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Six Mouse α-Tubulin mRNAs Encode Five Distinct Isotypes: Testis-Specific Expression of Two Sister Genes

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Five mouse α-tubulin isotypes are described, each distinguished by the presence of unique amino acid substitutions within the coding region. Most, though not all of these isotype-specific amino acids, are clustered at the carboxy terminus. One of the α-tubulin isotypes described is expressed exclusively in testis and is encoded by two closely related genes (Mα3 and Mα7) which have homologous 3′ untranslated regions but which differ at multiple third codon positions and in their 5′ untranslated regions. We show that a subfamily of α-tubulin genes encoding the same testis-specific isotype also exists in humans. Thus, we conclude that (i) the duplication event leading to a pair of genes encoding a testis-specific α-tubulin isotype predated the mammalian radiation, and (ii) both members of the duplicated sequence have been maintained since species divergence. A second α-tubulin gene, Mα6, is expressed ubiquitously at a low level, whereas a third gene, Mα4, is unique in that it does not encode a carboxy-terminal tyrosine residue. This gene yields two transcripts: a 1.8-kilobase (kb) mRNA that is abundant in muscle and a 2.4-kb mRNA that is abundant in testis. Whereas the 1.8-kb mRNA encodes a distinct α-tubulin isotype, the 2.4-kb mRNA is defective in that the methionine residue required for translational initiation is missing. Patterns of developmental expression of the various α-tubulin isotypes are presented. Our data support the view that individual tubulin isotypes are capable of conferring functional specificity on different kinds of microtubules.

Microtubules are assembled from heterodimers of α- and β-tubulin together with microtubule-associated proteins. They function in a wide variety of ways in eucaryotic cells; for example, they have specific functions in the mitotic and meiotic spindle, in the centriole, in the manchette and flagellar axoneme of spermatozoa, in axonal transport in neurons, and in the marginal bands of platelets. Clearly, associated proteins such as kinesin (28) play a crucial role in conferring these and other specific functions on microtubules. Additionally, α- and β-tubulin proteins themselves show significant heterogeneity, and the tubulin isotypes described to date vary in their patterns of expression in an evolutionarily conserved manner (16). This heterogeneity in α- and β-tubulins offers the potential of contributing to diversity of microtubule function in eucaryotic cells, either through differential polymerization of the various tubulin subunits, or by virtue of unique interaction(s) with associated proteins.

In mammals, α- and β-tubulins are encoded by large multigene families (5). A significant fraction of the genes in these families are pseudogenes (5, 13), a fact that makes it difficult to distinguish functional from nonfunctional genomic tubulin sequences. To circumvent this problem and to study the important question of the tubulin repertoire of mammals, we decided to study expressed mouse tubulin genes at the mRNA level by exhaustive screening of cDNA libraries. We recently reported the sequence and regulated expression of three mouse β-tubulin and two mouse α-tubulin isotypes (16). Here we present the complete sequence of four additional mouse α-tubulin mRNAs and compare them with the completed sequences of the two previously isolated α-tubulins. Gene-specific probes were generated to study the developmental expression of these α-tubulins. We find that two sister genes are expressed exclusively in testis and that a third gene gives rise both to an α-tubulin protein that is abundant in muscle and a mysterious larger transcript abundant in testis that lacks the N-terminally encoded methionine. Finally, we describe a gene that is expressed ubiquitously at a low level. We discuss the implications of these results for the functional diversity of mammalian microtubules.

MATERIALS AND METHODS

cDNA cloning and sequencing. RNA was prepared from the testes and bone marrow of adult Swiss Webster mice by the method of Berk and Sharp (2) and fractionated on oligo(dT) cellulose. The poly(A)$^+$ mRNA was used as a template for the synthesis of cDNA as previously described (14), and the cDNA was used to construct libraries in λgt11 (32). These libraries were not amplified, to avoid a differential amplification and consequent skewed representation of the cloned cDNAs. The libraries were replicated onto nitrocellulose (1), and the filters were probed with a gel-purified insert from the chicken α-tubulin cDNA pT1 (4), ^{32}P labeled by nick translation. Duplicate nitrocellulose filters were hybridized with the mixed nick-translated inserts of the 3′ untranslated region subclones of Mα1 and Mα2 (16) to eliminate from further analysis cDNAs identifiable as those we had already characterized. Hybridizations were carried out at 42°C in a mixture of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 20 mM phosphate buffer (pH 6.5) and 1× Denhardt solution. The library filters were washed to a final stringency of 2× SSC at 50°C. Positive hybridizing plaques that did not hybridize to the 3′ probes

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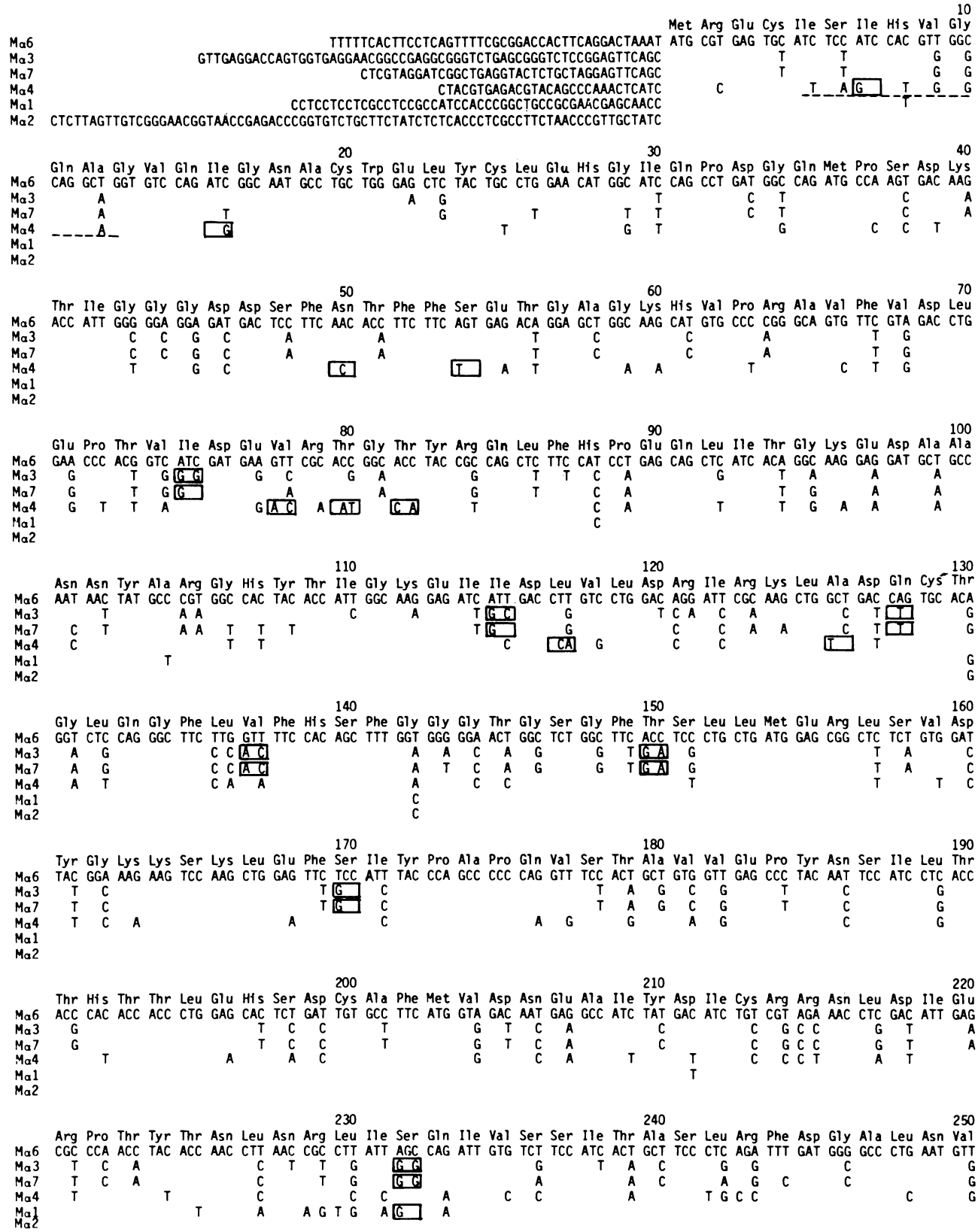
Mα6 TTTTTCACTTCCTCAGTTTTCGCGGACCACTTCAGGACTAAAT ATG CGT GAG TGC ATC TCC ATC CAC GTT GGC
Mα3 GTTGAGGACCAGTGGTGAGGAACGGCCGAGGCGGGTCTGAGCGGGTCTCCGGAGTTCAGC
Mα7 CTCGTAGGATCGGCTGAGGTACTCTGCTAGGAGTTCAGC
Mα4 CTACGTGAGACGTACAGCCCAAACTCATC
Mα1 CCTCCTCCTCGCCTCCGCCATCCACCCGGCTGCCGCGAACGAGCAACC
Mα2 CTCTTAGTTGTCGGGAACGGTAACCGAGACCCGGTGTCTGCTTCTATCTCTCACCCTCGCCTTCTAACCCGTTGCTATC
Met Arg Glu Cys Ile Ser Ile His Val Gly
10
Gln Ala Gly Val Gln Ile Gly Asn Ala Cys Trp Glu Leu Tyr Cys Leu Glu His Gly Ile Gln Pro Asp Gly Gln Met Pro Ser Asp Lys
20 30 40
CAG GCT GGT GTC CAG ATC GGC AAT GCC TGC TGG GAG CTC TAC TGC CTG GAA CAT GGC ATC CAG CCT GAT GGC CAG ATG CCA AGT GAC AAG
Thr Ile Gly Gly Gly Asp Asp Ser Phe Asn Thr Phe Phe Ser Glu Thr Gly Ala Gly Lys His Val Pro Arg Ala Val Phe Val Asp Leu
50 60 70
ACC ATT GGG GGA GGA GAT GAC TCC TTC AAC ACC TTC TTC AGT GAG ACA GGA GCT GGC AAG CAT GTG CCC CGG GCA GTG TTC GTA GAC CTG
Glu Pro Thr Val Ile Asp Glu Val Arg Thr Gly Thr Tyr Arg Gln Leu Phe His Pro Glu Gln Leu Ile Thr Gly Lys Glu Asp Ala Ala
80 90 100
GAA CCC ACG GTC ATC GAT GAA GTT CGC ACC GGC ACC TAC CGC CAG CTC TTC CAT CCT GAG CAG CTC ATC ACA GGC AAG GAG GAT GCT GCC
Asn Asn Tyr Ala Arg Gly His Tyr Thr Ile Gly Lys Glu Ile Ile Asp Leu Val Leu Asp Arg Ile Arg Lys Leu Ala Asp Gln Cys Thr
110 120 130
AAT AAC TAT GCC CGT GGC CAC TAC ACC ATT GGC AAG GAG ATC ATT GAC CTT GTC CTG GAC AGG ATT CGC AAG CTG GCT GAC CAG TGC ACA
Gly Leu Gln Gly Phe Leu Val Phe His Ser Phe Gly Gly Gly Thr Gly Ser Gly Phe Thr Ser Leu Leu Met Glu Arg Leu Ser Val Asp
140 150 160
GGT CTC CAG GGC TTC TTG GTT TTC CAC AGC TTT GGT GGG GGA ACT GGC TCT GGC TTC ACC TCC CTG CTG ATG GAG CGG CTC TCT GTG GAT
Tyr Gly Lys Lys Ser Lys Leu Glu Phe Ser Ile Tyr Pro Ala Pro Gln Val Ser Thr Ala Val Val Glu Pro Tyr Asn Ser Ile Leu Thr
170 180 190
TAC GGA AAG AAG TCC AAG CTG GAG TTC TCC ATT TAC CCA GCC CCC CAG GTT TCC ACT GCT GTG GTT GAG CCC TAC AAT TCC ATC CTC ACC
Thr His Thr Thr Leu Glu His Ser Asp Cys Ala Phe Met Val Asp Asn Glu Ala Ile Tyr Asp Ile Cys Arg Arg Asn Leu Asp Ile Glu
200 210 220
ACC CAC ACC ACC CTG GAG CAC TCT GAT TGT GCC TTC ATG GTA GAC AAT GAG GCC ATC TAT GAC ATC TGT CGT AGA AAC CTC GAC ATT GAG
Arg Pro Thr Tyr Thr Asn Leu Asn Arg Leu Ile Ser Gln Ile Val Ser Ser Ile Thr Ala Ser Leu Arg Phe Asp Gly Ala Leu Asn Val
230 240 250
CGC CCA ACC TAC ACC AAC CTT AAC CGC CTT ATT AGC CAG ATT GTG TCT TCC ATC ACT GCT TCC CTC AGA TTT GAT GGG GCC CTG AAT GTT
Mα6
Mα3
Mα7
Mα4
Mα1
Mα2

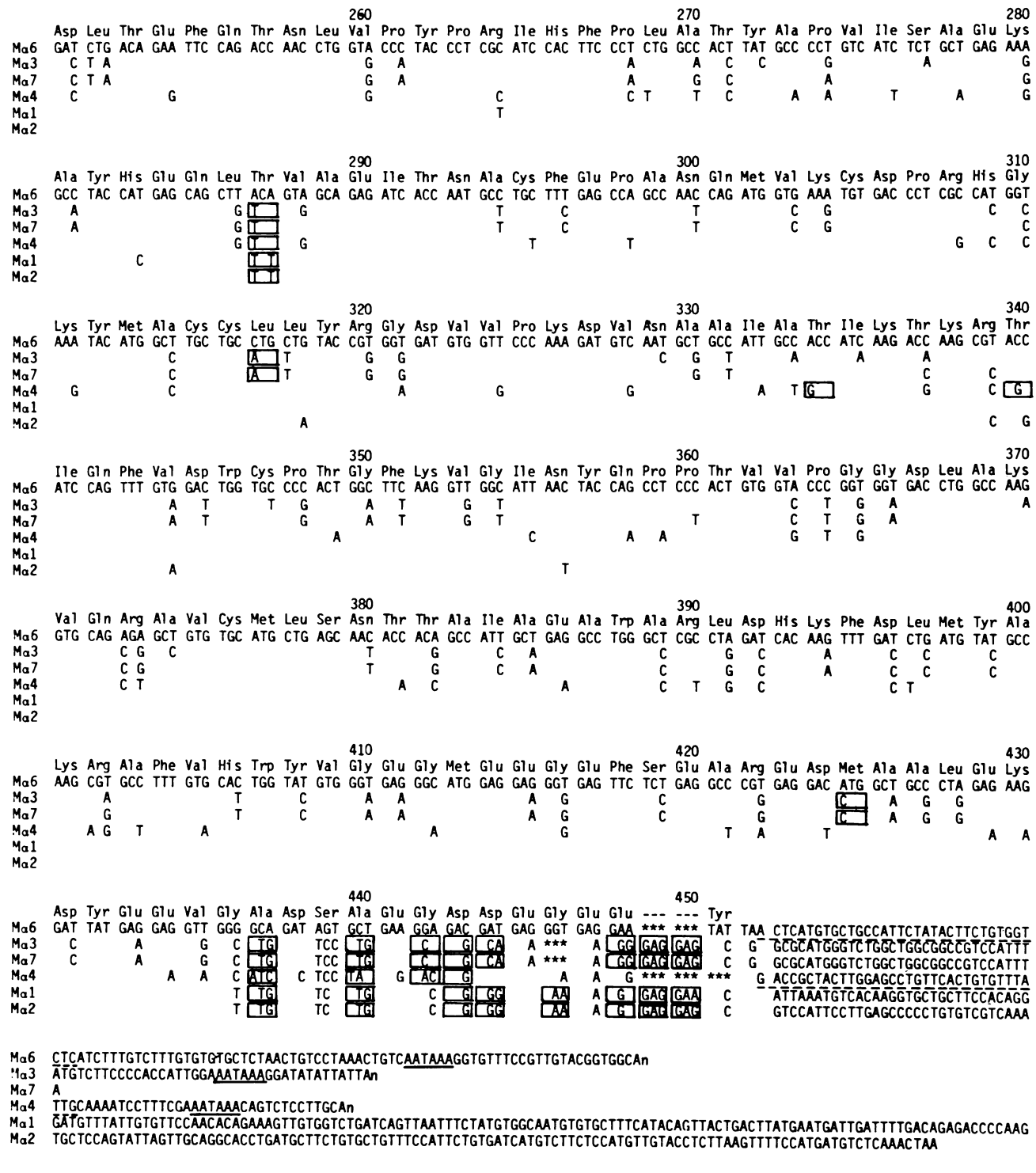

FIG. 1. Complete sequences of six α-tubulin cDNA clones, Mα6, Mα3, Mα7, Mα4, Mα1, and Mα2. Only the amino acid sequence of Mα6 is shown. Open boxes in the other sequences indicate amino acid changes with respect to Mα6. Asterisks denote amino acid deletions introduced into the carboxy-terminal region so as to maximize homology; spaces indicate sequence identity. Polyadenylation signals are underlined. Regions selected for the synthesis of antisense oligodeoxyribonucleotides for use as gene-specific probes are indicated by dashed lines. The sequence shown for Mα4 is that of the short (1.8 kb) transcript (see the text).

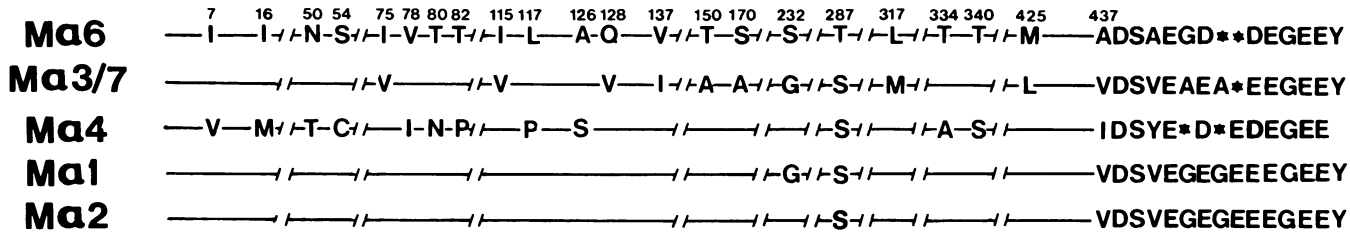

FIG. 2. Summary of isotype-specific amino acid substitutions. The reference sequence is Mα6. At positions where another isotype encodes different amino acids than those encoded by Mα6, those amino acids are indicated by the one-letter code. Only amino acid differences with Mα6 are indicated, with the exception of the carboxy-terminal region, where the complete sequence of each isotype is included.

from Mα1 or Mα2 were picked and purified. DNA was prepared from a 1-ml culture of each of these phages (S. A. Lewis and N. J. Cowan, Mol. Cell Biol., in press), cut with *Eco*RI, and resolved on 5% polyacrylamide gels. Each excised insert was subcloned into M13 and sequenced by the Sanger dideoxy chain termination method (22). Approximately 75 α-tubulin cDNA clones from each library were examined in this manner. Sequences of representative cDNAs were completed by subcloning *Bal* 31 exonuclease-deleted fragments into M13 as described elsewhere (15). The 3′ untranslated probe for Mα3/Mα7 and the 5′ probe for $M\alpha4_L$ (where L is the long transcript) were generated by this method. Antisense oligodeoxyribonucleotides were synthesized for use as gene-specific probes for Mα6 and Mα4; the sequences they hybridize to are indicated in Fig. 1.

RNA blot transfer experiments. RNA was prepared (2) from 10 different tissues dissected from mice of various ages (see figure legends). RNA concentrations were determined at A_{260}. Samples (10 or 20 μg) were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde (3). The RNA was transferred to nitrocellulose (24), and the blots were probed by the following methods: (i) with gel-purified insert from the subcloned 3′ untranslated region of Mα3, ^{32}P labeled by nick translation (21) or (ii) with one of the various oligodeoxyribonucleotides shown in Fig. 1, ^{32}P labeled with polynucleotide kinase. Hybridization and wash conditions for the nick-translated insert were as described above for the library screening. For synthetic oligonucleotides, hybridization was performed for 24 h at 42°C in a mixture of 20% formamide, 5× SSC, 5× Denhardt solution, and 20 mM phosphate buffer (pH 6.5). The blots were washed to a final stringency of 2× SSC at 60°C.

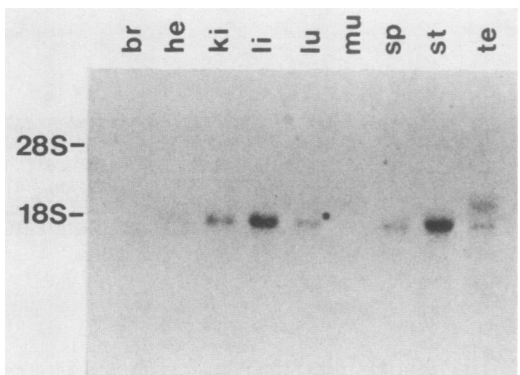

FIG. 3. Expression of Mα6 in tissues of the adult mouse. Total RNA was prepared from brain (br), heart (he), kidney (ki), liver (li), lung (lu), muscle (mu), spleen (sp), stomach (st), and testis (te) of adult mice. Portions (20 μg) of each sample were resolved on 1% agarose gels containing 2.2 M formaldehyde (3) and transferred to nitrocellulose (24). The blot was probed with the oligonucleotide complementary to the 3′ untranslated region of Mα6 (Fig. 1), ^{32}P labeled with polynucleotide kinase. The positions of 18S and 28S rRNAs are indicated as size markers. Weak but detectable expression of Mα6 in brain and muscle was evident upon longer exposure (data not shown).

RESULTS

Complete sequence of six α-tubulin cDNAs. To maximize the chance of identifying novel α-tubulin isotypes, two cDNA libraries [constructed with poly(A)$^+$ mRNA from mouse bone marrow and testis] were screened. The choice of these tissues was based on the observation that testis and lymphatic tissues (e.g., spleen and thymus) all express α-tubulin at relatively high abundance, as measured in RNA blot transfer experiments with a coding region probe. However, because this high abundance could not be accounted for by the expression of previously described α-tubulin isotypes alone (16), it seemed reasonable to expect the expression of other, novel α-tubulin isotypes in these tissues. In addition, the existence of unique kinds of microtubule structures (i.e., the manchette in spermatozoa and the marginal band in platelets) made testis and bone marrow particularly favorable sources in our search for novel tubulin isotypes. This search was facilitated by screening each library both with a chicken α-tubulin probe (to identify cloned α-tubulin coding sequences) (4) and with a

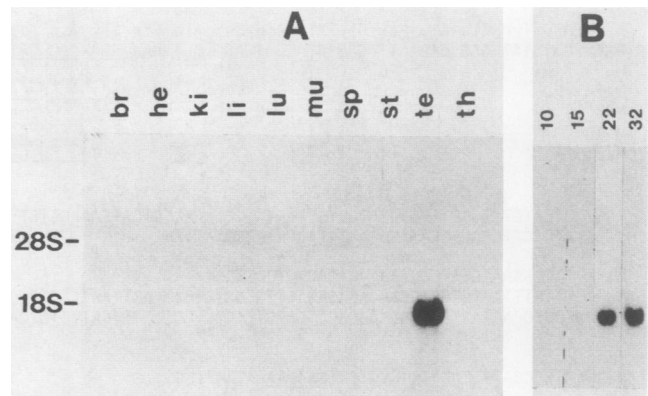

FIG. 4. Testis-specific expression of Mα3/Mα7. (A) An RNA blot identical to that described in the legend of Fig. 3 was probed with the excised insert of a subclone of Mα3 containing the entire 3′ untranslated region, ^{32}P labeled by nick translation (21). (B) Samples (10 μg) of RNA from mouse testis at days 10, 15, 22, and 32 were resolved on a formaldehyde-agarose gel (3), blotted to nitrocellulose (24), and probed with the same probe used in panel A.

mixed probe consisting of the subcloned 3′ untranslated regions of two previously described mouse α-tubulin isotypes, Mα1 and Mα2 (16). This latter probe served to identify (and thereby eliminate) many of those clones encoding previously characterized isotypes.

Four novel α-tubulin cDNAs were identified with this procedure. In each case, the complete sequence was determined from a series of extensively overlapping cloned fragments with identical sequences within the region of overlap. The sequence of each cDNA (Mα3, Mα4, Mα6, and Mα7) and the α-tubulin isotype that it encodes is shown in Fig. 1, together with the completed sequence of Mα1 and Mα2. Each cDNA represents a cloned copy of a distinct gene product, because each possesses unique 5′ and (with the exception of Mα3 and Mα7) unique 3′ untranslated regions. Also, each differs from the others in the coding region with regard to silent substitutions; more important (again with the exception of Mα3 and Mα7), each differs by virtue of a limited number of amino acid differences. Comparison of the amino acid sequences of the five α-tubulin isotypes shows that the majority of these differences lie within the 15 carboxy-terminal amino acids. Within this region, Mα1 and Mα2 are identical, as are Mα3 and Mα7. However, multiple differences exist between the carboxy-terminal regions of Mα1/Mα2, Mα3/Mα7, Mα4, and Mα6. Four of the five isotypes defined by the six cDNA clones contain an encoded carboxy-terminal tyrosine; the isotype encoded by Mα4 is

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Hα44   AGTTCTGGGTCCTCGGCCGCCCACAGGCGTCGGCGAAAG
Mα4L   C    A          T T    T G

Hα44   GCTGCCGCCCCGGCCGGGGACCAGGAAGCGTCAGGCAGC
Mα4L   C A  AA T             C G   G G T T

Hα44   TGGCAAGGGCTCCCCGGGGACGCGCCACAGCCTCACAGC
Mα4L     *T    G T G C  TTA T C    TG C A
           ▲
           G

Hα44   CGGCCCGAGTCTCCTGGGAGGCAGGGCTGGAAGGGCAGG
Mα4L    C      *** C       GAC     G  A  C

Hα44   GGTGAAGGCCAGCTGTGGCCGCTTGGGAAGGACCGCCTC
Mα4L    A       G GGACA       TT     TT T G

Hα44   GCCTGCTCCCGACCTAAGTCAGAACACCTGGATGACCGG
Mα4L   AG  T AT   C   A  G   T    C CC TGA

Hα44   TGCCTCCAGGACGCAGGTGCAGGTGAGACTCGCCCTGCC
Mα4L   C T  A  T    G     C A  C  *  T  *
                      TT

Hα44   ACAGCACCCTGCATCTCCGCGGAGGCCCTCGGGAGCCCA
Mα4L    C        G    T  TTC  A   TC   AT  G

Hα44   GCGTGTCTGCTCAAAACGAGGAAAGAATGGTTAAAGCCC
Mα4L    GACA  G         T    C    AT*********

Hα44   GAATCGCGACTCTTAATCCCAGCGGGACAG
Mα4L    GGC  T CT  *   G  A A CC
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FIG. 5. Homology between the 5′ end of the 2.4-kb transcript of Mα4 (i.e., $Mα4_L$, as discussed in the text) and the corresponding region of a human α-tubulin gene, Hα44. The sequence immediately upstream to the triplet-encoding amino acid 2 of the human gene Hα44 (Dobner et al., submitted) is shown together with the corresponding sequence at the 5′ end of $Mα4_L$. The sequences have been aligned for maximum homology, and only differences in $Mα4_L$ compared with Hα44 are indicated. Asterisks denote deletions. Termination codons in the sequence of $Mα4_L$ are underlined.

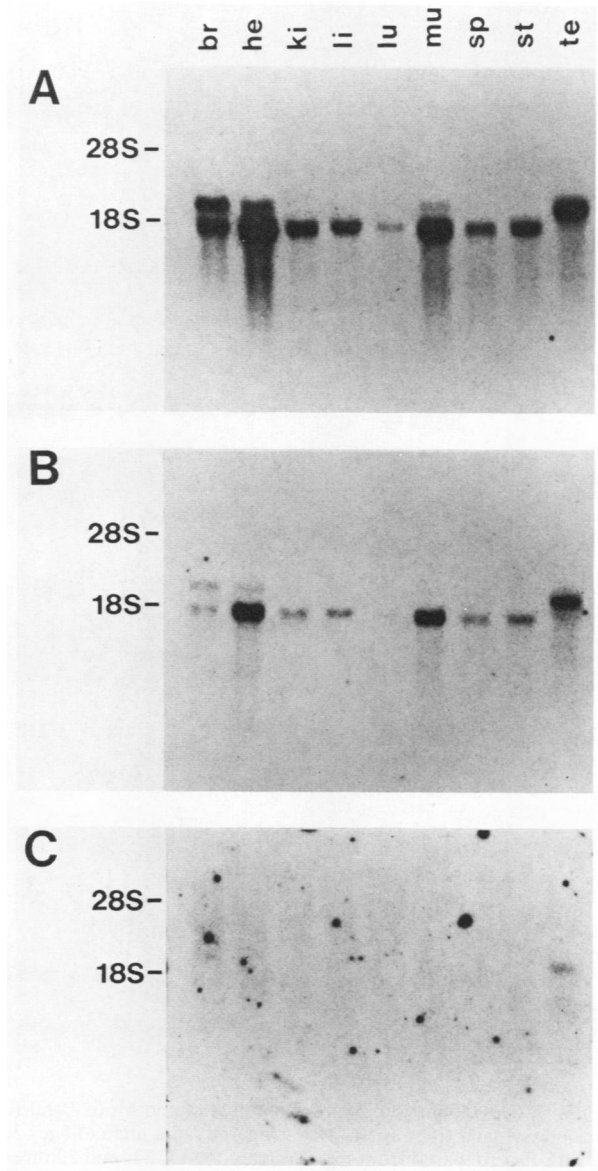

FIG. 6. Expression of Mα4 in adult mouse tissues. Blots of RNA from nine adult mouse tissues identical to those shown in Fig. 3 were used with the following probes from Mα4. (A) An antisense oligonucleotide complementary to the 3′ untranslated region of Mα4, ^{32}P-labeled with polynucleotide kinase. The extent of this probe is indicated in Fig. 1. (B) An antisense oligonucleotide complementary to 24 nucleotides of the α-tubulin coding region that uniquely recognizes Mα4 (also indicated in Fig. 1), ^{32}P-labeled with polynucleotide kinase. (C) The excised insert from a subclone of Mα4 encompassing sequences from 5′ to amino acid 2 (see Fig. 5 and the text), ^{32}P labeled by nick translation.

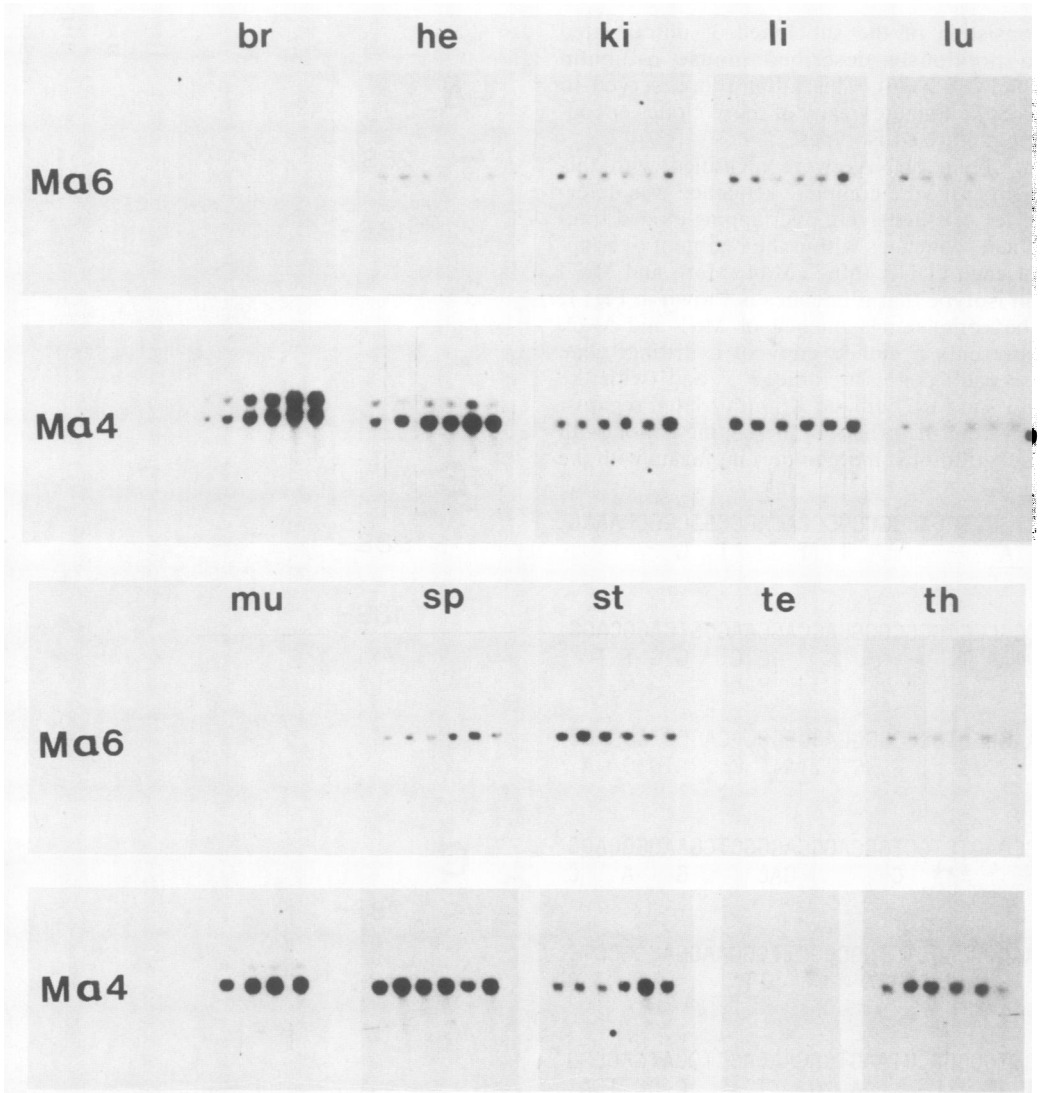

FIG. 7. Developmental expression of Mα4 and Mα6. Total RNA from brain (br), heart (he), kidney (ki), liver (li), lung (lu), spleen (sp), stomach (st), and thymus (th) was prepared from mice of ages 3, 6, 10, 15, 22, and 32 days (left to right). RNA from muscle (mu) and testis (te) was also prepared from mice of ages 10, 15, 22, and 32 days (left to right). Portions (10 μg) of each RNA sample were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde (3) and transferred to nitrocellulose. Duplicate blots were probed with the oligonucleotide complementary to the 3′ untranslated regions of Mα4 and Mα6 (see Fig. 1), ^{32}P labeled with polynucleotide kinase. A duplicate set of blots was also probed with the 3′ untranslated region probe for Mα3/Mα7, but the probe failed to detect bands in any of the RNA samples even after very long exposure times (data not shown) except those from testis (see Fig. 4B). Weak but detectable expression of Mα6 in developing brain and muscle is evident upon longer exposure (data not shown).

unusual in that it lacks a carboxy-terminal tyrosine. The amino acid substitutions characteristic of each isotype are summarized in Fig. 2.

Low-level expression of a ubiquitously expressed α-tubulin isotype (Mα6). To define the developmental and tissue-specific patterns of isotype-specific α-tubulin gene expression, probes were constructed for the unique 3′ untranslated regions of each isotype. In the case of the isotype encoded by Mα3 and Mα7, a probe was generated as a 3′ untranslated region subclone; in the case of Mα4 and Mα6, where the 3′ untranslated regions are relatively short, a synthetic antisense oligonucleotide was prepared. The regions encompassed by the gene-specific oligonucleotide probes are shown in Fig. 1. Their specificity was determined in control experiments in which each oligonucleotide was tested (i) for its ability to uniquely detect a cloned complementary sequence, and (ii) for its lack of cross-hybridization with noncomplementary cDNA clones (data not shown).

The ^{32}P-labeled Mα6-specific oligonucleotide was used in a blot transfer experiment to examine the relative abundance of Mα6 sequences in RNAs prepared from adult mouse brain, heart, kidney, liver, lung, muscle, spleen, stomach, and testis. The data (Fig. 3) show that, with the exception of brain and muscle, where Mα6 expression is barely detectable, the abundance of this isotype varies somewhat among the tissues examined. The size of the Mα6 transcript is 1.8 kilobases (kb); however, in testis, a second transcript of 2.4 kb is evident. The nature and significance of the large Mα6-specific transcript in testis is unknown.

Two genes encode an identical testis-specific isotype. Two of the cDNAs (Mα3 and Mα7) encode an identical α-tubulin isotype and indeed share an identical 3′ untranslated region,

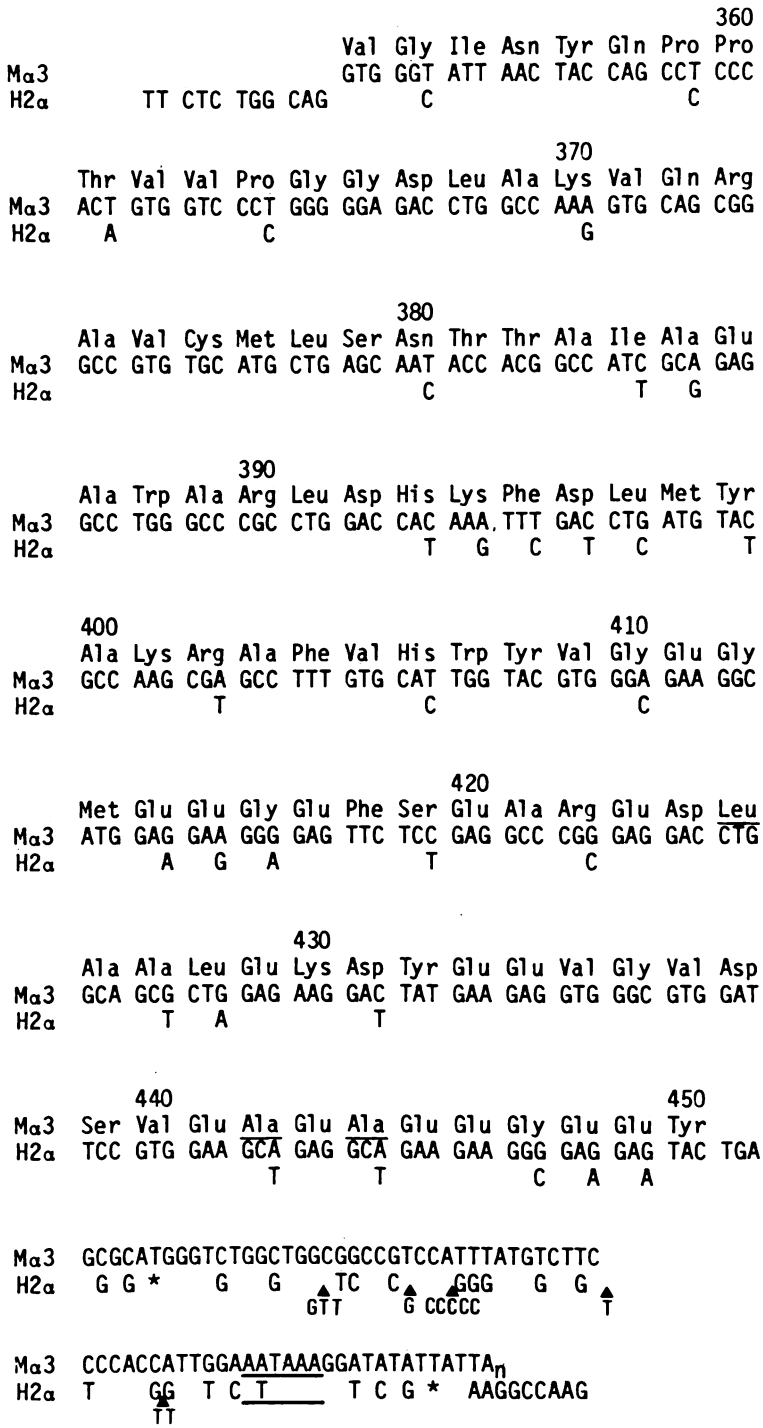

FIG. 8. A human α-tubulin gene, H2α, encodes the same isotype as Mα3/Mα7. The 3′ exon of the human α-tubulin gene, H2α, was sequenced and compared with the sequence of Mα3. Only sequence differences are indicated. Amino acids characteristic of this isotype are underlined. The 3′ untranslated regions are aligned for maximum homology; deletions are indicated by asterisks; polyadenylation signals are indicated with a thick underline.

at least within the extent of the overlapping clones examined (Fig. 1). However, multiple third codon position differences exist throughout the coding region, and the 5′ untranslated regions are somewhat dissimilar. Use of a ^{32}P-labeled subcloned 3′ untranslated region probe to determine the pattern of tissue-specific expression of Mα3 and Mα7 showed that these cDNAs are expressed exclusively in testis (Fig. 4A). Even after lengthy exposure, no Mα3/Mα7 transcript could be detected in somatic tissues at any stage of development, nor could one be detected in four stages of whole mouse embryo (data not shown). We conclude that expression of the genes encoding Mα3 and Mα7 is testis

TABLE 1. Summary of α-tubulin isotype-specific expression in mice

Gene	Approx relative levels[a] of expression in:									
	Brain	Heart	Kidney	Liver	Lung	Muscle	Spleen	Stomach	Testis	Thymus
Mα1[b]	+++	~	~	(~)	++	~	~	~	+	~
Mα2[b]	+++	+	+	+	++	~	++	+	+	+++
Mα3/Mα7									++++	
Mα6	(~)	~	~	+	~	(~)	~	+	~	~
Mα4[c]	+	++	+	+	~	++	+	+	~	+
$M\alpha4_L$[d]	+	~				~			++	

[a] ++++, Highest expression; ~, lowest expression; (~), trace.
[b] Data from Lewis et al. (16).
[c] Mα4 = 1.8-kb mRNA.
[d] $M\alpha4_L$ = 2.4-kb mRNA.

specific. The developmental regulation of these genes was examined in a blot transfer experiment by using RNA from testes of male mice of different ages. The data show barely detectable expression at day 15 and abundant expression at day 22, increasing thereafter until sexual maturity (Fig. 4B). Therefore, the expression of the genes encoding Mα3 and Mα7 appears linked to the process of spermatogenesis. The 3′ untranslated region probes used in these experiments recognize both Mα3 and Mα7 mRNAs. However, since Mα3 and Mα7 cDNAs were isolated in similar numbers from the testis cDNA library, we assume that the relative contributions of the genes to the total mRNA level are approximately equal.

A single gene yields two transcripts, one encoding an α-tubulin and one of unknown function. Multiple overlapping clones were isolated from the bone marrow and testis cDNA libraries that encode a novel α-tubulin isotype, Mα4 (Fig. 1 and 2). Curiously, sequence analysis of the cDNA clones encoding this isotype from testis ($M\alpha4_L$) showed that they lacked the initiator ATG codon. Instead, these clones included what appeared to be an amino-terminal extension. However, shortly upstream from the position expected for the initiator methionine, both the α-tubulin reading frame and all other reading frames were closed (Fig. 5). In contrast, clones encoding the identical isotype isolated from the bone marrow cDNA library did contain the initiator ATG codon in the expected position (Fig. 1). Because the Mα4-encoding cDNA clones from testis and bone marrow are absolutely identical in sequence 3′ to the position expected for the initiator methionine (including the 3′ untranslated regions), both transcripts almost certainly derive from the same gene. To investigate the tissue distribution of these transcripts, an RNA blot transfer experiment was performed with a ^{32}P-labeled antisense oligonucleotide derived from 3′ untranslated region sequences. This probe detected a 1.8-kb transcript that was particularly abundant in muscle and was present in all other somatic tissues examined; however, the 1.8-kb transcript was absent in testis, which exclusively expressed a 2.4-kb transcript, $M\alpha4_L$ (Fig. 6A). A 2.4-kb transcript was also detectable, albeit at lower abundance, in brain, heart, and striated muscle. To verify that the mRNAs recognized by the Mα4 3′ untranslated region probe encode α-tubulin, an oligonucleotide complementary to a heterologous region within the Mα4 coding sequence (Fig. 1) was synthesized and used as a gene-specific probe on a duplicate blot (Fig. 6B). Control experiments in which cDNA clones encoded all α-tubulin isotypes showed this oligonucleotide to be Mα4-specific (data not shown). The results are indeed identical to those obtained with the 3′ probe. Finally, a probe corresponding to the region of the testis Mα4 transcript (i.e., $M\alpha4_L$) 5′ to amino acid 2 was used on a duplicate blot (Fig. 6C). This probe hybridized only to the 2.4-kb transcript. The nature and significance of the bizarre 2.4-kb Mα4 testis transcript that lacks an initiator methionine is discussed below.

Developmental regulation of Mα4 and Mα6. The genes encoding Mα6 and Mα4 are expressed in a wide variety of adult mouse tissues (Fig. 3 and 6). To examine the pattern of developmental expression of these two isotypes, ^{32}P-labeled gene-specific antisense oligonucleotides were used as probes in blot transfer experiments, using equal samples of RNA from tissue dissected from mice aged 3, 6, 10, 15, 22, and 32 days. In brain and in muscle, where adult levels of Mα6 expression are barely detectable (Fig. 3), there are equally low levels of Mα6-specific mRNA during early development (Fig. 7). In other tissues showing more significant levels of adult Mα6 expression, the relative abundance of Mα6-specific mRNA does not change dramatically as a function of postnatal age. However, the 2.4-kb Mα6 transcript expressed in adult testis (Fig. 3) does not appear in testis RNA from mice aged 32 days or less. In the case of Mα4, where two transcripts are derived from a single gene (see above), some distinct developmental changes are evident. Whereas the level of Mα4 expression stays relatively unchanged in developing kidney, liver, lung, spleen, and immature thymus, there is a marked developmental increase in the level of expression of both the 1.8-kb and the 2.4-kb transcripts in

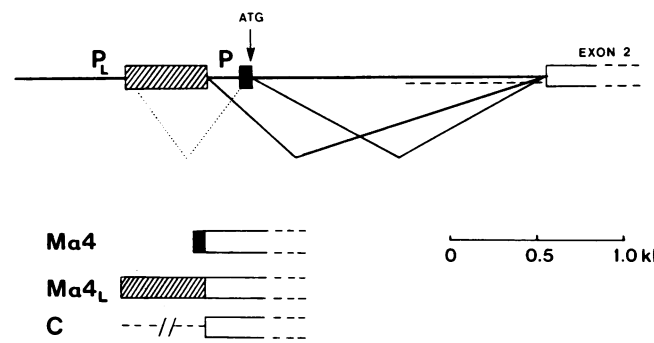

FIG. 9. Three classes of Mα4 and their relationship to the human α-tubulin gene, Hα44 (Dobner et al., submitted). Homologies between Hα44 and three Mα4 cDNA classes (Mα4, $M\alpha4_L$, and C; see the text) are shown as hatched, solid, and open boxes and the dashed line. The figure shows the probable mechanism for the generation of mRNAs represented by the cloned cDNAs. P_L and P are two promoters that yield transcripts spliced as shown. Alternatively, it is conceivable that Mα4 and $M\alpha4_L$ are derived from a transcript from P_L alone, and that this transcript is differentially spliced (as shown by the dotted line).

brain (Fig. 7). In testis, where the 2.4-kb transcript is exclusively expressed in the adult (Fig. 6), this transcript is undetectable before 32 days; instead, a very low level of the 1.8-kb transcript is evident at early developmental stages (Fig. 7).

A human α-tubulin gene subfamily encodes the same isotype-specific carboxy-terminal amino acids as do mouse cDNAs Mα3 and Mα7 and shares 3′ untranslated region homology. In an earlier report, we described the isolation of a subfamily of closely related human α-tubulin genes (31). The identification of these genes as representing of a related subfamily rested on restriction mapping data: whereas the majority of restriction sites were common to each gene, there were a large number of differences, primarily outside the coding regions, that could not be explained solely in terms of allelic differences. The isolation of the two closely related mouse α-tubulin cDNAs Mα3 and Mα7 prompted us to examine the sequence of the human α-tubulin gene subfamily described above. The sequence of the last exon of one of the members of this family, H2α, is presented in Fig. 8 and is compared with the homologous region in the mouse cDNA clone Mα3. The amino acid sequence encoded by H2α, including the characteristic carboxy-terminal region, is identical to that encoded by Mα3 and Mα7, and there is significant (58%) conservation of the 3′ untranslated regions as well. We therefore surmise that this human α-tubulin subfamily encodes the testis-specific α-tubulin isotype, and that the existence of multiple genes encoding a testis-specific α-tubulin is a feature common to all mammalian species.

DISCUSSION

In this paper, we describe the characteristics and differential expression of five α-tubulin isotypes in the developing mouse. The amino acid differences between these isotypes are summarized in Fig. 2, and their approximate relative levels of expression in 10 different tissues are summarized in Table 1. One isotype, encoded by two genes, Mα3 and Mα7, is absolutely testis specific. A second, Mα6, is a minor α-tubulin in all tissues. A third gene, Mα4, gives rise to two transcripts, the shorter of which encodes an α-tubulin abundant in muscle, and the longer of which lacks an initiation codon in the expected position. The other two α-tubulins, Mα1 and Mα2, are ubiquitously expressed, although the former is expressed primarily in lung and brain (16).

Like the β-tubulins in mouse and other vertebrate species (10, 16, 26), the α-tubulins described here differ most from each other in the sequence of the carboxy-terminal 15 amino acids (Fig. 2). As in β-tubulins (16, 25), other amino acid differences occur throughout the length of the protein, although not noticeably as clusters. All of these amino acid differences must have real significance (and not result from neutral sequence drift), because they are completely conserved in mammals in each case in which a cross-species comparison of the primary structure of the same isotype can be made. For example, two human α-tubulins, bα1 and kα1, have amino acid sequences (and expression patterns) identical to those of Mα1 and Mα2, respectively (6). Similarly, an α-tubulin cDNA encoding the same carboxy terminus and bearing 3′ untranslated region homology to Mα6 has been isolated from Chinese hamster ovary cells (9). Mα4 also has a structurally identical counterpart in monkeys and humans (P. R. Dobner, E. Kislanskis, B. M. Wentworth, and L. Villa-Komaroff, submitted for publication). Finally, we show here that a human α-tubulin gene encodes the same isotype as Mα3/Mα7. The carboxy terminus of both α- and β-tubulins is thought to be on the outer surface of the microtubule (29), and there is evidence that it is this domain that modulates microtubule assembly and binds microtubule-associated proteins (23). It seems likely, therefore, that these α-tubulin isotypes contribute to the diversity of microtubule function by differentially binding microtubule-associated proteins. Other, non-carboxy-terminal isotype-specific amino acid differences may also have subtle effects on the interaction between α- and β-subunits or on the dynamics of microtubule assembly.

Microtubules form the basis of many specialized structures in testis, such as the mitotic and meiotic spindles and the flagellar axoneme of the spermatozoan. In *Drosophila melanogaster*, a mutation in a β-tubulin gene has been described that disrupts all these functions (11, 12). (Recently, however, it has been reported that the expression of this gene is not testis specific; it is apparently expressed in the early stages of embryogenesis as well [19].) It does not follow that the mammalian testis-specific α-tubulin described here (encoded by Mα3 and Mα7) is an α-tubulin equivalent of the *D. melanogaster* β-tubulin. Indeed, the time course of appearance of the isotype encoded by Mα3/Mα7 would be consistent with postmeiotic expression, suggesting that this mammalian α-tubulin might be especially tailored for either the flagellar axoneme or the manchette.

In 1984, the isolation of a cDNA clone for a testis-specific mouse α-tubulin was reported (7). The sizes of the transcripts and 3′ untranslated region corresponding to this cDNA are completely inconsistent with all of the α-tubulins described here, including those encoded by the testis-specific cDNAs Mα3 and Mα7. Although it is possible that we failed to find an example of the previously reported cDNA in our screening experiments, we feel that this is highly unlikely, given the exhaustive nature of our search in which about 75 α-tubulin cDNAs from testis were sequenced. A more likely explanation (given that this previously reported cDNA was a single isolate) is that it represents a cloning artifact.

We present data here that demonstrate the existence of a multigene subfamily encoding the testis-specific α-tubulin isotype in both humans and mice (Fig. 8). Thus, the duplication of an ancestral, testis-specific α-tubulin gene must have occurred at some time before the mammalian radiation, and the function of (minimally) two of the resulting copies has been maintained since then, at least in mice. An interesting feature of this subfamily is the occurrence of a fourth intervening sequence at amino acid 352, in addition to the three introns common to all other vertebrate α-tubulin genes described to date (5, 30). A similar situation exists in the vertebrate α-actins, for example, where the smooth-muscle-specific α-actin has one intron in addition to those it shares with all other α-actins (27). It is possible that this extra intron plays some functional role: for example, it could contain a tissue-specific transcriptional enhancer sequence, or it could act to prevent gene conversion between these genes and the non-testis-specific α-tubulins.

Mα4 was represented in our libraries by three classes of cDNA, all of which were identical downstream from the codon encoding amino acid 2. One transcript, Mα4 (Fig. 1), represents the 1.8-kb mRNA and encodes a divergent α-tubulin, abundant in muscle and heart but absent in testis. A second transcript, $M\alpha4_L$ (the extended 5′ end of which is shown in Fig. 5), represents the 2.4-kb mRNA expressed in mature testis and more weakly in brain, heart, and muscle (Fig. 6). A third transcript contains approximately 1 kb of

semirepetitive DNA 5′ to amino acid 2 (data not shown). Because all these transcripts are identical 3′ to amino acid 2, we conclude that they each derive from the same gene. A comparison with the corresponding human α-tubulin gene, Hα44 (Dobner et al., submitted), clarifies the relationship of these three transcripts to one another (Fig. 5 and 9). Mα4 and $M\alpha4_L$ appear to be transcripts from two different promoters, both of which splice to the triplet encoding amino acid 2 of the gene, which is the start of the second exon of this and all other hiterto described vertebrate α-tubulin genes (5), whereas the third cDNA (designated C) (Fig. 9) is a copy of an unspliced transcript. Because the long mRNA ($M\alpha4_L$) has (i) no initiation codon in frame with the α-tubulin coding region, (ii) many stop codons in the region upstream from amino acid 2, and (iii) a seemingly random set of mutations relative to the human gene Hα44 in this upstream region (Fig. 5) which preserve neither reading frame nor amino acids, we conclude that, at least in mice, this transcript is unlikely to be translated. The intron-exon structure of vertebrate α-tubulin genes may facilitate the occurrence of misspliced or unspliced transcripts such as those described above. The first exon of such a gene has as its 3′ end the triplet encoding the initiator methionine ATG. This sequence is imperfect with respect to the consensus donor splice signal sequence (17). Such a gene structure thus lends itself to the use of alternative promoters, although in this case, such use may be adventitious. Mα6 also gives rise to two transcripts in testis (Fig. 3), perhaps by a similar mechanism.

The α-tubulin encoded by the short (1.8 kb) transcript of Mα4 is the most divergent of the five we describe and is unique in having no encoded carboxy-terminal tyrosine residue. In this regard, it is interesting to note that an enzyme has been previously characterized that is particularly abundant in brain and muscle and that specifically tyrosinylates the carboxy terminus of some but not all α-tubulins (20). Mα4 was expressed in all somatic tissues examined, at the highest level in striated, smooth, and cardiac muscle, where it appears to be the dominant α-tubulin. Microtubules, while not abundant in muscle, act as a part of the cytoskeleton and in the organization and movement of organelles (8). Perhaps Mα4 has evolved to play a specialized role in one of these nonmitotic functions, in organelle transport, for example.

Although tubulin is a heterodimer of α- and β-tubulin subunits, α- and β-tubulin genes do not appear to be expressed in pairs. For example, we found no brain-specific α-tubulin corresponding to the brain-specific β-tubulin, Mβ4 (16). Similarly, in an extensive seach of our testis cDNA library, we found no testis-specific β-tubulin sequences analogous to Mα3 or Mα7 (although we did isolate a testis-abundant β-tubulin [unpublished data]. Furthermore, despite evidence for a hematopoetic tissue-specific β-tubulin (18; unpublished data), we found no such α-tubulin cDNA in an exhaustive search of our bone marrow cDNA library. Thus, although ubiquitous α- and β-tubulins may be coordinately expressed (16), the same does not seem to be true for these highly specialized tubulins in testis, brain, and bone marrow. We conclude that the incorporation of one of these unique isotypes, either α or β, is sufficient to confer specialization of function on a microtubule.

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