Complex Regulation and Functional Versatility of Mammalian α- and β-Tubulin Isotypes during the Differentiation of Testis and Muscle Cells

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Abstract. In the accompanying paper (Gu, W., S. A. Lewis, and N. J. Cowan. 1988. *J. Cell Biol.* 106: 2011–2022), we report the generation of three antisera, each of which uniquely recognizes a different mammalian α-tubulin isotype, plus a fourth antibody that distinguishes between microtubules containing the tyrosinated and nontyrosinated form of the only known mammalian α-tubulin gene product that lacks an encoded carboxy-terminal tyrosine residue. These sera, together with five sera we raised that distinguish among the known mammalian β-tubulin isotypes, have been used to study patterns of tubulin isotype-specific expression in muscle and testis, two tissues in which characteristic developmental changes are accompanied by dramatic rearrangements in microtubule structures. As in the case of cells in culture, there is no evidence to suggest that there is subcellular sorting of different tubulin isotypes among different kinds of microtubule, even in a cell type (the developing spermatid) that simultaneously contains such functionally distinct structures as the manchette and the flagellum. On the other hand, the patterns of expression of the various tubulin isotypes show marked and distinctive differences in different cell types and, in at least one case, evidence is presented for regulation at the translational or posttranslational level. The significance of these observations is discussed in terms of the existence of the mammalian α- and β-tubulin multigene families.

VIRTUALLY all eukaryotic cells require microtubules to perform essential cellular functions such as mitosis and the maintenance of asymmetric shape via dynamic modulation of the interphase cytoskeleton. In addition, specialized kinds of microtubule have evolved that mediate particular functions in differentiated cell types. Examples of such specialized microtubules include those in neuronal axons that are involved in the bidirectional transport of organelles; the flagellar axoneme of spermatozoa; and the manchette, a pyramidal sheath of microtubules that surrounds the nucleus of the developing spermatid and is thought to contribute to the asymmetry of the sperm nucleus.

We recently reported the generation of sera that discriminate among most of the naturally occurring mammalian β-tubulin isotypes. In the accompanying paper (Gu et al., 1988), three α-tubulin isotype-specific antisera are described and data is presented that, as in the case of β-tubulin isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987), there appears to be free intermingling of these isotypes among functionally distinct microtubules in cultured cells. These results, in conjunction with genetic experiments in yeast (Shatz et al., 1986; Adachi et al., 1986), *Aspergillus* (May et al., 1985; Weatherbee et al., 1985), and *Drosophila* (Kemphues et al., 1982; Fuller et al., 1987), argue strongly against the occurrence of subcellular sorting of different tubulin gene products. Therefore, the explanation for the existence of the tubulin multigene family and the interspecies conservation of its expressed members must lie elsewhere. We propose that multiple tubulin genes have evolved to accommodate the quantitative demand for tubulin in different differentiating cell types and that conserved differences among isotypes became established as a result of their coevolution with different cell type-specific microtubule-associated proteins with which they must interact. Testis and muscle cells provide good paradigms to investigate this hypothesis, as both undergo well-characterized developmental changes involving the reorganization of microtubules for specialized purposes. Here we present the results of a study of these tissues using our α- and β-tubulin isotype-specific sera. Three conclusions emerge from this study. First, as expected, we found no evidence for the segregation of any isotype in any cell type. Second, the different tubulin gene products are expressed in the various cell types present in testicular tissue in a complex and distinctive pattern. Third, we show that in muscle, a high level of transcription of a gene encoding a particular α-tubulin isotype (Mα4) is induced upon myoblast fusion. However, the corresponding protein is detectable only weakly and only in a subset of myotubules, implying some type of translational or posttranslational regulation of its expression.

Materials and Methods

Growth, Induced Fusion, and Immunofluorescence Examination of Mouse C2 Cells

Mouse C2 cells (Yaffee and Saxel, 1977) were grown in DME containing 20% FCS and 0.5% chicken embryo extract (Gibco, Grand Island, NY). For immunofluorescence, they were grown on glass coverslips. At 70% confluence, the medium was changed to DME containing 10% horse serum, resulting in cell fusion, the process being essentially complete in 3 d. Whole protein extracts were prepared by boiling cells in SDS gel loading buffer (Laemmli, 1970). Western blotting was performed as described (Towbin et al., 1979). Cells were fixed and examined by indirect immunofluorescence with isotype-specific antibodies as described in the accompanying paper (Gu et al., 1988).

RNA Preparation and Analysis

Cultures of uninduced or induced C2 cells were used for the preparation of total RNA by the method of Chirgwin et al. (1979). The yield of RNA was determined by absorption at 260 nm. Samples (10 μg) were subjected to electrophoresis on 1% agarose gels containing 2.2 M formaldehyde (Boedtker, 1971). The RNA was transferred to nitrocellulose, and the blot prehybridized and probed with an antisense oligonucleotide specific to Mα4 (Villasante et al., 1986) that had been ^{32}P-end labeled with polynucleotide kinase. Prehybridization and hybridization conditions were 20% formamide, 5× SSC (SSC is 150 mM NaCl, 15 mM Na citrate), 5× Denhardt's solution (Denhardt, 1966), 20 mM Na phosphate, pH 6.8, for 16 h. After hybridization, the blot was washed to a final stringency of 52°C, 0.5× SSC.

Detection of Tubulin Isotypes in Mouse Testis

Adult male Swiss Webster mice were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3, containing 10 mM EGTA. The testis was excised, the tunica albuginea removed, and the tissue fixed for an additional 2 h at room temperature in the same fixative solution. 6-μm-thick cryostat sections were prepared. Sections were permeabilized for 5 min in 0.5% Triton-PBS and blocked for 15 min in TBST-3% BSA (TBST is 50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20). All antisera were diluted into TBST-3% BSA and allowed to react at 37°C for 2 h. The sera used are described in the accompanying paper and in Lewis et al. (1987). Sections were mounted as described (Johnson and Araujo, 1981) and viewed and photographed using a Zeiss Axiophot immunofluorescence microscope.

Results

Specificity of β-Tubulin Isotype-Specific Antisera

We previously described the generation of antisera that distinguish among the six known mammalian β-tubulin isotypes (Lewis et al., 1987; Burgoyne, R. D., M. A. Cambray-Deakin, S. A. Lewis, S. Sarkar, and N. J. Cowan, manuscript submitted for publication). The specificity of each of these sera was established using Western blots of cloned fusion proteins corresponding to each of these isotypes, as well as Western blots of whole extracts of HeLa cells. To demonstrate that the sera specific for Mβ2, Mβ3/4, Mβ5, and Mβ6 recognized only β-tubulin in mouse testis (and other tissues), a further Western blot experiment was performed (Fig. 1). The data show that β-tubulin is indeed the only protein recognized by these sera in whole extracts of mouse testis, ovary, spleen, and brain.

Relative Affinities of Isotype-Specific Sera

To assess the relative affinities of our α-tubulin isotype-specific antisera, as well as the relative affinities of the β-tubulin isotype-specific sera previously generated in our laboratory (Lewis et al., 1987), serial dilutions of cloned fusion proteins corresponding to each isotype were spotted onto strips of nitrocellulose. After reaction with each specific antiserum at the dilutions used in the immunofluorescence experiments described in this work, the strips were washed and the bound antibody detected by reaction with ^{125}I-labeled protein A. The relative affinities of the α-tubulin isotype-specific sera measured in this way were in the ratio Mα6:Mα4:Mα4+Y:Mα3 = 1:2:2:3; for our β-tubulin isotype-specific sera, the relative affinities were Mβ5:Mβ6:Mβ3/4:Mβ2 = 1:1.5:2:2.

Induction of Mα4 Gene Expression upon Fusion of C2 Cells

To assess changes in isotype composition that might accom-

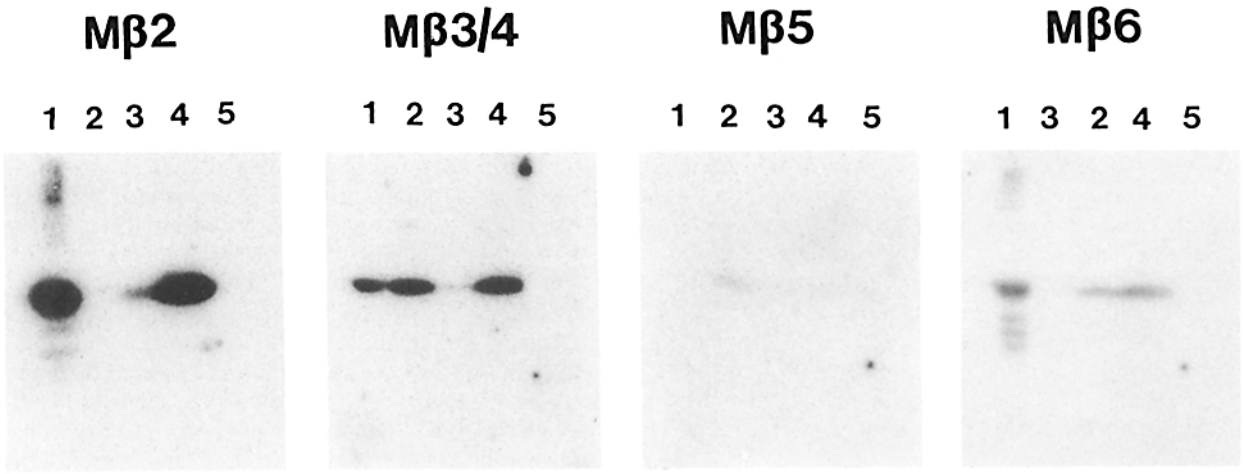


Figure 1. Specificity of β-tubulin isotype-specific sera on whole extracts of mouse tissues. Whole extracts of adult mouse testis (lanes 2), ovary (*lanes 3*), brain (lanes *4*), and spleen (lanes *5*) were resolved on 8.5% stacking SDS-polyacrylamide gels together with 5 μg marker bovine brain tubulin (lanes *1*). The gel contents were electrophoretically transferred to nitrocellulose, and the blots probed with antisera-specific Mβ2, Mβ3/4, Mβ5 (Lewis et al., 1987), and Mβ6 (Burgoyne, R. D., M. A. Cambray-Deakin, S. A. Lewis, S. Sarkar, and N. J. Cowan, manuscript submitted for publication). Detection of bound antibody was by reaction with ^{125}I-labeled protein A.

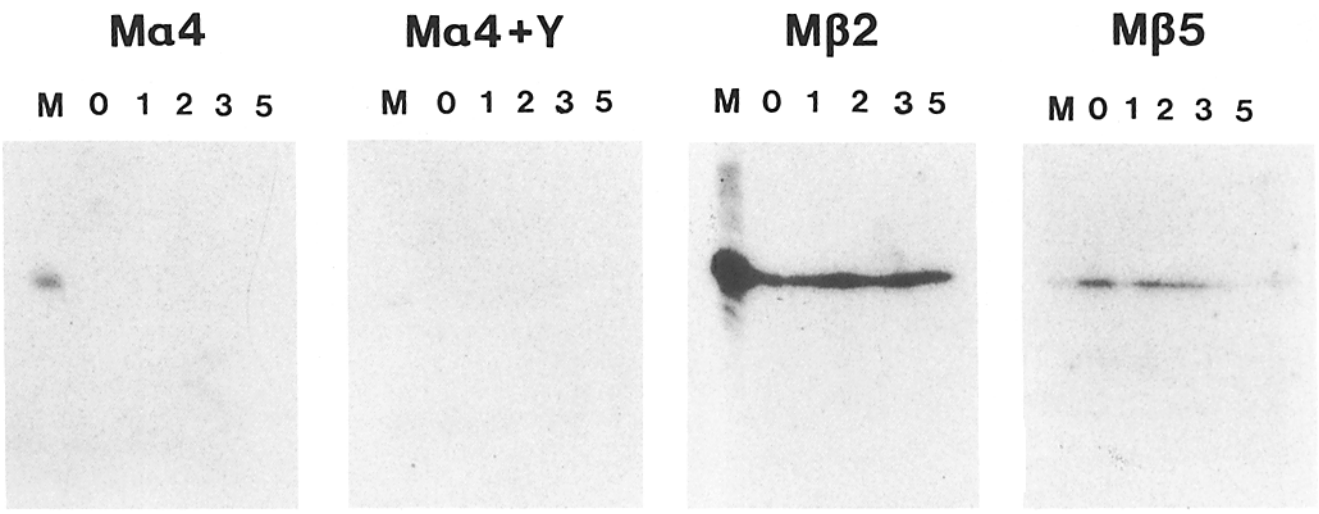


Figure 2. Expression of specific tubulin isotypes during induced fusion of C2 cells. Whole extracts of mouse C2 cells were prepared at 0, 1, 2, 3, and 5 d after induction of fusion, and equal loadings of each extract were resolved on stacking 8.5% SDS–polyacrylamide gels. *M*, marker bovine brain tubulin (5 μg). After electrophoretic transfer of the gel contents to nitrocellulose, duplicate blots were probed with α- and β-tubulin isotype–specific antisera (Gu et al., 1988). Detection of bound antibody was by reaction with ^{125}I-labeled protein A.

pany myoblast fusion, a series of Western blot experiments was performed in which each of our isotype-specific sera was used to probe whole extracts of C2 cells (Yaffe and Saxel, 1977) at daily intervals after the induction of fusion. The antibody specific for Mβ2 detects a high level of this isotype that does not appreciably change during myoblast fusion, while the Mβ5 antibody detects a lower level of expression that declines beyond the second day after the induction of differentiation (Fig. 2) at which time fusion is almost complete. Other β-tubulin isotype–specific sera (i.e., Mβ3/4 or Mβ6) as well as the Mα6-specific antibody were negative in identical Western blot experiments (data not shown). In addition, the antisera specific for the untyrosinated form of Mα4 or for the tyrosinated form of the same isotype (Mα4+Y) both failed to detect the presence of these polypeptides in whole extracts of either unfused C2 cells or differentiated fixed myotubules (Fig. 2). This observation was puzzling in view of the fact that among the mRNAs encoding α-tubulin isotypes expressed in mouse muscle, the Mα4 mRNA appears to be the most abundant (Villasante et al., 1986). We therefore performed an RNA blot transfer experiment in which a probe specific for Mα4 was used to measure the relative levels of Mα4-specific RNA during the induced fusion of C2 cells. The result of this experiment is shown in Fig. 3. The mRNA that encodes Mα4 is undetectable until 2 d after the induction of fusion, and by day 3, it is comparable to that found in adult mouse muscle (Fig. 3, lane *m*). This is in spite of the fact that the Mα4 polypeptide is undetectable in either tyrosinated or untyrosinated form on Western blots of extracts made from C2 cells during the course of induced fusion (Fig. 2). To explore this issue further, we looked at tubulin isotype expression in fixed preparations of unfused and fused C2 cells.

Free Intermingling of β-Tubulin Isotypes during the Reorganization of Microtubules That Occurs during Myoblast Fusion

Because of the complex cellular and biochemical events that accompany myoblast fusion, we decided to investigate corresponding changes in microtubule organization using sera that discriminate between the two most prominently expressed β-tubulin isotypes; i.e., Mβ2 and Mβ5. In these ex-

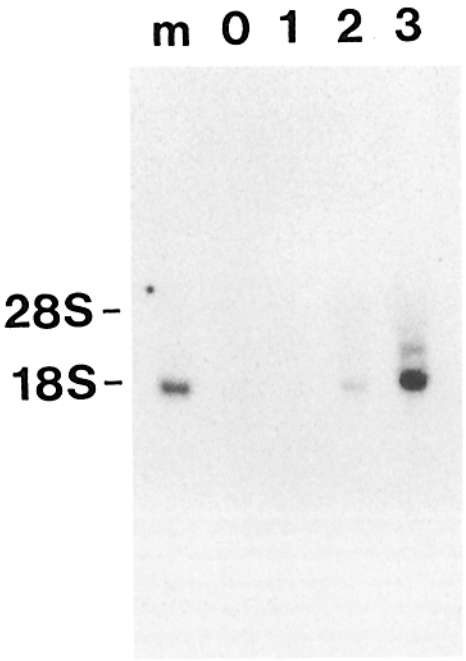


Figure 3. Expression of Mα4-specific mRNA during induced fusion of C2 cells. Total RNA was prepared from 32-d-old mouse muscle (*m*) and from mouse C2 cells at 0, 1, 2, and 3 d after induction of fusion, and aliquots (10 μg) were resolved on a 1% agarose gel containing 2.2 M formaldehyde. Equal loading of each track was confirmed by inspection of the gel under shortwave UV light at the end of the run. The gel content was transferred to nitrocellulose, and the blot hybridized with an Mα4-specific antisense oligonucleotide (Villasante et al., 1986) ^{32}P-labeled with polynucleotide kinase.

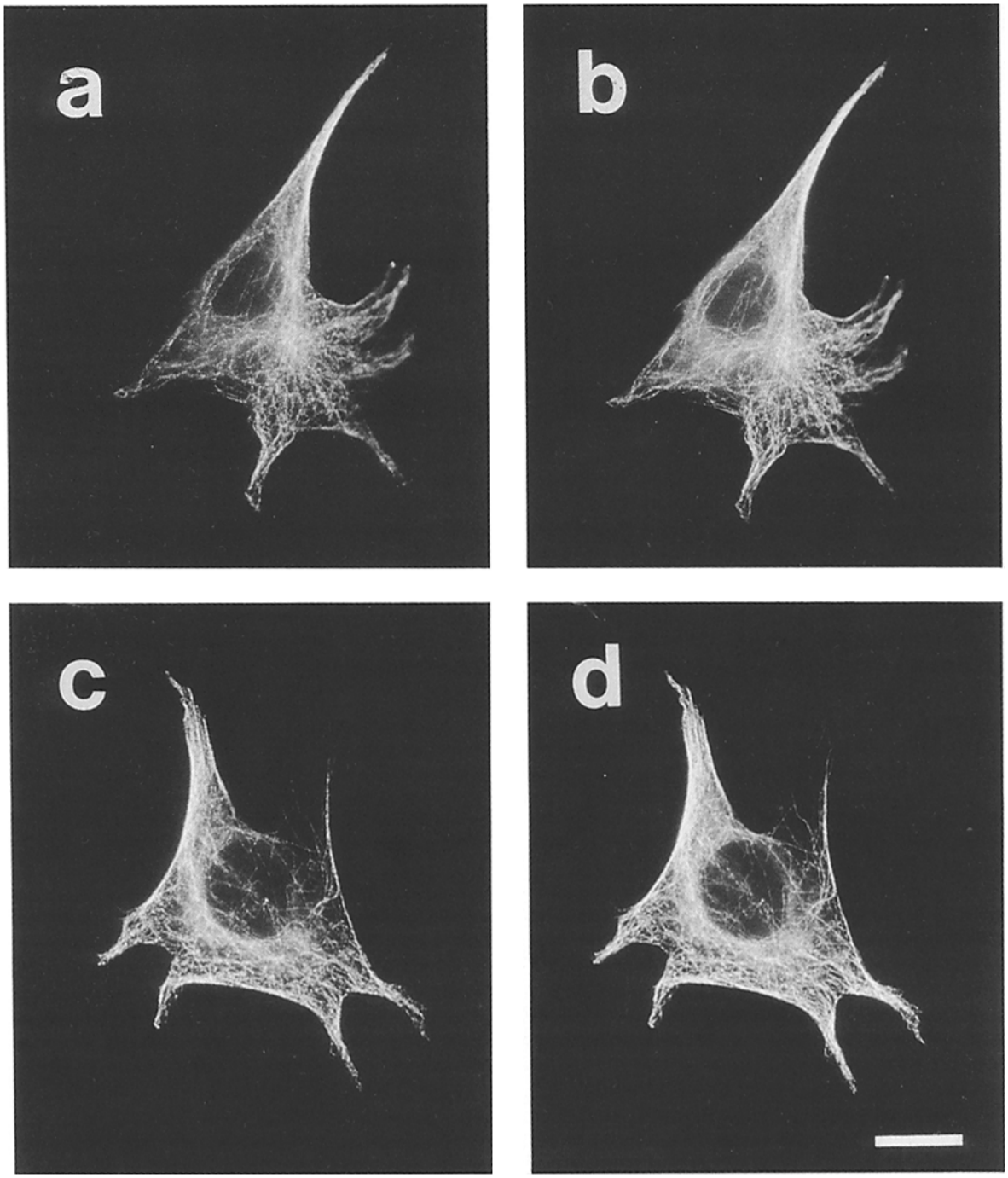


Figure 4. Coassembly of specific tubulin isotypes in unfused C2 myoblasts. Fixed, detergent-extracted preparations of uninduced C2 cells were examined by indirect double-label immunofluorescence using the antisera specific for Mβ2 (shown in *a*) or Mβ5 (shown in *c*), together with a general α-tubulin antibody that recognizes all microtubules without regard to isotype content (shown in *b* and *d*). Note the coincidence of interphase microtubules labeled with isotype-specific and general tubulin antisera (compare *a* with *b*, *c* with *d*). Spindle microtubules were also labeled coincidently (data not shown). Bar, 10 μm.

periments, fixed preparations of either unfused C2 cells or fully fused syncitia were examined by double-label indirect immunofluorescence using both an isotype-specific antibody (Mβ2 or Mβ5) and a general α-tubulin antibody to identify the entire microtubule population without regard to isotype composition. The results of these experiments are shown in Figs. 4 and 5. In unfused C2 cells, both Mβ2 and Mβ5 are present in a characteristic cytoplasmic microtubule network that is indistinguishable from the network detected by the general α-tubulin antibody (Fig. 4, *a–d*). Upon fusion into myotubules, however, there is a dramatic rearrangement of microtubule structures. The intensely labeled multidirectional microtubule bundles characteristic of unfused C2 cells is completely replaced by very fine parallel arrays of micro-

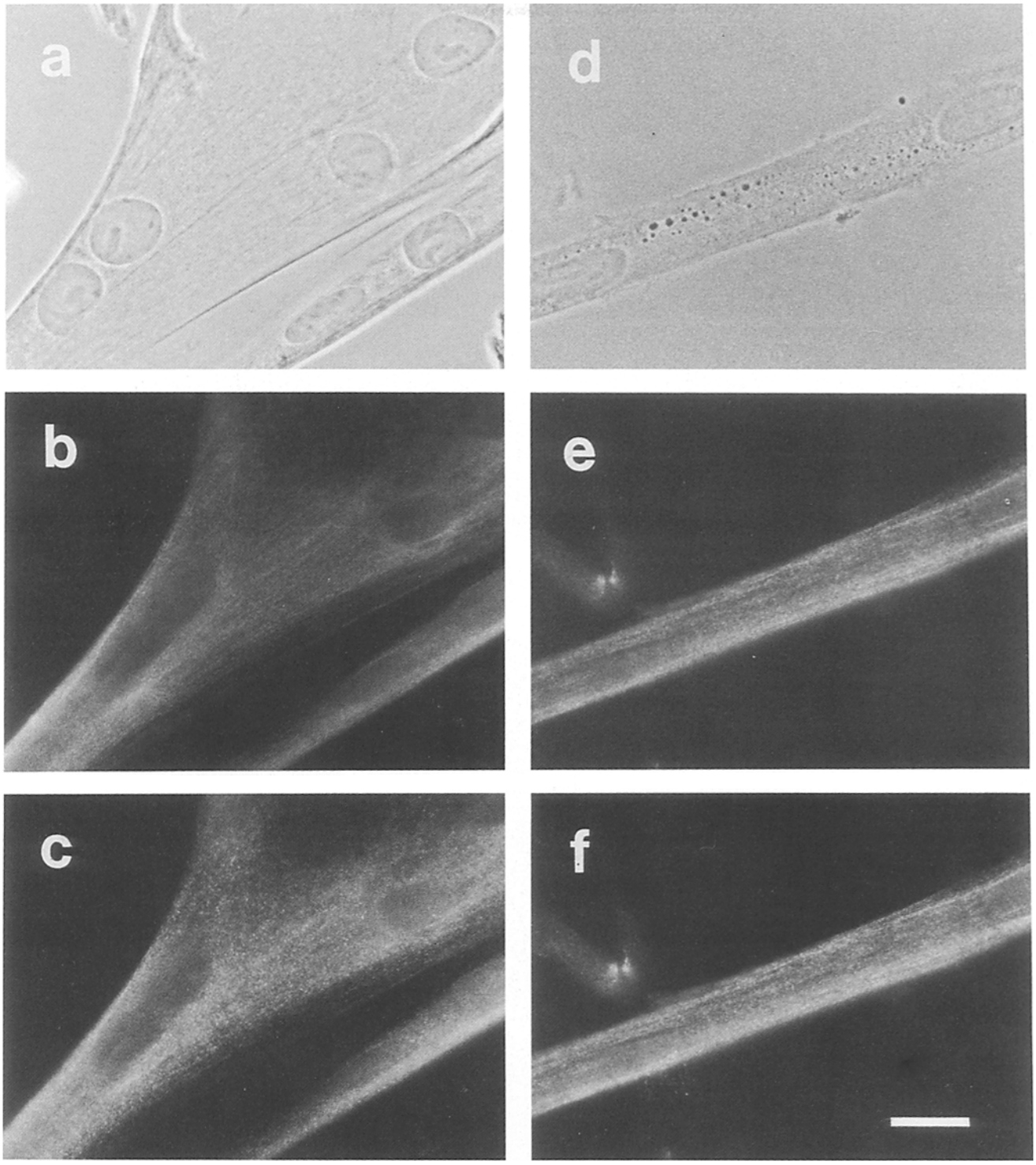


Figure 5. Coassembly of specific tubulin isotypes after myoblast fusion. Fixed, detergent-extracted preparations of fused C2 cells were examined by indirect double-label immunofluorescence using the antisera specific for Mβ2 (shown in *b*) or Mβ5 (shown in *e*) together with a general α-tubulin antibody that recognizes all microtubules without regard to isotype content (shown in *c* and *f*). *a* and *d* show the same field in *b* and *c*, and *e* and *f*, respectively, photographed using phase-contrast optics. Nuclei in the fused muscle syncitium are clearly visible (*a* and *d*). Bar, 10 μm.

tubules at the surface of the myotubules that are barely resolvable at the level of light microscopy (Fig. 5, *b*, *c*, *e*, and *f*). Nonetheless, within the degree of resolution afforded by these experiments, there appears to be complete coincidence between microtubules identified by the β-tubulin isotype-specific sera (Fig. 5, *b* and *e*) and by a general tubulin antibody in the same preparation (Fig. 5, *c* and *f*). Thus, there is no evidence for functional segregation of β-tubulin iso-

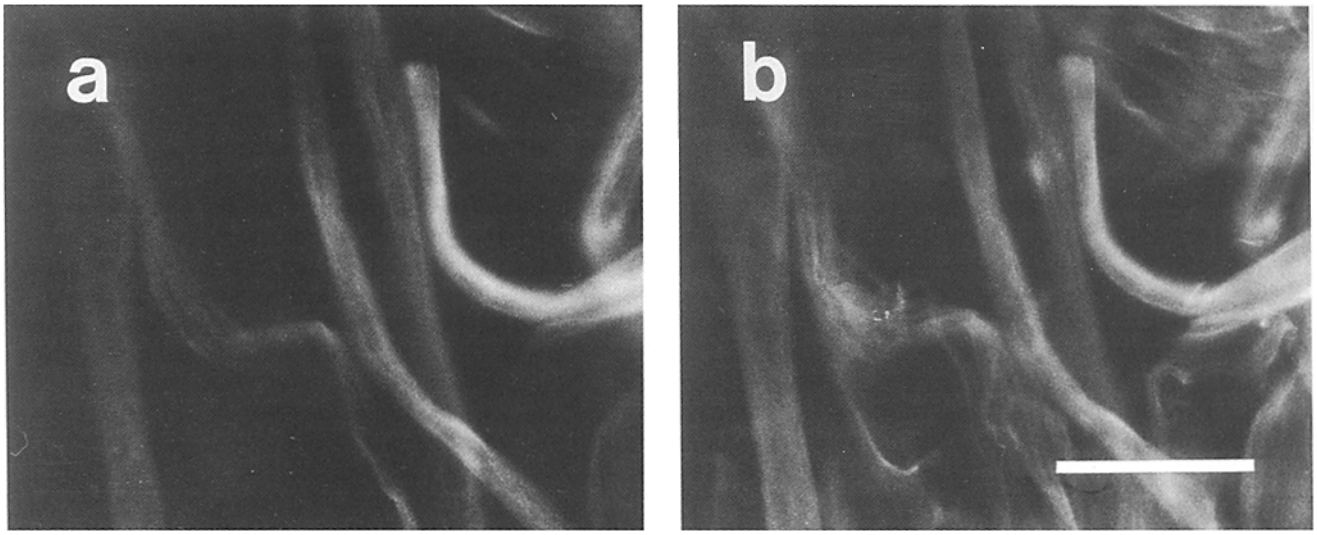
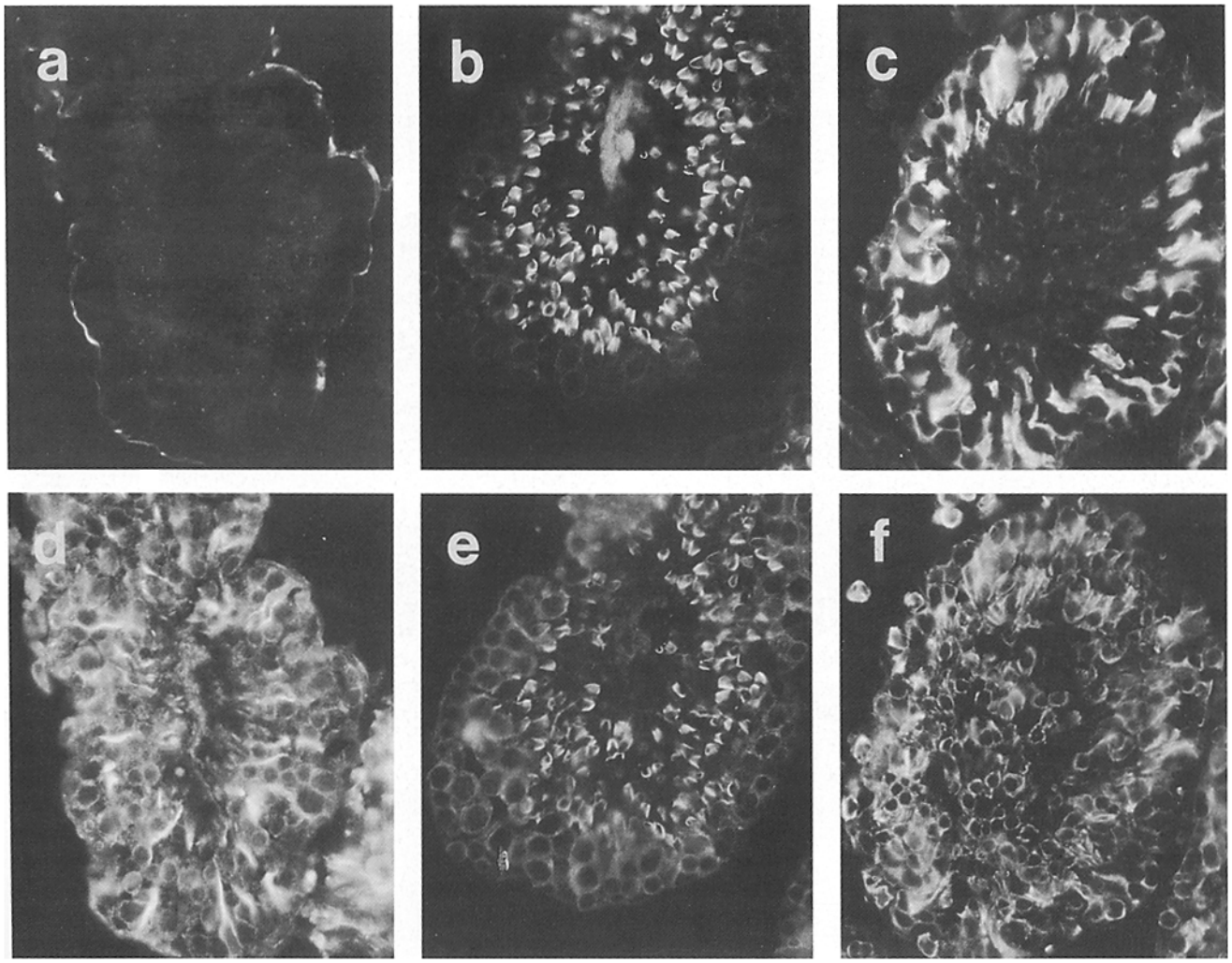


Figure 6. Expression of Mα4 in fused C2 cells. Fixed, detergent-extracted preparations of fused C2 cells (3 d after induction) were examined by indirect double-label immunofluorescence using the antibody specific for the nontyrosinated form of Mα4 together with a β-tubulin antibody that detects microtubules without regard to isotype composition. *a* shows fluorescence using the Mα4-specific antibody and *b* shows the same field detected with the general β-tubulin antibody. Note that the Mα4-specific antibody detects microtubules in some but not all of the myotubules that react with the general antibody (compare *a* and *b*). Similar experiments using the Mα4+Y antibody resulted in no detectable fluorescence (data not shown). Bar, 50 μm.



types in either unfused or fused C2 cells. As on Western blots, the sera specific for Mβ3/4, Mβ6, and Mα6 were negative on fused and unfused C2 cells.

Low Level Expression of Mα4 in Fused Myoblasts

The expression of Mα4 was undetectable in Western blots of whole extracts of unfused or fused C2 cells (Fig. 2). Nevertheless, double-label immunofluorescence experiments using the Mα4 antiserum (specific for the nontyrosinated form of Mα4) on fixed preparations of fused C2 cells did result in weak fluorescence of a subset of some myotubules (Fig. 6 *a*). Control experiments using the second (FITC- or rhodamine-labeled) antibodies alone were negative (data not shown), so that the labeling seen with the Mα4 antibody is not artefactual. Thus, a modest level of Mα4 expression is detectable at the protein level in fused C2 myotubules by immunofluorescence, though this level is too low to allow detection on Western blots of whole extracts of identical cells. The same preparation shown in Fig. 6 *a* labeled with a general tubulin antibody (Fig. 6 *b*) reveals a more extensive network of labeled myotubules, suggesting that the Mα4 polypeptide is present in some, but by no means all, of these structures. Because it is difficult or impossible to resolve individual microtubules in fused preparations of C2 cells at the level of light microscopy (see Fig. 5, *b*, *c*, *e*, and *f*), we were unable to unequivocally show whether there was consistent coincidence between microtubules labeled with Mα4 and a general tubulin antibody. However, the general appearance of the fluorescence patterns is very similar in those myotubules in which Mα4 is expressed. Curiously, corresponding experiments with the Mα4+Y antiserum failed to detect the presence of the tyrosinated form of Mα4 in any microtubules in either fused or unfused C2 cells. Thus, Mα4 is induced to

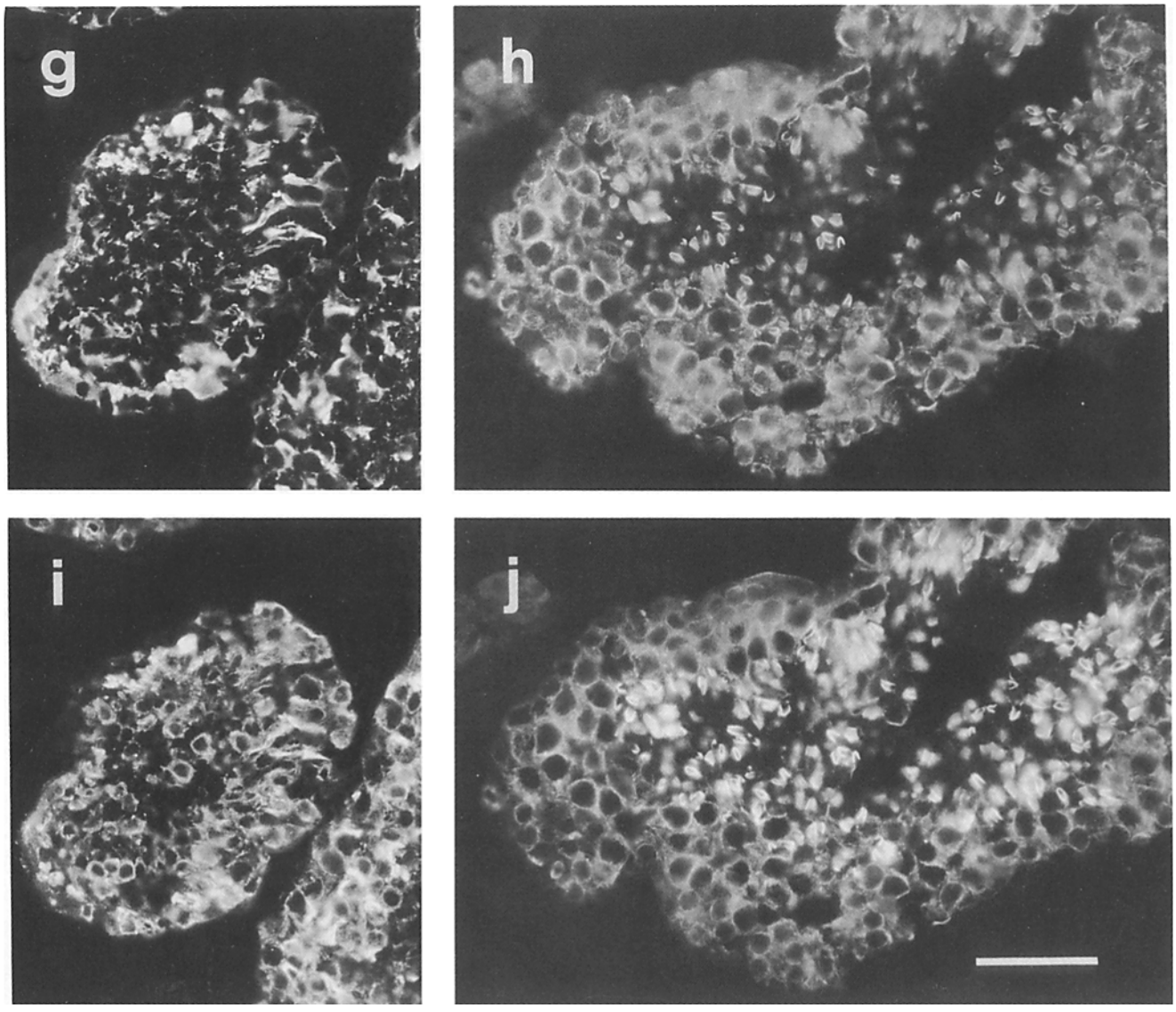


Figure 7. Expression of Mβ2, Mβ3, Mβ5, Mβ6, and Mα3 in mouse testis. Cryostat sections (6 μm) of paraformaldehyde-fixed mouse testis tissue were analyzed by indirect double-label immunofluorescence with the antisera specific for Mβ2 (*a*), Mβ3/4 (*b*), Mβ5 (*c*), Mβ6 (*g*), or Mα3/7 (*h*), together with a general α-tubulin antibody that detects all microtubules irrespective of their isotype content (shown in *d–f*, *i*, and *j*). Bar, 50 μm.

high levels during myoblast fusion, while the protein is found only at low levels, is wholly or mostly nontyrosinated, and is expressed only in a subset of myotubules. Therefore, the induced expression of the Mα4 gene that occurs upon fusion appears subject to control at the translational or posttranslational level. The implications of these observations are discussed below (see Discussion).

Expression of α- and β-Tubulin Isotypes in Mammalian Testis

The diversity of cell types present within mammalian testis provides an opportunity to examine both differences in the distribution of tubulin isotypes among diverse cell types and the distribution of these isotypes among functionally distinct microtubules within the same cell type. We therefore performed double-label immunofluorescence experiments on sections of fixed mouse testis tissue using sera specific for six β- and three α-tubulin isotypes. Representative data from those sera that were positive are shown in Fig. 7. Mβ2, which is expressed at a very low level in mouse testis (as measured at the RNA level [Lewis et al., 1985]) is entirely restricted in its expression to cells of the fibrous capsule that surrounds each seminiferous tubule (Fig. 7 *a*). This labeling is not artefactual, since control experiments in which only the fluorochrome-conjugated second antibody was applied to similar sections were completely negative (data not shown) and since the Mβ2 antibody recognizes only β-tubulin on Western blots of whole testis extracts (Fig. 1). The pattern obtained with the Mβ2-specific antibody contrasts sharply with the overall pattern of microtubules detected in the same section with a general tubulin antibody (Fig. 7 *d*) in which most, if not all, cell types present in the tubule are labeled. Mβ3 is the dominant β-tubulin mRNA expressed in testis (Wang et al., 1986). The antibody to Mβ3 also recognizes Mβ4 but since this isotype is entirely brain specific (Lewis et al., 1985), any fluorescence observed with the Mβ3/4 antibody represents expression of the Mβ3 isotype alone. Sections containing developing spermatozoa showed strong labeling of manchette microtubules with the Mβ3/4-specific antibody, each manchette sheath being apparent as a V-shaped structure (Fig. 7 *b*). Weaker labeling of microtubules is observed in the cytoplasm of the round cells (which are resting or developing germ cells) and in the tails of immature spermatozoa. No labeling is observed in Sertoli cells or in cells of the fibrous capsule. The Mβ5-specific antibody is strongly expressed in the microtubules of Sertoli cells that surround and interdigitate between developing spermatids (Fig. 7 *c*); there is only very weak (if any) expression of this isotype in germ-line cells. Immunofluorescence of dissociated testis confirmed the identification of Mβ5-positive cells as Sertoli cells since only cells in which the heads of developing spermatozoa are embedded label with the Mβ5-specific antiserum (data not shown). Labeling of Sertoli cell microtubules is also evident in sections probed with the Mβ6-specific antibody, though microtubules belonging to other cell types are also detected (Fig. 7 *g*).

Neither Mα4 nor Mα6 mRNAs are expressed at appreciable levels in testis (though there is expression of an aberrant Mα4 transcript that is incapable of translation to yield a functional polypeptide [Villasante et al., 1986]). On the other hand, two mRNAs, Mα3 and Mα7, which encode an identical isotype (Mα3/7), are the dominant α-tubulin gene

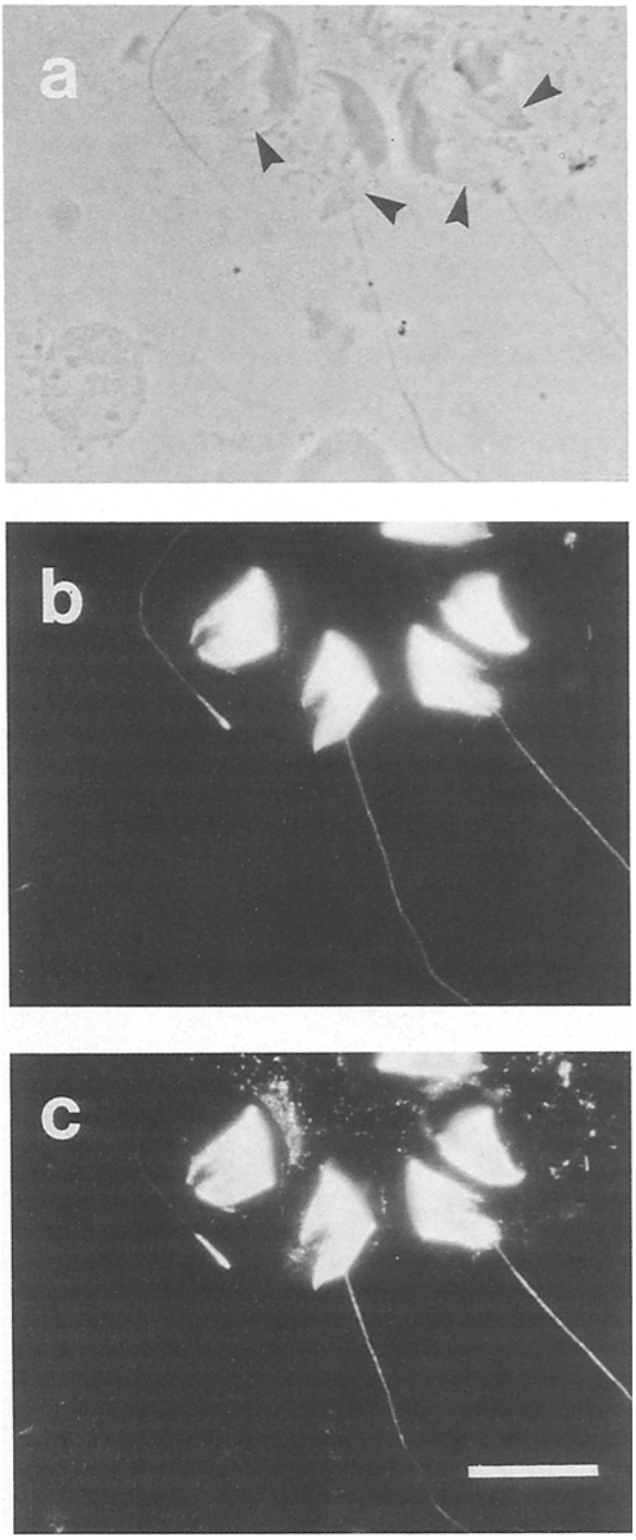


Figure 8. Coassembly of Mβ3 into manchette and flagellar microtubules in developing spermatozoa. Fixed, detergent-extracted cytospin preparations of dissociated mouse testis tissue were analyzed by indirect double-label immunofluorescence using the Mβ3/4-specific antibody plus a general tubulin antibody. (*a*) Immature spermatozoa photographed using phase-contrast optics. The dark crescent-shaped nuclei are clearly visible and are surrounded by the microtubule sheath known as the manchette (*arrowheads*). (*b* and *c*) Same field as that shown in *a* probed with the Mβ3/4-specific antibody (*b*) or a general tubulin antibody (*c*). Bar, 10 μm.

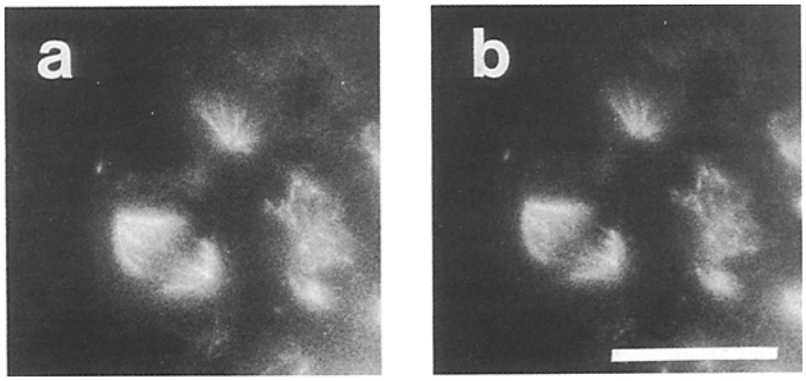


Figure 9. Assembly of Mβ3 into spindle microtubules in dividing testis cells. Double-label immunofluorescence of spindle microtubules contained in cryostat sections of mouse testis detected with the Mβ3/4-specific antibody (*a*) are shown. (*b*) Same field as that photographed in *a* showing fluorescence with a general tubulin antibody. Bar, 2 μm.

transcripts in adult testis (Villasante et al., 1986). Immunofluorescence experiments on fixed sections of mouse testis with the Mα3/7-specific antiserum showed that, like Mβ3, its expression is restricted to the germ-line cells (Fig. 7 *h*). However, the fluorescence obtained with the Mα3/7 antibody appears weaker in the manchette and tails of spermatids relative to cells in earlier stages of spermatogenesis (in contrast to Mβ3/4), possibly due to masking by microtubule-associated proteins, or to posttranscriptional modifications of α-tubulin in these structures, or both.

A Single β-Tubulin Isotype (Mβ3) Contributes to Several Functionally Distinct Kinds of Microtubule

The data described above show that there are distinct and contrasting patterns of cell type–specific expression of different tubulin isotypes within the testis. Some of these cell types (e.g., the developing spermatid) contain at least two distinct types of microtubule simultaneously; i.e., flagellar microtubules and the microtubules of the manchette. To study these structures in more detail, cytospin preparations of dissociated mouse testis cells were examined using the Mβ3/4 antibody, which strongly labels manchette microtubules (Fig. 7 *b*). The results of this experiment are shown in Fig. 8. The dark curved nuclei of the spermatids are clearly visible using phase-contrast optics and are surrounded by the microtubular sheath of the manchette (Fig. 8 *a*, *arrows*); the developing flagella are also clearly visible. Reaction with the Mβ3/4-specific antibody clearly identifies both manchette and flagellar microtubules (Fig. 8 *b*) in a manner essentially indistinguishable from the labeling pattern observed with an antibody (in this case, a monoclonal anti–β-tubulin antibody) that does not discriminate among the various β-tubulin isotypes (Fig. 8 *c*). Thus, there is no evidence for any subcellular sorting of the Mβ3 isotype between functionally distinct microtubules. Indeed, Mβ3 is also assembled into the microtubules of dividing cells in testis tissue where, once again, there is no detectable distinction between the labeling patterns observed with Mβ3 (Fig. 9 *a*) or a general antitubulin antibody (Fig. 9 *b*). Similar results were obtained for the testis-specific isotype Mα3/7 (data not shown).

Discussion

In this study, we use our newly generated α- and β-tubulin specific antisera to examine the expression and use of tubulins in two very different differentiating systems. In both cases, microtubules undergo extensive rearrangements to perform many different functions during the differentiation process. However, in these systems, as in cultured cells (Lewis et al., 1987; Lopata and Cleveland, 1987; Bond et al., 1986) we found no evidence for the subcellular sorting of tubulin isotypes. Nevertheless, the dramatically different patterns of expression of α- and β-tubulin we observed among the diverse cell types present in testis and the regulated expression of two different tubulin isotypes during myoblast fusion are both suggestive of different functional roles for distinct α- and β-tubulin gene products.

In testis, the seminiferous tubules are bounded by a fibrous capsule and contain developing germ cells and Sertoli cells which support their growth. The germ cells (spermatogonia, spermatocytes, and spermatids) are round until after meiosis when their nuclei become crescent shaped, their cytoplasm is shed, and they grow a flagellum. The heads of the developing spermatozoa are embedded in the cytoplasm of the flame-shaped Sertoli cells, with the more mature cells toward the lumen of the tubule (Rugh, 1968). Microtubules can be found in the meiotic and mitotic spindles, in the interphase cytoskeleton of all the different cells, as part of the manchette (a conical array of microtubules surrounding the spermatid nucleus that is thought to be involved in its elongation [Docher and Bennet, 1974]), and in the flagella of the spermatozoa. Using our isotype-specific antisera we find that Mβ3 and Mα3/7, the two major tubulins expressed in mature testis, are coexpressed only (or overwhelmingly) in germ cells and contribute to all the microtubule structures (i.e., manchette, spindles, interphase cytoskeleton, and flagella) in these cells (Fig. 7). Indeed, since our collection of β-tubulin sera is probably complete with respect to the isotypes expressed in testis (Wang et al., 1986), we conclude that Mβ3 is the only (or overwhelmingly predominant) β-tubulin isotype expressed postmeiotically. Therefore, it must necessarily be incorporated into many different microtubule structures, demonstrating its functional versatility. The same conclusion has been reached for a *Drosophila* testis β-tubulin isotype through the analysis of mutants (Kemphues et al., 1979, 1982; Fuller et al., 1987). Three other β-tubulin isotypes are expressed in very different patterns: Mβ2, a major brain β-tubulin, is expressed only in the fibrous capsule of the seminiferous tubules (Fig. 7 *a*); Mβ5, a widely expressed isotype, is expressed in testis mainly in Sertoli cells (Fig. 7 *c*); and Mβ6, an isotype that is primarily expressed in brain,

is found in Sertoli cells, and possibly weakly in early stages of germ cell maturation (Fig. 7 *g*). The microtubules in the cytoplasm of Sertoli cells are arranged in a highly polarized fashion, lying radially with respect to the seminiferous tubules (Fig. 7, *c*, *d*, and *g*) and converging toward the lumen.

Because of the greater homology between α-tubulin isotypes, our collection of specific antisera is less complete than it is for β-tubulin isotypes. We lack sera for Mα1 and Mα2, which differ by only a single amino acid; both are expressed in testis tissue, as demonstrated by RNA blot analysis (Lewis et al., 1985). As discussed above, Mα3/7, the major developmentally regulated testis-specific α-tubulin (Villasante et al., 1986), is restricted in its expression to the germ line (Fig. 7 *h*; Gizang-Ginsberg, E., and D. Wolgemuth, personal communication). Mα4 is not expressed in testis and Mα6 is found only in a few cells located between seminiferous tubules—probably blood cells (data not shown).

The immunofluorescence data on fixed sections of testis show marked differences in the patterns of expression of the various tubulin isotypes. However, we have made no attempt to quantitate the relative levels of expression of these isotypes at the cellular level. Nonetheless, the overall levels of expression in whole testis tissue have been examined at the level of mRNA and show that Mα3/7 and Mβ3 are by far the most abundant transcripts (Wang et al., 1986; Villasante et al., 1986).

When myoblasts fuse into myotubules, the microtubules rearrange to form long parallel fibers beneath the surface of the syncitium (Tassin et al., 1985), which are probably involved in maintaining the highly asymmetric shape of the cell and perhaps in organizing and moving its organelles and receptors (Bischoff and Holzer, 1968; Prives et al., 1982; Fernandez and Herman, 1982). We used the mouse myoblast cell line C2 (Yaffe and Saxel, 1977) to study this process with respect to the differential expression of tubulin isotypes. C2 cells undergo a rapid fusion (2–3 d) in culture when they near confluence and are switched from a growth medium containing FCS to one containing horse serum. They behave in culture much like explanted myoblasts in the time course of fusion, changes in gene expression, receptor clustering, and contraction of the resulting fibers (Yaffee and Saxel, 1977; Inestrosa et al., 1983; Bains et al., 1984). We found that the level of one β-tubulin isotype, Mβ2, remained constant during this process while the amount of the other β-tubulin present, Mβ5, decreased (Fig. 2). Most important, the transcription of the most heterologous α-tubulin isotype, Mα4, which is expressed mainly in muscle and brain, is induced to very high levels during fusion of C2 cells (Fig. 3). However, the protein itself is induced only very weakly, and interestingly, only in a subset of myotubules (Fig. 6). Thus, some translational or posttranslational mechanism must be acting differently from myotubule to myotubule to restrict expression of this isotype. What distinguishes these myotubules and what happens in vivo merits further investigation. Two Mα4 transcripts are induced in C2 cells in response to fusion. The relative abundance of these two Mα4 mRNAs is more similar to that found in cardiac rather than skeletal muscle (Fig. 3; Villasante et al., 1986), although C2 cells are derived from thigh muscle from a 2-mo-old mouse. Similarly, it has been shown that cardiac actin is the actin isotype induced in C2 differentiation (Bains et al., 1984) suggesting that these two cytoskeletal genes may be coordinately regulated.

From the results in this and the accompanying paper, as well as previous work by ourselves and others (e.g., Lewis et al., 1987; Lopata and Cleveland, 1987; Cowan et al., 1987; Wang et al., 1986; Villasante et al., 1986; Cleveland and Sullivan, 1985), a clear picture of both mammalian α- and β-tubulin isotype structure, evolution, expression, and use is emerging. Isotypes are defined by their amino acid sequences and patterns of expression, both of which have been conserved over the course of evolution (in many cases absolutely) since the mammalian radiation. Although the patterns of expression vary tremendously from cell type to cell type and during differentiation, when isotypes are coexpressed in a single cell, either naturally or in transfection experiments, all the microtubules in that cell are mixed copolymers of the available isotypes. However, the fact that coexpressed isotypes invariably coassemble does not imply that they interact in the same way with other cellular components. It seems probable that isotypes have coevolved with microtubule-associated proteins and other components present in the cell types in which they are expressed, and that the interaction between isotypes and microtubule-associated proteins creates a selective pressure that conserves isotype-defining amino acid sequence differences over eons. In this model, tubulin genes duplicated in ancestral organisms, allowing divergence of regulatory sequences and a consequent expression of different amounts of tubulin in different differentiated cell types, as has been suggested by Raff (1984). Concomitantly, the encoded isotypes have evolved to specifically or preferentially interact with MAPs in those particular cell types in which they are expressed. The striking variation in patterns of isotype-specific α- and β-tubulin expression in the diverse cell types present in testis and brain tissue (Burgoyne, R. D., M. A. Cambray-Deakin, S. A. Lewis, S. Sarkar, and N. J. Cowan, manuscript submitted for publication), as well as the developmental changes in tubulin gene expression that accompany myoblast fusion, are consistent with this model. The availability of isotype-specific sera and the expression of individual isotypes as cloned proteins should provide a basis for the study of the importance of specific tubulin–MAP interactions in vivo and in vitro.

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