MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 996–1000
0270-7306/88/020996-05$02.00/0

Vol. 8, No. 2

Localization of a Highly Divergent Mammalian Testicular α Tubulin That Is Not Detectable in Brain

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Received 17 September 1987/Accepted 6 November 1987

Sequence analysis of a mouse testicular α-tubulin partial cDNA, pRDαTT1, reveals an isotype that differs from both the somatic and the predominant testicular α tubulins at approximately 30% of the 212 amino acid residues determined. Although this mouse testicular cDNA retains the highly conserved sequence, Glu-Gly-Glu-Glu, found in the carboxyl termini of many α tubulins, the protein extends substantially beyond this sequence and does not terminate with a C-terminal tyrosine. Using rabbit antiserum prepared to a novel synthetic peptide predicted from this mouse testis α-tubulin cDNA, we have detected by immunoblot and indirect immunofluorescence an antigenic epitope present in testicular α tubulin that is not detectable in brain α tubulins. We find that the antiserum specifically binds to the manchettes and meiotic spindles of the mouse testis but not with neural fibers or tubulin extracts of the adult mouse brain. These results demonstrate that at least one of the multiple α-tubulin isotypes of the mammalian testis is expressed and used in male germ cells but not in the brain.

Microtubules, formed from heterodimers of α and β tubulin, constitute the primary structural components of mitotic and meiotic spindles, eucaryotic cilia and flagella, and elongated neuronal processes (3). The differential expression of specific tubulins during development in higher eucaryotes (3), the identification of a testis-specific β tubulin in *Drosophila melanogaster* (6), testis-specific α tubulins in mouse (4, 16) and chicken (13) testes, and a male-specific *Drosophila* α tubulin (14) suggest that tissue- or cell-type-specific microtubules could exist in organs such as the testis.

Microtubules play an important role in the dramatic morphological changes in cell structure and shape that occur during the differentiation events of spermatogenesis. Of the microtubular structures peculiar to spermatogenesis, the meiotic spindle and the manchette are highly notable. The manchette forms during the haploid phase of spermatogenesis, when round spermatids first begin to lose their spherical shape, and disappears as the male germ cell elongates during late spermatid differentiation (2). The manchette structure consists of a parallel arrangement of up to 1,100 microtubules attached to a circumnuclear, dense protein plate. Although the function of the manchette is not known, it effectively partitions the cytoplasm of the spermatid, may play a role in shaping the nucleus of the spermatozoon, and is likely involved with transport of cytoplasmic components from the anterior to the posterior portion of the developing male gamete.

Identification of a divergent testicular α tubulin. A 1,650-nucleotide cDNA insert that contains coding and 3′-untranslated sequences of the rat brain α tubulin, pILαT1 (10), was used to isolate a novel α-tubulin cDNA from a mouse testis cDNA library (4). Approximately 20,000 colonies were screened by colony hybridization. Filters were hybridized with the ^{32}P-labeled rat α-tubulin insert DNA at 65°C in a solution of 0.4 M sodium phosphate buffer (pH 8), 2.0 mM EDTA, 5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.1% sodium dodecyl sulfate), and 100 μg of sonicated salmon sperm DNA per ml for 12 to 18 h. Filters were subsequently washed at room temperature and at 65°C to a final stringency of 0.25 M sodium phosphate–1% sodium dodecyl sulfate. The mouse testis α-tubulin cDNA, pRDαTT1, identified in the screening, was found to contain the carboxyl half of an α tubulin and part of the 3′-untranslated region. Although only one clone was identified in the preliminary screen, a second partial cDNA clone with an identical coding-region sequence has been isolated from another mouse testis cDNA library (a gift from K. Kleene). To isolate the second cDNA, approximately 400,000 plaques were screened in duplicate by hybridization to a probe from the pRDαTT1 coding region, followed by washes at low and high stringencies. pRDαTT1 represents an apparently rare α-tubulin transcript, since only two isolates have been obtained after screening over 400,000 recombinants. Sequence data based upon analysis of both DNA strands, together with the predicted amino acid sequence of the partial polypeptide, are shown in Fig. 1.

A comparison of the predicted amino acid sequences of the carboxyl terminus of the coding region of pRDαTT1 with testis-specific α tubulins from mouse (Mα3/7) and chicken testes, the male-specific *Drosophila* α tubulin, a ubiquitous mouse α tubulin (mα6), and a rat brain α tubulin is shown in Fig. 2. The first amino acid in the mouse testis cDNA corresponds to amino acid 256 of the rat α-tubulin cDNA, pILαT1 (10). All of the sequences compared here, except for that of *D. melanogaster*, contain an *Eco*RI site at this point. To maximize homology, it was necessary to create two interruptions in the mouse testis tubulin sequence. One proline residue was deleted from the mouse testis cDNA at amino acid 360, and a 12-amino-acid region was deleted between amino acids 410 and 423. On both sides of the larger deletion, there is a direct repeat of 12 nucleotides, GTGAGG$^{A}_{G}$CATGG, in the rat brain α tubulin (10). Similar repeat sequences leading to deletions have been described in

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          10                  20                  30                  40
CAG ACC AGC CTG GTG CTC TAC CCT CAC ATC CAC TTC CCT CTC GCC ACT
Gln Thr Ser Leu Val Leu Tyr Pro His Ile His Phe Pro Leu Ala Thr

50                  60                  70                  80                  90
TAT GCT CCT GTC ATC TCT GCT GAG AAA GCC TAC CAG GAG CAG CTT TCT
Tyr Ala Pro Val Ile Ser Ala Glu Lys Ala Tyr Gln Glu Gln Leu Ser

  100                 110                 120                 130                 140
ATA GAA AAG ATT ACT AGT GCC TGC TTT GAG CCA GCC AAC CAG AAG GTG
Ile Glu Lys Ile Thr Ser Ala Cys Phe Glu Pro Ala Asn Gln Lys Val

    150                 160                 170                 180                 190
AAA TGT GAT TCT TGT CAT GGT AAA TAC ATG GCT TGC TGC CTA TTG TAC
Lys Cys Asp Ser Cys His Gly Lys Tyr Met Ala Cys Cys Leu Leu Tyr

        200                 210                 220                 230                 240
CAC AGT GGT ATG ATT CCC AAA GAT ATC CAT GCT GCT ATT GCC ACC ACC
His Ser Gly Met Ile Pro Lys Asp Ile His Ala Ala Ile Ala Thr Thr

          250                 260                 270                 280
AAG ACC AGG TGC AGC ATC CAG CTT GTG GAC TGG TAC CCC ACT GGC TTC
Lys Thr Arg Cys Ser Ile Gln Leu Val Asp Trp Tyr Pro Thr Gly Phe

290                 300                 310                 320                 330
AAG GTT GGC ATT AAT TAC CAG CCT CAC TGT GGT CCC TGG AGC AAC CTG
Lys Val Gly Ile Asn Tyr Gln Pro His Cys Gly Pro Trp Ser Asn Leu

  340                 350                 360                 370                 380
GCC AAG GTC CAG AGA GCT GTG TGC ATG CTG AGC AAC ACC ACA GCC ATT
Ala Lys Val Gln Arg Ala Val Cys Met Leu Ser Asn Thr Thr Ala Ile

    390                 400                 410                 420                 430
GCT GAG GCC TGG GCT CCT GTA GAC CAC AAG TTT GAT CTG TTG CAT GCC
Ala Glu Ala Trp Ala Pro Val Asp His Lys Phe Asp Leu Leu His Ala

      440                 450                 460                 470                 480
AAG CGT GCC TTT GTG CAC TGG TGT GTG GGT GAG GAC ATG GCT GCC CTA
Lys Arg Ala Phe Val His Trp Cys Val Gly Glu Asp Met Ala Ala Leu

          490                 500                 510                 520
GAG AAG GGT TAT GAG GAG GTT GGT ATG GGT TCT GTT GAA GCT GAG GGT
Glu Lys Gly Tyr Glu Glu Val Gly Met Gly Ser Val Glu Ala Glu Gly

530                 540                 550                 560                 570
GAG GAG GAG GAC AGG GAT ACT AGT TGC TGC ATC ATG TTC AGT TCC AGC
Glu Glu Glu Asp Arg Asp Thr Ser Cys Cys Ile Met Phe Ser Ser Ser

  580                 590                 600
ATT GGT AAC AGG CAT CCA TGC TAG
Ile Gly Asn Arg His Pro Cys
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FIG. 1. Nucleotide and predicted amino acid sequences of pRDαTT1. The nucleotide sequence of the cDNA insert of pRDαTT1 was determined for both strands by the method of Maxam and Gilbert (12). The DNA strands were separated and purified after end labeling by treatment with methylmercury hydroxide and electrophoresis on 5 to 10% acrylamide gels (7). The 3′ and 5′ termini of restriction fragments were labeled by using the Klenow fragment of *Escherichia coli* DNA polymerase 1 and T4 polynucleotide kinase, respectively. The nucleotides are numbered starting with the first nucleotide of the pRDαTT1 cDNA.

studies of human globin deficiencies (5). Comparison of the sequence of pRDαTT1 to the testis or male-specific α-tubulin isotypes and the two somatic α-tubulin isotypes reveals that the C terminus of pRDαTT1 differs markedly from other α-tubulin isotypes (Fig. 2). The difference in approximately 30% of the amino acids of this region of the α-tubulin molecule compared with the other α-tubulin isotypes suggested to us that this mouse testicular α tubulin may have a specialized function(s) in the testis.

An antigenic epitope in the divergent testicular α tubulin that is not detectable in brain α tubulin. Reasoning that a peptide unique to pRDαTT1 could be synthesized, allowing us to produce an antibody that could be used to localize this testicular tubulin, we used the method of Kyte and Doolittle (8) to predict possible hydrophilic sites from primary sequence data. It is important to be able to predict from the testis cDNA sequence the stretch of primary sequence most likely to elicit antibodies that will also bind to the native protein. Although this testicular α tubulin was mostly hydrophobic, as expected for α tubulins, one stretch of sequence from residues 355 to 370 was relatively more hydrophilic. In addition, part of this region, residues 360 to 370, when compared with the brain α tubulin contained a deletion and some rather significant amino acid substitutions as a result of a frameshift mutation (the deletion of two successive C residues in the mouse sequence) following amino acid 360 (Fig. 2).

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                                    260                                 270
pRDαTT1  Gln Thr Ser Leu Val Leu Tyr Pro His Ile His Phe Pro Leu Ala Thr
Mα3/7     *   *   Asn  *   *  Pro  *   *  Arg  *   *   *   *   *   *   *
Chicken   *   *   Asn  *   *  Pro Phe  *  Arg  *   *   *   *   *  Val  *
Drosoph.  *   *   Asn  *   *  Pro  *   *  Arg  *   *   *   *   *   *   *
Mα6       *   *   Asn  *   *  Pro  *   *  Arg  *   *   *   *   *   *   *
Rat       *   *   Asn  *   *  Pro  *   *  Arg  *   *   *   *   *   *   *

                                    280
pRDαTT1  Tyr Ala Pro Val Ile Ser Ala Glu Lys Ala Tyr Gln Glu Gln Leu Ser
Mα3/7     *   *   *   *   *   *   *   *   *   *   *  His  *   *   *   *
Chicken   *   *   *  Ile  *   *  Ser Asp Arg  *   *  His  *   *   *   *
Drosoph.  *   *   *   *   *   *  Val  *   *   *   *  His  *   *   *  Thr
Mα6       *   *   *   *   *   *   *   *   *   *   *  His  *   *   *  Thr
Rat       *   *   *   *   *   *   *   *   *   *   *  His  *   *   *   *

                290                                 300
pRDαTT1  Ile Glu Lys Ile Thr Ser Ala Cys Phe Glu Pro Ala Asn Gln Lys Val
Mα3/7    Val Ala Glu  *   *  Asn  *   *   *   *   *   *   *   *  Met  *
Chicken  Val Ala Glu  *   *   *  Ser  *   *   *   *  Asn  *   *  Met  *
Drosoph. Val Ala Glu  *   *  Asn  *   *   *   *   *   *   *   *  Met  *
Mα6      Val Ala Glu  *   *  Asn  *   *   *   *   *   *   *   *  Met  *
Rat      Val Ala Glu  *   *  Asn  *   *   *   *   *   *   *   *  Met  *

                                    310
pRDαTT1  Lys Cys Asp Ser Cys His Gly Lys Tyr Met Ala Cys Cys Leu Leu Tyr
Mα3/7     *   *   *  Pro Arg  *   *   *   *   *   *   *  Met  *   *
Chicken   *   *   *  Pro Gln Gln  *   *   *   *   *   *  Met  *   *
Drosoph.  *   *   *  Pro Arg Arg  *   *   *   *   *   *  Drosoph.  *
Mα6       *   *   *  Pro Arg  *   *   *   *   *   *   *   *   *   *
Rat       *   *   *  Pro Arg  *   *   *   *   *   *   *   *   *   *

                320                                 330
pRDαTT1  His Ser Gly Met Ile Pro Lys Asp Ile His Ala Ala Ile Ala Thr Thr
Mα3/7    Arg Gly Asp Val Val  *   *   *  Val Asn  *   *   *   *  Ile
Chicken  Arg Gly Asp Val Val  *   *   *  Val Asn Val  *   *   *  Ala Ile
Drosoph. Arg Gly Asp Val Val  *   *   *  Val Asn  *   *   *   *  Ile
Mα6      Arg Gly Asp Val Val  *   *   *  Val Asn  *   *   *   *  Ile
Rat      Arg Gly Asp Val Val  *   *   *  Val Asn  *   *   *   *  Ile

                                    340                                 350
pRDαTT1  Lys Thr Arg Cys Ser Ile Gln Leu Val Asp Trp Tyr Pro Thr Gly Phe
Mα3/7     *   *  Lys Arg Thr  *   *  Phe  *   *   *  Cys  *   *   *   *
Chicken   *   *  Asn Arg  *  Leu  *  Phe  *   *   *  Cys  *   *   *   *
Drosoph.  *   *  Lys Arg  *   *   *  Phe  *   *   *  Cys  *   *   *   *
Mα6       *   *  Lys Arg Thr  *   *  Phe  *   *   *  Cys  *   *   *   *
Rat       *   *  Lys Arg Thr  *   *  Phe  *   *   *  Cys  *   *   *   *

                                    360
pRDαTT1  Lys Val Gly Ile Asn Tyr Gln Pro --- His Cys Gly Pro Trp Ser Asn
Mα3/7     *   *   *   *   *   *   *   *  Pro Thr Val Val  *  Gly Gly Asp
Chicken   *   *   *   *   *   *   *   *  Pro Ile Pro Thr  *  Gly Gly Asp
Drosoph.  *   *   *   *   *   *   *   *  Pro Thr Val Val  *  Gly Gly Asp
Mα6       *   *   *   *   *   *   *   *  Pro Thr Val Val  *  Gly Gly Asp
Rat       *   *   *   *   *   *   *   *  Pro Thr Val Val  *  Gly Gly Asp

                370                                 380
pRDαTT1  Leu Ala Lys Val Gln Arg Ala Val Cys Met Leu Ser Asn Thr Thr Ala
Mα3/7     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
Chicken   *   *  Gln  *   *   *   *   *   *   *   *   *   *   *   *   *
Drosoph.  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
Mα6       *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
Rat       *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *

                                    390
pRDαTT1  Ile Ala Glu Ala Trp Ala Pro Val Asp His Lys Phe Asp Leu Leu His
Mα3/7     *   *   *   *   *   *  Arg Leu  *   *   *   *   *   *  Met Tyr
Chicken   *   *   *   *   *   *  Arg Leu  *   *   *   *   *   *  Met Tyr
Drosoph.  *   *   *   *   *   *  Arg Leu  *   *   *   *   *   *  Met Tyr
Mα6       *   *   *   *   *   *  Arg Leu  *   *   *   *   *   *  Met Tyr
Rat       *   *   *   *   *   *  Arg Leu  *   *   *   *   *   *  Met Tyr

                400                                 410
pRDαTT1  Ala Lys Arg Ala Phe Val His Trp Cys Val Gly --- --- --- --- ---
Mα3/7     *   *   *   *   *   *   *   *  Tyr  *   *  Glu Gly Met Glu Glu
Chicken   *   *   *   *   *   *   *   *  Tyr  *  Ser Glu Gly Met Glu Glu
Drosoph.  *   *   *   *   *   *   *   *  Tyr  *   *  Glu Gly Met Glu Glu
Mα6       *   *   *   *   *   *   *   *  Tyr  *   *  Glu Gly Met Glu Glu
Rat       *   *   *   *   *   *   *   *  Tyr  *   *  Glu Gly Met Glu Glu

                                    420                                 430
pRDαTT1  --- --- --- --- --- --- --- Glu Asp Met Ala Ala Leu Glu Lys Gly
Mα3/7    Gly Glu Phe Ser Glu Ala Arg  *   *  Leu  *   *   *   *   *  Asp
Chicken  Gly Glu Phe Ala Glu Ala Arg  *   *  Leu  *   *   *   *   *  Asp
Drosoph. Gly Glu Phe Ala Glu Ala Arg  *   *  Leu  *   *   *   *   *  Asp
Mα6      Gly Glu Phe Ser Glu Ala Arg  *   *   *   *   *   *   *   *  Asp
Rat      Gly Glu Phe Ser Glu Ala Arg  *   *   *   *   *   *   *   *  Asp

                                    440
pRDαTT1  Tyr Glu Glu Val Gly Met Gly Ser Val Glu Ala Glu Gly Glu Glu Glu
Mα3/7     *   *   *   *   *  Val Asp  *   *   *   *   *  Ala --- --- ---
Chicken   *  Asp  *   *  Ala Thr Asp Leu Phe  *  Asp  *  Asn  *  Ala Gly
Drosoph.  *   *   *   *   *  Ile Asp  *  Thr Thr Glu Leu  *   *  --- ---
Mα6       *   *   *   *   *  Ala Asp  *  Ala  *  Gly Asp --- --- Asp  *
Rat       *   *   *   *   *  Val Asp  *   *   *  Gly  *   *   *   *   *

                450
pRDαTT1  Asp Arg Asp Thr Ser Cys Cys Ile Met Phe Ser Ser Ser Ile Gly Asn
Mα3/7    Gly Glu Glu Tyr
Chicken   *  Ser
Drosoph. Asp Glu Glu Tyr
Mα6      Gly Glu Glu Tyr
Rat      Gly Glu Glu Tyr

pRDαTT1   Arg His Pro Cys
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FIG. 2. Comparison of the predicted amino acid sequence of pRDαTT1 with other testis-specific or predominant testicular α-tubulin isotypes from mouse (Mα3/7) (16), chicken (13), and *D. melanogaster* (14) testes. Two somatic α tubulins, mouse Mα6 (16) and rat brain α-tubulin pILαT1 (10), are also shown. pRDαTT1 begins at amino acid 256, using an alignment to the rat brain α tubulin. Asterisks indicate homology with pRDαTT1; differences are noted. Dashes indicate gaps used to optimally align the sequences. The numbers used are those for the rat protein starting with methionine as amino acid 1 (10). The sequence chosen for the synthetic peptide is boxed.

A Cys355-370 peptide, designated αTT1, was synthesized with the following sequence: Cys-Ile-Asn-Tyr-Gln-Pro-His-Ala-Gly-Pro-Trp-Ser-Asn-Leu-Ala-Lys. (The proline at amino acid position 360 is lacking in the testicular tubulin [Fig. 2].) The amino-terminal cysteine was added to facilitate conjugation to the carrier protein for immunization. Residue 362, a cysteine in the natural product sequence, was replaced by alanine to prevent cyclization and simplify synthesis. The product we obtained was 87% peptide by weight and was used without further purification. The peptide was conjugated through its amino-terminal sulfhydryl group. Two female rabbits each received primary immunizations of 1.4 mg of peptide conjugate emulsified in Freund complete adjuvant at multiple intradermal sites. Secondary immunizations, consisting of 1 mg of peptide-carrier conjugate in Freund incomplete adjuvant, were administered in the same manner 4 weeks after the primary immunizations. Sera were collected at weekly intervals, heated at 56°C for 1 h to inactivate complement, and stored at −20°C until assayed. Preimmune sera from these rabbits were treated in the same way, pooled, and used as control sera. Both rabbits responded to immunization with diptheria toxoid (DT)–Cys355-370 conjugate by producing antibodies to DT and to DT–Cys355-370 (Table 1). Rabbit anti-somatic α-tubulin antibody from pig brain did not react with either DT or the peptide.

To establish the identity of the protein recognized by the αTT1 sera, immunoblot analysis was performed. The brain and testis were each ground in a Dounce homogenizer in a solution of 0.2 M NaCl and 0.002 M $CaCl_2$ buffered with 0.05 M Tris hydrochloride at pH 7.4 (1:5 [wt/vol]). The extract was clarified by centrifugation for 20 min at 5,000 × *g* at 4°C, and aliquots were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (1, 13). Immunoblotting of a mouse testis extract revealed a single band of 58 kilodaltons reactive with the anti-peptide αTT1 sera diluted 1:100 (Fig. 3, lane A). The anti-peptide αTT1 sera were not immunoreactive by Western blotting (immunoblotting) with a mouse brain extract (Fig. 3, lane B). However, an anti-somatic tubulin serum did react with the 58-kilodalton band in both the testis and brain extracts (Fig. 3, lanes C and D). In vitro translation products directed by total testis poly(A)$^+$ RNA also yielded an immunoreactive 58-kilodalton protein detected in the supernatant with the anti-peptide αTT1 sera (data not shown).

TABLE 1. Peptide-specific antibodies in sera from rabbits immunized with DT-Cys[a]

Test antiserum and dilution	Antibody binding[b] at day after secondary immunization:			
	Cys355-370		DT	
	7	14	7	14
Antiserum 841				
1:10	235	622	398	495
Antiserum 842				
1:10	ND	1,072	231	1,414
1:100	ND	393	248	1,318
Antibody against pig brain tubulin (1:50)		0		0

[a] DT (Connaught Laboratories) was activated with 6-maleimidocaproic acyl *N*-hydroxy succinimide (9). A solid-matrix radioimmunoassay was used to test samples for antibodies to the peptide (17). Microdilution wells of polyvinyl chloride plates were incubated with the antigens. Specific antibody was detected by reaction with ^{125}I-labeled goat anti-rabbit immunoglobulin.

[b] Results are expressed as counts per minute for ^{125}I-labeled goat anti-rabbit immunoglobulin bound, corrected for background binding of pooled preimmune sera (150 cpm). ND, Not determined.

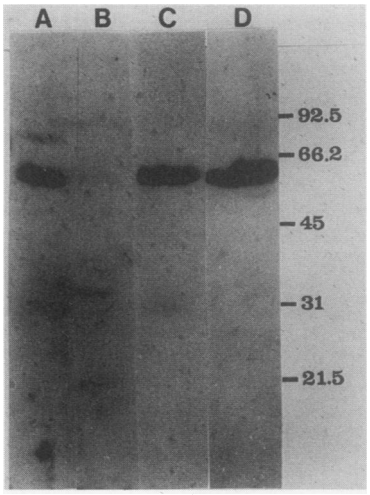

FIG. 3. Immunoblot of mouse testis and brain extracts. Extracts of mouse testis (lanes A and C) and brain (lanes B and D) were prepared from mature SJL male mice. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western transfer, lanes A and B were probed with rabbit anti-αTT1 and lanes C and D were probed with mouse anti-somatic α tubulin. The immunoblots were developed by reaction with protein A-purified rabbit anti-Cys355-370 (αTT1) (212 μg/3 ml) or mouse anti-chicken brain α tubulin (monoclonal) (1:1,000). Protein A or rabbit anti-mouse secondary antibodies labeled with ^{125}I were used at a dilution of 1:10,000 and 1:50,000, respectively. Autoradiography was used to localize immunoreactive sites on the nitrocellulose in a 4-day exposure at −70°C. The gel was calibrated with molecular mass markers (in kilodaltons) (Bio-Rad Laboratories, Richmond, Calif.) stained with amido black.

To localize the α tubulin in the testis, indirect immunofluorescence staining of cell smears was performed. Decapsulated testes from adult BALB/cBy mice, trimmed into small pieces, were incubated in warm RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% fetal calf serum (TC buffer), and the cells were dispersed by shaking in a 37°C water bath for 30 min. The cell suspension was filtered through nylon gauze and washed twice in warm TC buffer by centrifugation at 700 × *g* for 15 min. Cell smears, made by cytocentrifugation, were rapidly fixed in 3% fresh paraformaldehyde in phosphate-buffered saline, followed by chilled acetone for 10 s. Antiserum to αTT1 peptide reacted with filamentous bundles attached to the nuclei of elongated spermatids (Fig. 4A). When costained with anti-sperm acrosome antiserum, the filamentous bundles and the sperm acrosomes (arrows labeled a in Fig. 4C) were found to be located at the opposite poles of the nuclei. The filamentous-bundle immunofluorescence staining therefore represents localization of tubulin antigens in the manchettes of elongated spermatids. Monoclonal antibody to chick brain tubulin also reacted with the spermatid manchette (Fig. 4D). In addition to the manchette, the antiserum to αTT1 peptide and the monoclonal antibody to chick brain both reacted with metaphase spindles of large spermatogenic cells most likely undergoing meiotic cell division (Fig. 4D, insert). Similar immunofluorescence reactions of both antibodies were found against guinea pig and hamster testis cells, indicating that the αTT1 epitope is not species specific (data not shown).

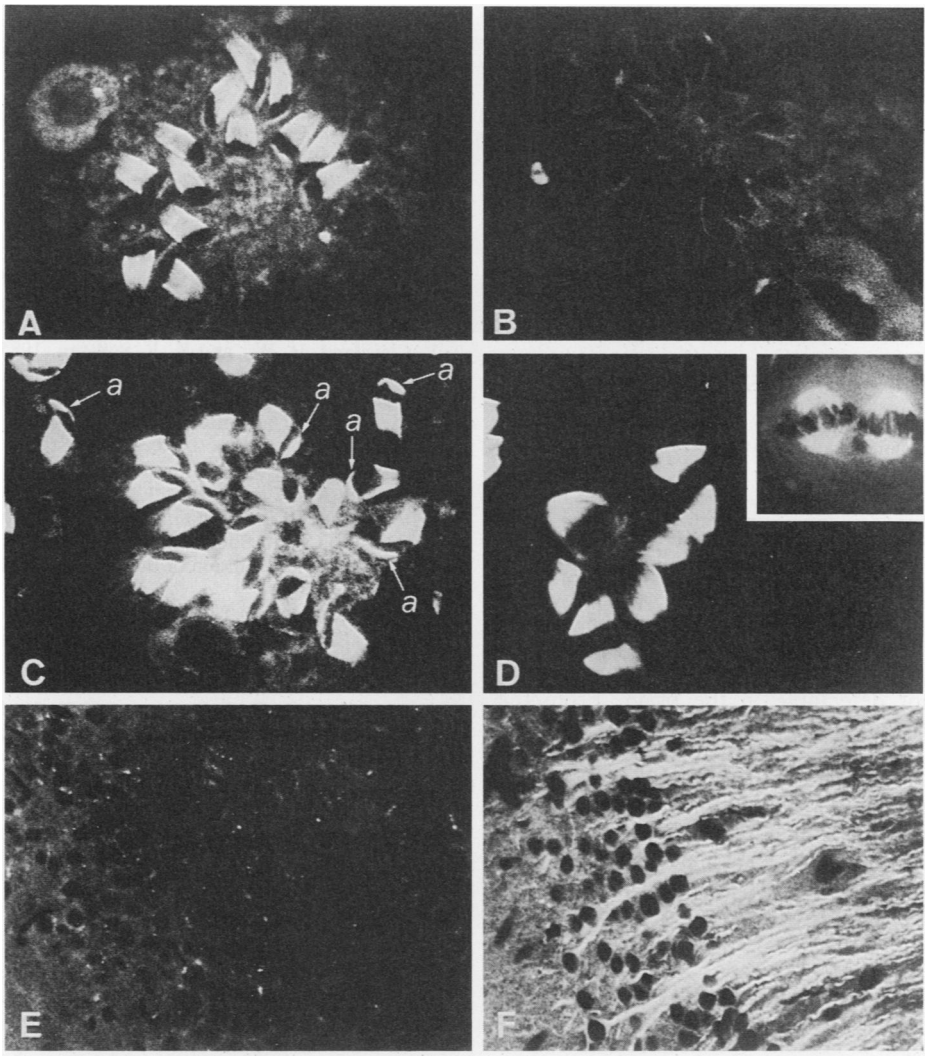

FIG. 4. Indirect immunofluorescence staining of mouse testis and cerebellum sections. Cell smears of testis (A through D) and cerebellum sections (E and F) were stained with the following antisera: anti-testis peptide αTT1 (A), anti-testis peptide αTT1 absorbed with 300 μg of peptide αTT1 (B), anti-testis peptide αTT1 and anti-mouse acrosome (C), anti-chick brain α tubulin (D and insert), anti-testis peptide αTT1 (E), and anti-chick brain α tubulin (F). Magnification, ×256. Tubulin antigen in mouse testis was detected by indirect immunofluorescence according to Cherry and Hsu (2). The cell smears and brain sections were stained with (i) rabbit antiserum immunoglobulin G (IgG) (at 1:50) against the testis peptide αTT1, with or without prior absorption with tissue or peptide antigens (in some experiments, in combination with a rabbit antiserum to mouse sperm acrosome), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antiserum IgG (Organon Teknika, Malvern, Pa.) or (ii) monoclonal antibody to chick brain α tubulin (Amersham Corp., Arlington Heights, Ill.) (at 1:500), with or without absorption with tissue or peptide antigens, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG antiserum IgG (Organon Teknika). After indirect immunofluorescence staining, the slides were stained with hematoxylin for 1 h, covered with buffered glycerol and a cover slip, and studied with the Leitz microscope with UV and visible light.

To localize the α tubulin in the brain, brain cerebellum sections were examined. A normal male BALB/cBy mouse was anesthetized with tribromoethanol and fixed by intravascular perfusion with 10% neutral formaldehyde. The brain was embedded in paraffin at 37°C, and 5-μm-thick sections of the cerebellum were deparaffinized. In contrast to the results with the testis, the αTT1 antibody did not react with the mouse brain antigen (Fig. 4E). The monoclonal antibody to chick brain did, however, react strongly with neural fibers in the mouse brain (Fig. 4F).

The finding that anti-testis peptide αTT1 reacted only with testicular tubulin whereas the monoclonal antibody to chick brain tubulin reacted with both brain and testis α tubulins was confirmed by the results of the immunoabsorption study (Table 2). (i) The reaction between anti-testis peptide αTT1 and the manchette was prevented after absorption with peptide αTT1 but not after absorption with cow brain tubulin. (ii) The reaction between the monoclonal antibody to chick brain α tubulin and the manchette and the mouse brain was effectively removed by absorption with cow brain tubulin but not by absorption with testis peptide αTT1. Finally, absorption of either of the antibodies with a nonhomologous six-residue peptide made from a different region of the testicular α tubulin (amino acids 389 to 394 in Fig. 1) had no effect on their reactions with the manchette or brain.

Possible significance of a divergent testicular α tubulin. Focusing on antigenic differences in mammalian α tubulins, we have identified an antigenic epitope for a mouse testicular

TABLE 2. Immunofluorescence reaction of anti-tubulin antibodies with testis and brain tubulin before and after absorption with antigens[a]

Reagents	Reaction with:	
	Testis manchette	Brain
Anti-testis peptide αTT1 antiserum IgG		
Alone	+++	−
Absorbed with peptide αTT1 (300 μg)	−	−
Absorbed with cow somatic tubulin (50 μg)	+++	−
Absorbed with a six-residue α-tubulin peptide (300 μg)	+++	−
Monoclonal anti-somatic tubulin antibody		
Alone	+++	+++
Absorbed with peptide αTT1 (300 μg)	+++	+++
Absorbed with cow somatic tubulin (12 μg)	−	−
Absorbed with a six-residue α-tubulin peptide (300 μg)	+++	+++

[a] For absorption, 200 μl of each of the antisera was incubated with 100 μg of peptide αTT1 or a six-residue α-tubulin peptide at 4°C overnight; this was repeated twice. To absorb anti-testis peptide αTT1 antiserum (at 1:50) and the monoclonal antibody to chick brain α tubulin (at 1:500) with the cow brain tubulin, these antisera were incubated once at 37°C for 60 min with 50 and 12 μg of the antigen, respectively.

α tubulin that is not detectable in the brain. From the immunofluorescence staining patterns, the antigen recognized by the αTT1 antiserum is associated with the manchettes of spermatids and the meiotic spindles. Six additional distinct mouse α-tubulin cDNAs encoding five tubulin isotypes have been isolated from a testis cDNA library (16). Of this group, two predominant testicular α tubulins have not diverged greatly from the consensus somatic α-tubulin sequence, and the absence of an initiator ATG codon in one cDNA suggests that one of these α-tubulin mRNAs may not be translated.

The data presented here establish that at least one of the testicular α-tubulin cDNAs, the sequence containing the αTT1 antigenic epitope, is actually synthesized and can be detected in certain microtubule structures. Moreover, this highly divergent testicular α-tubulin isotype is associated with the manchette and the meiotic spindle, two structures unique to germ cells. We do not know whether the same α tubulin is present in both structures or whether the unusual epitope is present in more than one α tubulin in the testis. The absence of a nucleotide sequence coding for a C-terminal tyrosine in pRDαTT1 and in a chicken testis α tubulin (13) suggests that these α tubulins could produce a more stable microtubule unable to undergo the cyclic addition and removal of tyrosine.

Two hypotheses have been proposed to explain the existence of multiple tubulin isotypes. One proposes that the polypeptides are functionally equivalent but are regulated differentially by specific regulatory signals. Recent evidence for a free intermingling of mammalian β tubulins in transfected cells supports this hypothesis (11). Alternatively, novel isotypes may produce functionally distinct microtubules needed for specialized cell structures. The existence of pRDαTT1 and the divergent chicken testicular α tubulin, two α-tubulin isotypes with highly divergent sequences and limited tissue or cellular localization, suggests this possibility.

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