable detyrosinated microtubules in these instances.

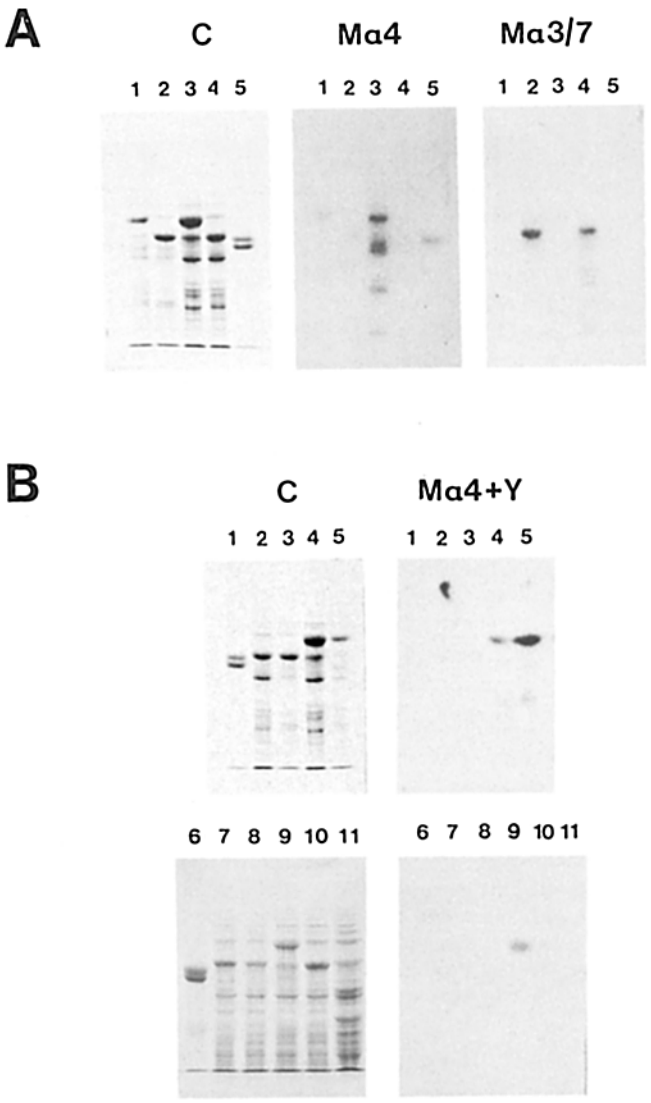


Figure 3. Specificity of an antiserum with respect to the tyrosination of α-tubulin isotypes. Cloned cDNA sequences encoding the carboxy-terminal portions of Mα4 and Mα3/7 were modified by site-directed mutagenesis so as to add (Mα4+Y) or remove (Mα3/7–Y) the encoded carboxy-terminal tyrosine residue (see Fig. 1). Each of these modified sequences was expressed in host *E. coli* cells as a fusion protein coupled to *E. coli* trp E sequences. In the case of Mα4+Y, the fusion protein was purified and used to generate an (Mα4+Y)-specific antiserum. (*A*) Panel *C*, Coomassie Blue stain of an 8.5% SDS–polyacrylamide gel loaded with whole extracts of bacteria expressing fusion proteins encoding Mα4+Y (lane *1*), Mα3/7–Y (lane *2*), Mα4 (lane *3*), Mα3/7 (lane *4*), and 5 μg marker bovine brain tubulin (lane *5*). Panels designated *Mα4* and *Mα3/7* show autoradiographs of Western blots of gels identical to that shown in *C*, in which the antisera specific for these isotypes were used. Detection was with ^{125}I-labeled protein A. (*B*) Panel *C*, Coomassie Blue stain of 8.5% SDS-polyacrylamide gels loaded with whole extracts of bacteria expressing fusion proteins encoding Mα3/7 (lanes *2* and *8*), Mα3/7–Y (lane *3*), Mα4 (lanes *4* and *9*), Mα4+Y (lane *5*), Mα1/2 (lane *7*), Mα6 (lane *10*), and *E. coli* trp E alone (lane *11*). Marker bovine brain tubulin is in lanes *1* and *6*. Panel marked *Mα4+Y* shows autoradiographs of Western blots prepared from gels identical to those shown at the left, reacted with the antiserum raised against the Mα4+Y fusion protein (see Fig. 1 *B*). Detection was with ^{125}I-labeled protein A.

Expression of Mα3/7, Mα4, and Mα6 in HeLa and NIH 3T3 Cells

The specific sera that we raised recognize three distinct isotypes: one (Mα3/7) is expressed exclusively in testis; a second (Mα4) is expressed most abundantly in muscle and brain; and a third (Mα6) is expessed in several tissues, though at a very low level (Villasante et al., 1986). To examine whether these three isotypes assemble into microtubules or affect microtubule function outside the context of their normal pattern or level of expression, the cDNAs encoding Mα3/7, Mα4, and Mα6 were each cloned into a vector designed to express these sequences upon transfection into cultured cells via transcription from the SV-40 T-antigen promoter (see Materials and Methods). In each case, the transfected culture was analyzed by double-label immunofluorescence using an isotype-specific antibody (to detect the expression of transfected sequences) and a general tubulin antibody (to detect all microtubules irrespective of their content of tubulin isotypes). The data show that expression of Mα3/7 and Mα6 in transfected cells results in the incorporation of these isotypes into all interphase microtubules of either HeLa cells (Fig. 4, *a*, *b*, *e*, and *f*) or NIH 3T3 cells (Fig. 4, *c*, *d*, *g*, and *h*), since labeling with the specific and general tubulin antisera is in all cases coincident. Furthermore, these experiments provide additional evidence concerning the specificity of the Mα3/7 antiserum, since untransfected cells are not detected. The presence of weakly labeled untransfected cells in the case of Mα6 is due to an extremely low level of endogenous Mα6 expression in HeLa and NIH 3T3 cells, and not to cross-reactivity with other α-tubulin isotypes.

Parallel transfection experiments performed using a cDNA sequence encoding Mα4 cloned into the SV-40 expression vector are shown in Figs. 5 and 6. When transfected cells were analyzed using the Mα4+Y antiserum, this form of the Mα4 isotype was found to be assembled into all interphase microtubules in a manner indistinguishable from that detected by a general tubulin antibody (Fig. 5, *a–d*). In contrast, identical transfection experiments analyzed using the Mα4 antiserum showed the presence of this (untyrosinated) isotype in a distinct subset of interphase microtubules that have a prominently perinuclear localization and tend to be curved (Fig. 6, *a–d*). However, in some transfected cells, the nontyrosinated form of this isotype occurs in all interphase microtubules in the cell (Fig. 6, *e* and *f*). Such cells appeared completely normal and had divided, as evidenced by their appearance in pairs on the coverslip (Fig. 6 *e*). As with the serum specific for Mα3/7 (Fig. 4, *a* and *c*), the specificity of the Mα4 and Mα4+Y antisera is confirmed by the presence of surrounding untransfected cells which are essentially unlabeled (Figs. 5 and 6). While the sera raised against the fusion proteins Mα4 and Mα4+Y show slight cross-reactivity on Western blots, little, if any, is apparent on preparations of fixed microtubules (see Fig. 6 and accompanying paper). The detection of two subsets of interphase microtubules by the antisera specific for Mα4 and Mα4+Y closely parallels the observations on tubulin tyrosination made by Gundersen et al. (1984, 1987). The implications of these data are discussed below.

Presence of Mα3/7, Mα4, and Mα6 in Spindle Microtubules

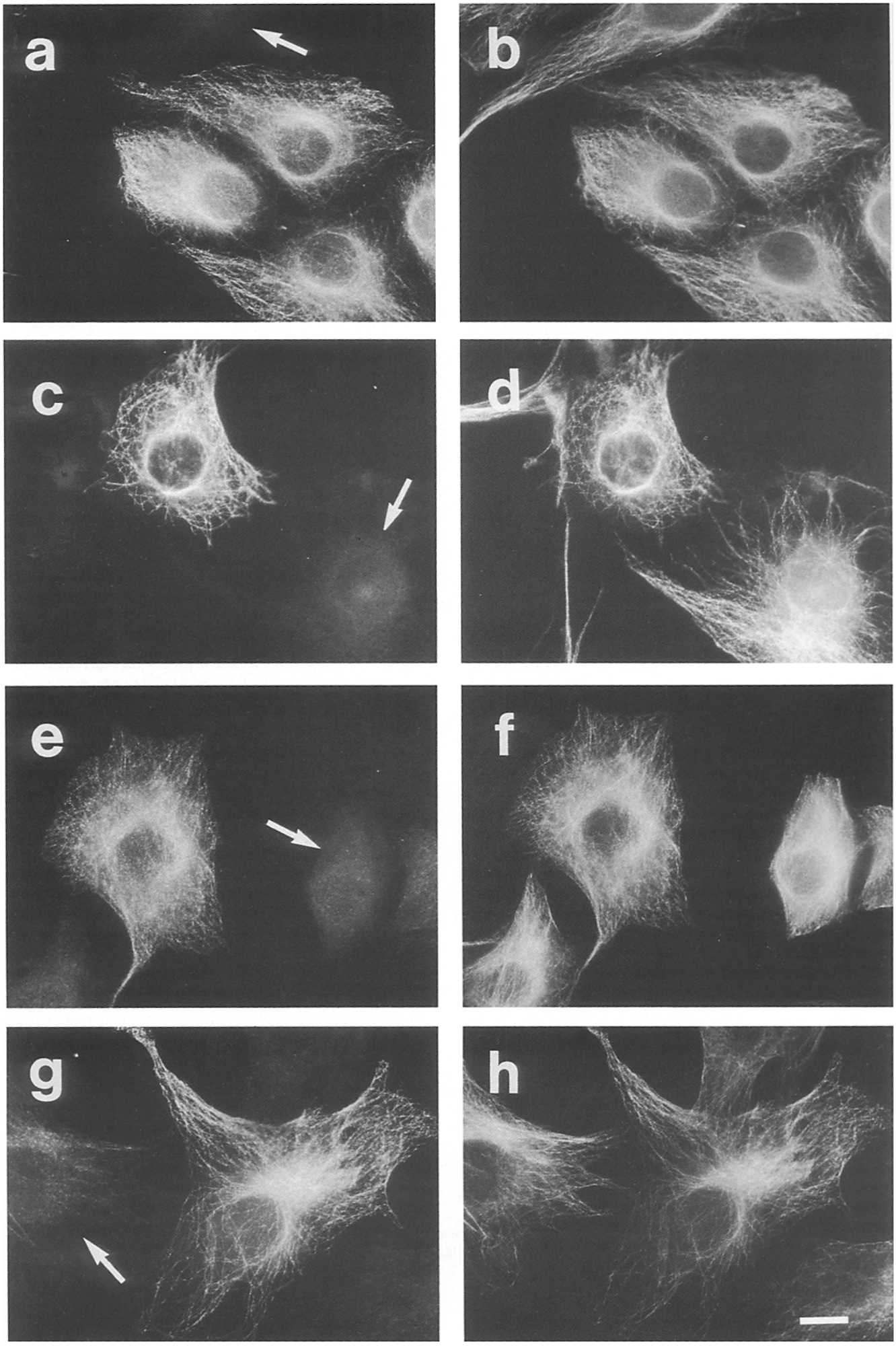
The capacity of Mα3/7, Mα4, and Mα6 to assemble into spindle microtubules was determined in cytospin preparations of unattached (i.e., mitotic) transfected HeLa cells. As with interphase cells, the selected mitotic populations were double labeled with isotype-specific and general tubulin antisera. In the case of Mα3/7 and Mα6, the data show no significant difference in microtubule labeling, irrespective of whether the isotype-specific or general tubulin antibody was used (Fig. 7). However, in the case of the Mα4 isotype, only the serum raised against the tyrosinated form of this polypeptide detected spindle microtubules, and these were (like Mα3/7 and Mα6) coincident with the microtubules detected by a general tubulin antibody. The serum that recognized only the nontyrosinated form of Mα4 failed to detect any spindle microtubules in the same transfection experiment, even though this is the form of the protein being translated in the transfected cells. Thus, the isotypes represented by Mα3/7, Mα4, and Mα6 competently assemble into all interphase and spindle microtubules upon transfection into tissue culture cells, while the detyrosinated form of Mα4 is usually found only in a limited subset of interphase microtubules and not at all in spindle microtubules.

Coassembly of Chimeric α-Tubulin Isotypes

The principal differences that distinguish one α-tubulin isotype from another lie within the carboxy-terminal 15 amino acids. Nevertheless, other characteristic differences also exist scattered throughout the polypeptide chain. Both the carboxy-terminal and internal sequences that distinguish each isotype are rigidly conserved among mammalian species. To assess the influence that different regions of individual α-tubulin isotypes might have with respect to each other vis-a-vis coassembly and/or phenotype, we spliced together different segments of cloned cDNAs so as to generate chimeric α-tubulin molecules. Two chimeric constructs were assembled: one consisted of NH_2-terminal sequences corresponding to Mα6 coupled to COOH-terminal sequences corresponding to Mα3; and a second, complementary construct consisting of NH_2-terminal Mα3 sequences coupled to Mα6 COOH-terminal sequences. In each case, the breakpoint was an Eco RI site at amino acid 254. The chimeric constructs were inserted into the SV40 expression vector and tested for their ability to direct the synthesis of assembly-competent α-tubulin after transfection into HeLa cells. In each experiment, the assembly of chimeric isotypes was monitored using the α-tubulin isotype-specific antibody corresponding to the carboxy-terminal segment; i.e., the exact segment originally used for the elicitation of each isotype-specific immune response (see Fig. 1). The result of this experiment showed that conjoining of NH_2-terminal and COOH-terminal regions belonging to different α-tubulin isotypes has no apparent influence on microtubule assembly (Fig. 8). Similar results have been obtained in experiments involving chimeric β-tubulins (Bond et al., 1986; Fridovich-Keil et al., 1987).

Discussion

We describe here the generation of immune sera that are capable of discriminating among three of the five known mammalian α-tubulin isotypes, as well as sera that distinguish the tyrosinated from the nontyrosinated form of a single α-tubulin isotype. To generate these sera, we used cloned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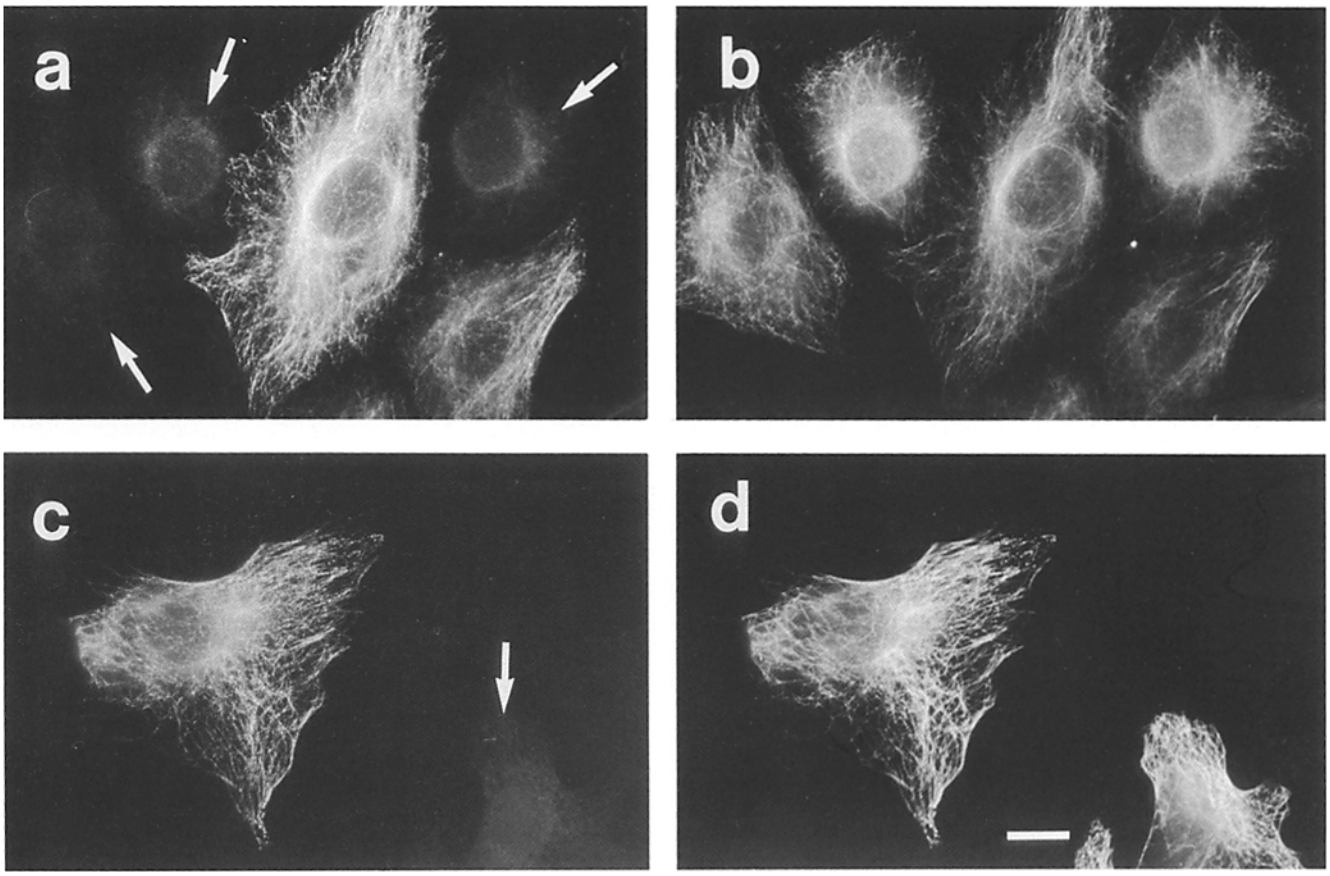

Figure 5. Localization of the tyrosinated form of a single α-tubulin isotype (Mα4) in the interphase microtubules of tissue culture cells. A cloned cDNA sequence encoding Mα4 was inserted into the eukaryotic expression vector pSVdhfr (see Materials and Methods). This construct was introduced into either HeLa cells (*a* and *b*) or NIH 3T3 cells (*c* and *d*) by calcium phosphate precipitation. Transfected cells expressing the Mα4 isotype were examined in fixed, detergent-extracted preparations by double-label indirect immunofluorescence using rabbit antisera specific for the tyrosinated form of Mα4 (*a* and *c*), together with a guinea pig β-tubulin–specific antibody that detects all microtubules (shown in *b* and *d*). Surrounding untransfected cells in *a* and *c* are indicated by arrows. Bar, 10 μm.

fusion proteins expressed in bacterial cells consisting of the first 35,000 D of the *E. coli* trp E protein fused to the carboxy-terminal region of the various α-tubulin isotypes (Fig. 1). These fusion proteins therefore include the carboxy-terminal α-tubulin region containing the characteristic and divergent sequences that distinguish most of the isotypes. To avoid eliciting an immune response to common (i.e., shared) epitopes, including those present on the trp E portion of each fusion protein, animals were first rendered tolerant to all but one of the fusion proteins. Thus, upon challenge with the remaining fusion protein, only epitopes that are unique can be recognized. This method of raising antisera to discriminate among related molecules that contain one or more epitopes in common has been previously used to increase the probability of obtaining mAbs specific to unique epitopes (Matthew and Patterson, 1983), as well as for the generation of polyclonal antisera that specifically recognize distinct but highly homologous mammalian β-tubulin isotypes (Lewis et al., 1987). The method offers certain advantages over the use of synthetic peptide haptens: potential problems relating to peptide purification and solubility are avoided, and it is not necessary to attempt the selection of the optimal peptide(s).

Figure 4. Expression of Mα3/7 or Mα6 in transfected tissue culture cells results in their coassembly into interphase microtubules without disruption of function. Cloned cDNA sequences encoding Mα3/7 or Mα6 were inserted into the eukaryotic expression vector pSVdhfr (see Materials and Methods) and introduced into either HeLa cells (*a*, *b*, *e*, and *f*) or NIH 3T3 cells (*c*, *d*, *g*, and *h*) by calcium phosphate precipitation. Transfected cells expressing Mα3/7 or Mα6 were examined in fixed detergent-extracted preparations by double-label indirect immunofluorescence using antisera specific for Mα3/7 (shown in *a* and *c*) or Mα6 (shown in *e* and *g*) and a β-tubulin–specific antibody that detects all microtubules (shown in *b*, *d*, *f*, and *h*). Controls in which both second antibodies were used only with one or the other first antibody showed both second antibodies to be completely specific (data not shown). Note the coincident labeling of microtubules with isotype-specific and general antitubulin antisera (compare *a* with *b*, *c* with *d*, *e* with *f*, and *g* with *h*) and the presence of surrounding (untransfected) cells in *a* and *c* that do not express Mα3 (*arrows*); similar surrounding untransfected cells in *e* and *g* show very weak fluorescence because of a low level of endogenous expression of Mα6 in HeLa and NIH 3T3 cells. Bar, 10 μm.

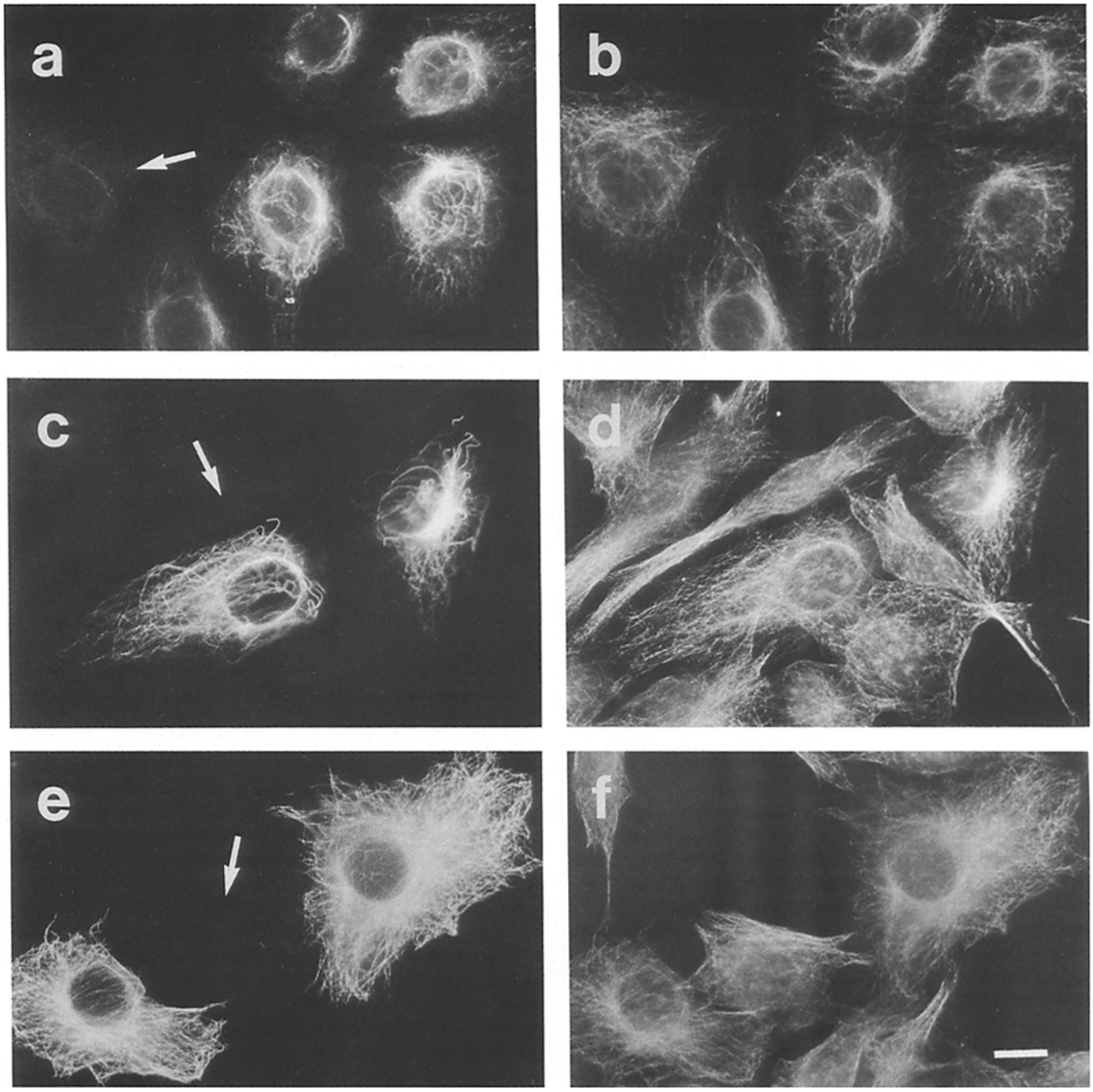

Figure 6. Distribution of the untyrosinated form of Mα4 in transfected tissue culture cells. A transfection experiment identical to that described in Fig. 5 was analyzed using the antibody specific for the untyrosinated form of Mα4 (*a*, *c*, and *e*). (*a* and *b*) HeLa cells; (*c–f*) NIH 3T3 cells. *b*, *d*, and *f* show the same fields as *a*, *c*, and *e*, respectively, detected with a general tubulin antibody. Note the presence of unlabeled untransfected cells in *a*, *c*, and *e* (*arrows*). Bar, 10 μm.

This could be particularly important in situations where multiple but randomly scattered amino acid substitutions distinguish various members of an otherwise highly homologous family of proteins.

Three criteria were used to establish the specificity of the anti-α-tubulin antisera. First, each antibody was tested in Western blot experiments for its ability to discriminate among cloned fusion proteins representing each of the known mammalian α-tubulin isotypes. Second, the sera were tested in Western blot experiments of whole extracts of mouse tissues to assess their specificity for epitopes unique to α-tubulin. Finally, the ability of each antibody to discriminate among different α-tubulin isotypes in fixed microtubules was ascertained in transfection experiments in which cells expressing specific transfected α-tubulin isotypes showed strongly fluorescent microtubules, whereas surrounding untransfected cells remained negative. Using these criteria, we generated three α-tubulin isotype-specific sera that uniquely recognize

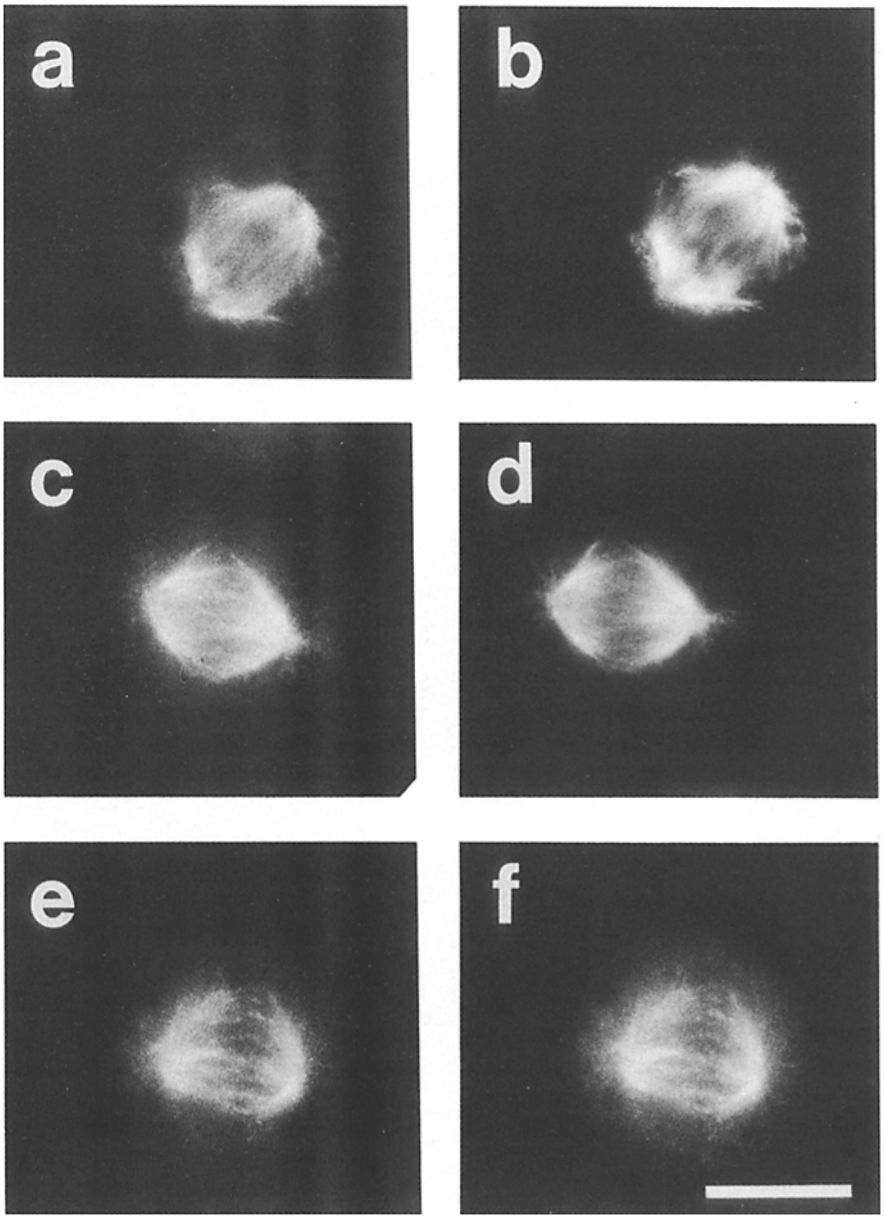

Figure 7. Assembly of Mα3/7, Mα4, and Mα6 into spindle microtubules. Mitotic cells from HeLa cell cultures transfected with constructs designed to express Mα3/7, Mα4, or Mα6 (see Materials and Methods) were examined in fixed, detergent-extracted cytospin preparations by double-label indirect immunofluorescence using the antisera specific for Mα3/7 (*a*), Mα4+Y (*c*), or Mα6 (*e*) plus a β-tubulin-specific antibody that detects all microtubules (*b*, *d*, and *f*). No spindles were labeled with the Mα4 antibody, which recognizes only the nontyrosinated form of this isotype. Bar, 10 μm.

Mα3/7, Mα4, or Mα6. In addition, two sera that distinguish between the tyrosinated and detyrosinated form of a single α-tubulin isotype (Mα4) were generated. Although the two sera cross react slightly with their respective antigens on Western blots (Fig. 3 *B*), they are clearly discriminatory in immunofluorescence experiments on fixed microtubules (Fig. 6 and accompanying paper).

Of the three α-tubulin isotypes for which we raised specific antisera, two are expressed in tissues that undergo distinct developmental changes involving the formation and/or reorganization of microtubule structures (Villasante et al., 1986). Mα3/7 is expressed exclusively in testis, where specialized microtubule structures such as the flagellum and manchette are associated with spermatogenesis, while Mα4 is abundantly expressed in muscle and brain, in which great asymmetries are produced in cell morphology during development. On the other hand, Mα6 is expressed in many tissues but at a very low level. The existence of distinct α-tubulin isotypes in tissues containing specialized microtubule structures might be explained in terms of structural requirements conferred by distinct amino acid differences in the polypeptide chain. Such differences could, for example, be important for interaction with specific microtubule-associated proteins. Though this may indeed be the case, our data show that Mα3/7, Mα4, and Mα6 are capable of assembly into interphase and spindle microtubules in cell types (Mα4, Mα3/7) or at levels (Mα6) at which they are not normally expressed. Thus, in common with mammalian β-tubulin isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987), there is no evidence for segregation of these isotypes among functionally different microtubules.

In addition to the encoded sequence heterogeneity that exists among tubulin isotypes, both α- and β-tubulin polypeptides can undergo posttranslational modification. In the case

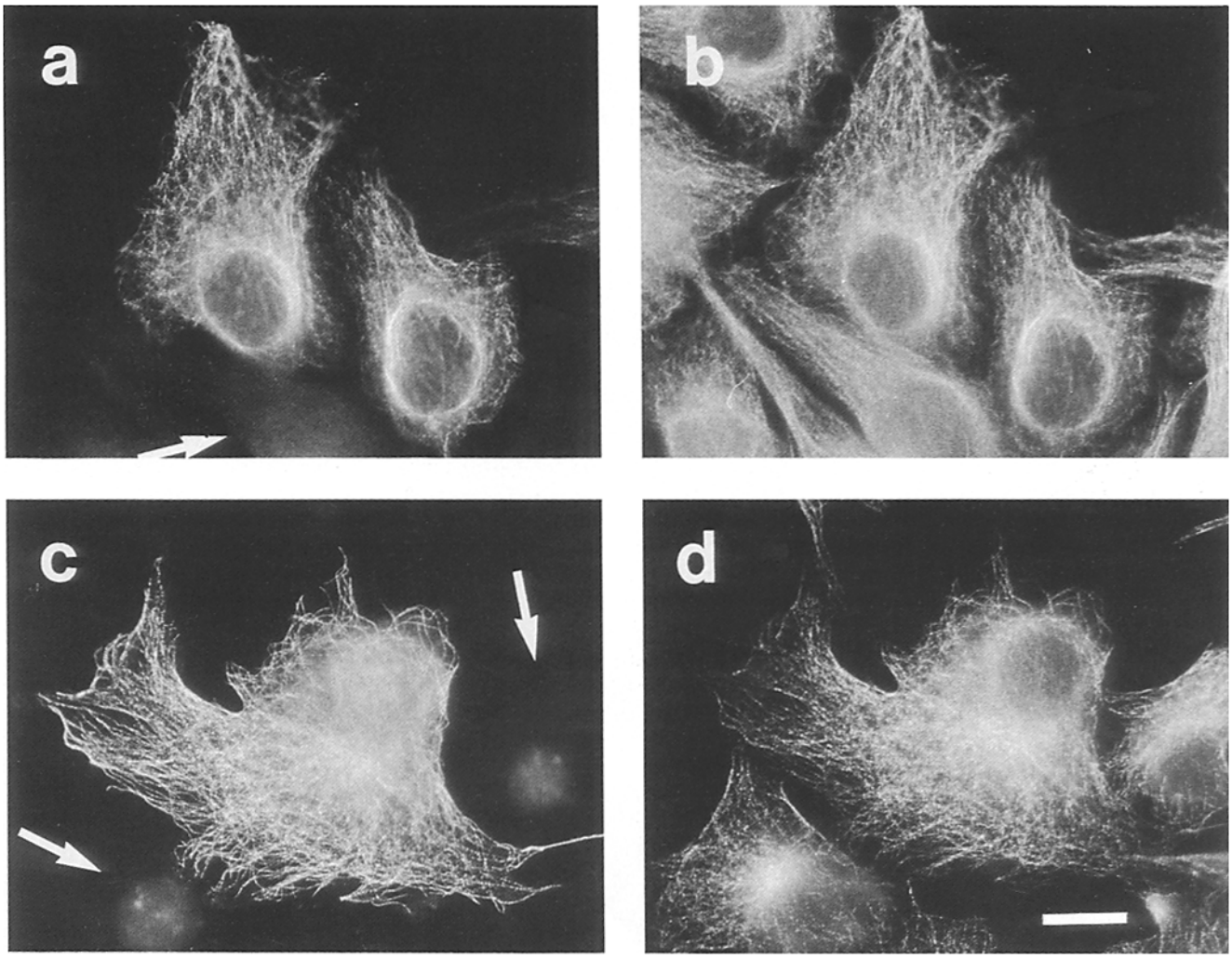

Figure 8. Assembly of chimeric α-tubulin isotypes into interphase microtubules. Two chimeric isotypes, one consisting of NH_2-terminal sequences derived from Mα6 coupled to COOH-terminal sequences corresponding to Mα3 and a second consisting of NH_2-terminal Mα3 sequences coupled to Mα6 COOH-terminal sequences were constructed. Each was inserted into the pSVdhfr expression vector (Mulligan and Berg, 1981) and introduced into HeLa cells or NIH 3T3 cells by calcium phosphate precipitation (Wigler et al., 1979). Transfected cells were detected by indirect double-label immunofluorescence using the α-tubulin isotype-specific antibody corresponding to the isotype encoded by the carboxy-terminal segment, plus a β-tubulin–specific antibody that detects all microtubules. (*a* and *b*) HeLa cells; (*c* and *d*) NIH 3T3 cells. (*a* and *c*) Cells transfected with the Mα6/Mα3 chimeric isotype detected with the Mα3/7-specific antibody (note the presence of surrounding untransfected cells [*arrows*]); (*b* and *d*) the same field shown in *a* and *c*, respectively, detected with a general tubulin antibody. Microtubules detected with either antibody appear coincident. Coincident microtubules were also observed in spindles in the same transfected cultures, and identical results were obtained using the Mα3/Mα6 chimeric isotype (data not shown).

of α-tubulins, these modifications include the acetylation of lysine (at position 40 [LeDizet and Piperno, 1986]), and the removal or addition of the carboxy-terminal tyrosine residue. The cycle of α-tubulin tyrosination and detyrosination has been well studied in vivo and in vitro. Tubulin tyrosine ligase rapidly tyrosinates the tubulin heterodimer (Raybin and Flavin, 1977; Gundersen et al., 1987), while tubulin tyrosine carboxypeptidase detyrosinates assembled microtubules slowly compared to the half-life of a microtubule in a cultured cell (Kumar and Flavin, 1981; Gundersen et al., 1987; Webster et al., 1987). Antibodies that distinguish between tyrosinated and detyrosinated microtubules have been prepared by Gundersen et al. (1984) and used to show that in cultured cells a small subset of microtubules is enriched in nontyrosinated α-tubulin. These microtubules are distinguished from the bulk of the cell's interphase microtubules by their wavy morphology and the fact that they are not growing (Gundersen et al., 1987). Schulze and Kirschner (1987) have shown that such wavy microtubules are 3–5 times more stable than average. However, several lines of evidence suggest that the detyrosination of these microtubules is a consequence, rather than a cause of their enhanced stability: tyrosinated and detyrosinated tubulin behave in a dynamically similar way in vitro (Raybin and Flavin, 1977; Kumar and Flavin, 1982); the drug-induced stabilization of microtubules in vivo results in their increased detyrosination (Gundersen et al., 1987); and spindle microtubules, which turn over more rapidly than interphase microtubules, are extremely

poor in detyrosinated α-tubulin (Gundersen and Bulinski, 1986).

Thus, these more stable microtubules probably become detyrosinated because the tyrosine-carboxypeptidase has more time to act on them. The function of this detyrosination remains a mystery. What role it may play in differentiated cells is also unclear, although tubulin tyrosine ligase has been shown to be enriched in developing brain and muscle (Deanin et al., 1977), and in neurons, axonal microtubules have been shown to be detyrosinated compared to dendritic microtubules (Cambray-Deakin and Burgoyne, 1987). Acetylation of α-tubulin is similar to detyrosination in many respects: it is reversible (L'Hernault and Rosenbaum, 1985*b*), nonessential for cell growth and division (Piperno et al., 1987), and acts mainly on more stable microtubules, such as the wavy interphase microtubules of some cultured cells (Piperno et al., 1987), axonal microtubules (Cambray-Deakin and Burgoyne, 1987), and microtubules of cilia and flagella (LeDizet and Piperno, 1986; L'Hernault and Rosenbaum, 1983).

The work described above on α-tubulin tyrosination was performed using antisera directed against two short peptides derived from a single carboxy-terminal α-tubulin sequence and differing only by the presence or absence of the tyrosine residue. The sequence of these peptide haptens, GEEEGEE (Y), corresponds to Mα1/2 (Fig. 1). Both Mα1 and Mα2 are widely expressed, and are particularly abundant in cultured cells, where Mα3/7, Mα4, and Mα6 are expressed at very low levels or not at all. The α-tubulin isotype Mα4 differs from Mα1, Mα2, and other mammalian α-tubulins in that it lacks an encoded carboxy-terminal tyrosine residue. We were therefore particularly interested in investigating how this isotype behaves in the cycle of detyrosination/tyrosination. Here we describe two sera raised against this isotype: one that recognizes only the detyrosinated form of Mα4 in fixed microtubules and another that recognizes only or predominantly the tyrosinated form (Mα4+Y). We have used these sera to examine the behavior of this isotype when expressed (by transfection) in cultured cells, in particular, in regard to how it fits into the cycle of tubulin detyrosination/tyrosination. Several conclusions can be drawn. Mα4 is a substrate for tubulin tyrosine ligase. When it is expressed in (transfected) cultured cells at low or moderate levels, it is found predominantly in tyrosinated form in all microtubules (Fig. 5), and in its detyrosinated form in only a subset of wavy microtubules (Fig. 6, *a–d*). Thus it behaves in the cycle of tyrosination/detyrosination like the bulk of α-tubulin, as described by Gundersen et al. (1984, 1987). However, in any transient transfection experiment, the level of expression varies immensely from one cell to another, due to variation in the number of copies of transfected DNA molecules that are taken up. Thus, when we transfected an Mα4-expressing construct into NIH 3T3 cells, in some cases the Mα4 isotype was incorporated into all the cells' microtubules in its nontyrosinated form; i.e., without prior posttranslational tyrosination (Fig. 6, *e* and *f*). This presumably occurs because there is not enough tyrosine ligase in the cell to handle all of the nontyrosinated α-tubulin present due to the overexpression of Mα4. The overabundance of untyrosinated tubulin has no apparent effect on cell morphology or division (since overexpressing cells [like all transiently transfected cells] are usually found in groups of two or four on the coverslip, showing that they have divided since transfection). This experiment reinforces the conclusions of others (Webster et al., 1987; Kumar and Flavin, 1982) that nontyrosinated α-tubulin is dynamically similar to tyrosinated α-tubulin, and therefore that the enhanced stability of naturally occurring detyrosinated microtubules is probably a cause rather than an effect of detyrosination.

These data, taken together with numerous other studies that have sought to address issues relating to the expression of multiple α- and β-tubulin genes both in lower and higher eukaryotes, all point to an apparent functional interchangeability within each family of isotypes. This presents us with a curious paradox, at least in regard to the mammalian tubulin multigene families: on the one hand, the isotypes appear functionally interchangeable but on the other, there has been rigid interspecies conservation of the amino acid sequences that distinguish one isotype from another, at least since the mammalian radiation (about 100 million years ago). One possible resolution of this paradox is that specific isotype sequences may indeed be required for interaction with microtubule-associated proteins. In that event, the ability of all isotypes to coassemble into functionally distinct microtubules could be regarded as adventitious: only the expression of certain isotype sequences (rather than their segregation) in concert with specific microtubule-associated proteins may be required for the proper functioning of specific kinds of microtubules. In the accompanying paper, these ideas are explored further by using our α- and β-tubulin isotype-specific sera to study tubulin isotype expression and use in cells and tissues in which reorganization and assembly of functionally different kinds of microtubule form an integral part of the differentiation process.

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