

Isotypes of α -Tubulin Are Differentially Regulated during Neuronal Maturation

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Abstract. The mRNAs for two isotypes of α -tubulin, termed T α 1 and T26, are known to be expressed in the rat nervous system. We have compared the expression of these two α -tubulin mRNAs during neural development, using RNA blotting and in situ hybridization techniques with probes directed against unique sequences of each mRNA. T α 1 mRNA is highly enriched in the embryonic nervous system but is markedly less abundant in the adult brain; T26 mRNA is expressed in many embryonic tissues with little change in abundance during development. Within the nervous system, T α 1 mRNA is enriched in regions with neu-

rons actively undergoing neurite extension, such as the cortical plate, whereas T26 mRNA is relatively homogeneous in distribution, with some enrichment in proliferative zones. Expression of T α 1 mRNA is also increased in PC12 cells induced to differentiate and extend neurite processes by nerve growth factor. Taken together, the data indicate that T α 1-tubulin mRNA is expressed at high levels during the extension of neuronal processes. The abundant expression of T α 1-tubulin mRNA may therefore reflect either a means to increase the available pool of α -tubulin or a specific requirement for the T α 1 isotype for neurite extension.

MICROTUBULES, which are assembled from α - and β -tubulins, together with microtubule-associated proteins, play an important role during neuronal process extension (Daniels, 1972), as well as providing the substrate for axonal transport in the mature neuron (Vale et al., 1985). In addition to these specialized neuronal functions, microtubules also function in the mitotic and meiotic spindles, in the centriole, and as part of the cytoskeletal framework common to all eukaryotic cells. The diversity of microtubule function may result from associated proteins such as kinesin (Vale et al., 1985) or through structural heterogeneity inherent to the tubulins. In mammals, a large number (10–20) of genes have been detected for both α - and β -tubulins (Cowan, 1984; Cleveland and Sullivan, 1985). Although several of these genes are pseudogenes, at least six different α -tubulin genes (Villasante et al., 1986), and five different β -tubulin genes (Wang et al., 1986) are expressed in the mouse. In addition to this genetic heterogeneity, there are also posttranslational modifications of tubulin polypeptides (Cleveland and Sullivan, 1985), resulting in significant microheterogeneity of tubulins.

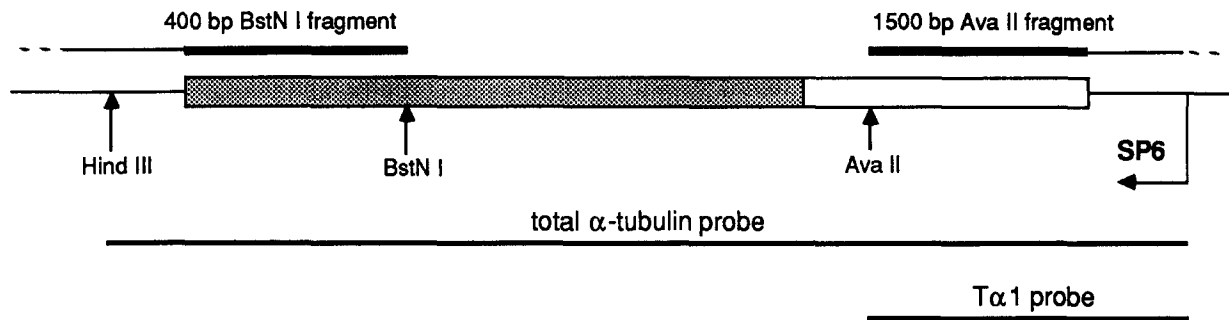
Tubulin microheterogeneity appears to be most extensive in the mammalian brain. In the rat, seven forms (isotypes) of α -tubulin can be detected by isoelectric focussing throughout development but with variations in their relative abundance, while the number of β -tubulin species increases from

three in embryonic brain to 14 in the adult (Gozes and Littauer, 1978; Wolff et al., 1982). It is likely that at least some of this heterogeneity is generated at the level of transcription: two distinct α -tubulin mRNAs (Lemischka et al., 1981; Ginzburg et al., 1981) and three β -tubulin mRNAs (Bond et al., 1984) are expressed in rat brain. The two α -tubulin mRNAs that are known to be expressed in rat brain, T α 1 (Lemischka et al., 1981) and T26 (Ginzburg et al., 1986), are homologous to the human $\beta\alpha$ 1 and $\kappa\alpha$ 1 (Cowan et al., 1985) and the mouse M α 1 and M α 2 mRNAs (Lewis et al., 1985), respectively, by virtue of their homologous amino acid sequences and their conserved 3' noncoding regions. A third mouse α -tubulin mRNA, M α 4, which has no known rat homologue, is also expressed in the postnatal brain (Villasante et al., 1986).

Tubulin mRNAs display a variety of temporal and spatial patterns of expression during development of the nervous system, suggesting that different tubulin isotypes play different functional roles during neural development or that different tubulin genes are programmed for expression during different developmental processes. In the mouse, for example, M α 1 α -tubulin mRNA decreases in abundance during embryogenesis and postnatal development of the brain, while M α 2 mRNA remains relatively constant (Lewis et al., 1985). In the rat, there is a similar general decrease in α -tubulin mRNA abundance during brain development (Bond and Farmer, 1983) but the expression of specific α -tubulin mRNAs has not been determined. We have previously reported that levels of α -tubulin mRNA are highly enriched in regions of the developing rat brain where neuronal process

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Ava II

Tα1 GAAGGAGAGGAATACTAAATTTAAATGTCACAAGGTGCTGCTTTACAGGGATGTTTATTCTGGTCCAACATAGAAAAGTTGTGGGCTGATCAGTTAATTTGTATGTG
 GluGlyGluGluTyr***

T26 GAAGGAGAGGAATACTAGTTCCTCCTGCAGTCCCTGTATCATGTCAAACAACCTCCAGCTCCAGCACTAGCTGCAGGCATCGATGCTTCTATGCTGTTCCTCCTT
T26 OLIGONUCLEOTIDE

Tα1 GCAATGTGTGCTTTTCATACAGTTACTGACTTTAAGTGTGAATGATTGTGTCAGAGACCCGAGCCGTCCACTTCACTGATGGGTTTTAAATAAAAATACCTCCCTGTCTT (A)_n

T26 CTGTGATCATGTCTTCTCCATGTGTACCTCTTAAGTTTCCAT (A)_n

Figure 1. Generation of hybridization probes for rat α -tubulin mRNAs. The structure of the T α 1-tubulin cDNA pDevo 1 in the RNA transcription vector pGEM-4 is shown at the top: the coding region is stippled and the 3' noncoding region is represented by the open box; flanking vector sequences are shown as a thin line. Anti-sense RNA probes to total α -tubulin mRNA or T α 1 mRNA were generated by SP6 transcription after linearization with the restriction endonucleases Hind III or Ava II, respectively. The location of the fragments used in the Southern blot experiment shown in Fig. 3 are indicated above: the thicker bars represent cDNA sequences within these fragments. Shown below are the nucleotide sequences of the 3' regions of T α 1 mRNA (taken from our data and Lemischka et al., 1981) and T26 mRNA (Ginzburg et al., 1981): the position of the sequence complementary to the oligonucleotide used to detect T26 mRNA is indicated.

extension is occurring (Miller et al., 1987). To extend these observations, we have defined the temporal and spatial patterns of expression of the two known rat α -tubulin mRNAs, T α 1 and T26, in developing neuronal populations. This study demonstrates that expression of T α 1 α -tubulin mRNA is correlated both temporally and spatially with process extension in the developing nervous system, whereas expression of T26 α -tubulin mRNA is constitutive, with some degree of enrichment in cells in germinal zones.

Materials and Methods

Hybridization Probes

The cDNA clone (pDevo 1) of rat α -tubulin mRNA, isolated and characterized as previously described (Miller et al., 1987), corresponded to the 3' 700 nucleotides of the T α 1 mRNA sequence described by Lemischka et al. (1981). The cDNA insert of pDevo 1 was subcloned (Fig. 1) into the RNA transcription vector pGEM-4 (Promega-Biotec, Madison, WI). Radiolabeled anti-sense RNA probes were generated from this clone with SP6 RNA polymerase (Boehringer Mannheim Diagnostics, Houston, TX) and [³⁵S]UTP (New England Nuclear, Boston, MA; 1,000 Ci/mmol) or 32P-GTP (New England Nuclear, 800 Ci/mmol) under conditions described by the manufacturer (Promega Biotec) and Melton et al. (1984). The T α 1 α -tubulin subclone was linearized with Hind III to provide a template for transcription of the entire cloned insert, including coding region sequences (Fig. 1). To prepare an RNA probe that was specific for the T α 1 α -tubulin mRNA, the same subclone was digested with Ava II, the fragments separated by electrophoresis on a 1.0% agarose gel, and the appropriate fragment excised. The purified fragment, which contained the SP6 promoter and ~150 nucleotides of the 3' noncoding region of the T α 1 α -tubulin isotype, was used as a template for RNA transcription (Fig. 1). To detect T26 mRNA, an oligonucleotide (GCATCGATGCCTGCAGCTAGTGCTG-GAGCT) was synthesized to be complementary to part of the unique 3' noncoding region of the T26 mRNA (Fig. 1). Radiolabeled oligonucleotide probes were generated either by end-labeling with [³²P]ATP and poly-

nucleotide kinase or by addition of [³⁵S]dATP using terminal deoxynucleotide transferase. Approximately five labeled residues were added to the oligonucleotide by the latter procedure.

RNA Isolation and Analysis

Timed pregnant Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) provided a source of fetal and neonatal brains of precise gestational and chronological age. Embryonic rats at the 14th (E14) and 16th (E16) days of gestation were dissected into head and body, and brain and body, respectively. The brains of neonatal rats were left whole or dissected as previously described (Lenoir et al., 1986). PC12 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum; neurite extension was induced by the addition of 50 ng/ml nerve growth factor (NGF,¹ Collaborative Research, Waltham, MA). Total cytoplasmic RNA was isolated from the freshly dissected tissue samples or from the PC12 cells by extraction with phenol/chloroform/isoamyl alcohol (Schibler et al., 1980). Alternatively, total cellular RNA was extracted by homogenization in guanidine isothiocyanate and CsCl gradient ultracentrifugation (Chirgwin et al., 1979). Poly (A)⁺ RNA was prepared by chromatography on oligo(dT) cellulose (Collaborative Research) (Aviv and Leder, 1972).

RNA samples were fractionated by electrophoresis on 1.5% agarose gels in the presence of 1 M formaldehyde (Rave et al., 1979) and transferred to nitrocellulose (Thomas, 1980). Anti-sense RNA probes labeled with ³²P were hybridized to the immobilized RNA as previously described for probes prepared by nick-translation (Lenoir et al., 1986). The ³²P end-labeled oligonucleotide probe specific to T26 mRNA was hybridized to the immobilized RNA as described by Woods et al. (1982). Blots were rehybridized with the clone pB15, which corresponds to the ubiquitous mRNA for the protein cyclophilin (P. Danielson, S. Forss-Petter, M.A. Brow, L. Calavetta, J. Douglas, R. J. Milner, and J. G. Sutcliffe, manuscript in preparation), to control for degradation and amount of the RNA samples loaded.

The relative abundances of tubulin mRNAs were estimated by hybridization of radiolabeled RNA samples to fragments of pDevo 1 on Southern blots. The clone was digested with Ava II to generate a 1,500-nucleotide

1. Abbreviation used in this paper: NGF, nerve growth factor.

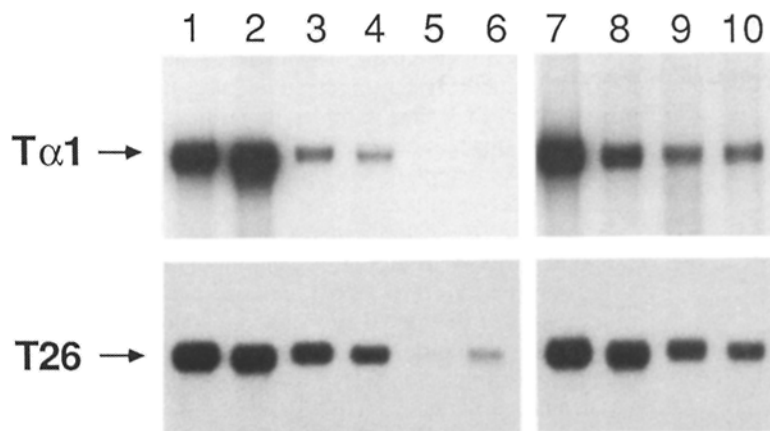


Figure 2. Expression of *Ta1* and *T26* α -tubulin mRNAs in the developing embryo (E) and neonate (P). Aliquots (1 μ g) of poly (A)⁺ RNA prepared from E16 brain (lanes 1 and 7), P1 brain (lane 2), P23 brain (lane 3), adult brain (lane 4), P1 kidney (lane 5), P1 liver (lane 6) E14 head (lane 8), E16 body (lane 9), and E14 body (lane 10) were separated by electrophoresis, blotted to nitrocellulose, and hybridized with the indicated probes labeled with ³²P. Rehybridization of the same blots with the clone pIB15 demonstrated that similar amounts of RNA were present in each lane.

fragment containing 148 nucleotides of the unique 3' region of *Ta1*, as well as vector sequences. Digestion with the enzyme *Bst*NI produced a 400-nucleotide fragment containing 153 nucleotides of coding region sequence. The digests were separated by electrophoresis on agarose gels and the appropriate fragments excised. Aliquots (0.5–1 μ g) of the fragments were mixed, separated by electrophoresis on 1% agarose gels, and blotted to nitrocellulose. The blots were hybridized with samples of tissue RNAs [2 μ g poly (A)⁺] broken by treatment at pH 9.5 and 5' end-labeled with ³²P using T4 polynucleotide kinase to give a product of \sim 100 nucleotides (Milner and Sutcliffe, 1983). These conditions were adjusted so that the immobilized DNA is in at least 5–10-fold excess over any tubulin mRNAs in the probe population. The coding region sequence contained in the *Bst*NI fragment (corresponding to amino acids 314–364 of *Ta1*, Lemischka et al., 1981) differs in only six nucleotides from the equivalent sequence of *T26* mRNA (Ginzburg et al., 1981); under the hybridization conditions used *Ta1* and *T26* sequences will hybridize to this fragment with equal efficiency. The relative hybridization to the *Bst*NI and *Ava*II fragments is a measure of the relative abundance of total and *Ta1* mRNAs, respectively.

In Situ Hybridization

Neonatal rats were anesthetized with chloral hydrate and perfused with cold 4% paraformaldehyde in 120 mM phosphate buffer (pH 7.3), and the brain removed and postfixed in the same fixative for 6 h. Embryonic rats were treated by immersion in the same fixative overnight. The tissue was subsequently rinsed in several changes of cold graded sucrose solutions (12%, 16%, 18%) and frozen, and cryostat sections 16 μ m thick were cut and mounted on chrom-alum subbed slides. Sections were stored at 4°C for immediate use, or at –70°C for longer-term storage.

Protocols for pretreatment of tissue and *in situ* hybridization analysis were based on those of Higgins and Wilson (1987). Slides were placed in 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min, rinsed several times in PBS, and immersed in 50 mM Tris-HCl (pH 7.6), 5 mM EDTA containing 100 μ g/ml proteinase K for 7.5 min. This was followed by rinsing the slides in PBS and immersion in 0.02 N HCl for 10 min. After a final PBS rinse, the slides were again fixed in 4% formaldehyde for 5 min, and dehydrated in graded ethanols containing 0.33 M sodium acetate. 1 ml of prehybridization mixture containing 50% formamide, 0.75 mM NaCl, 25 mM 1,4-piperazinediethylenesulfonic acid (Pipes), pH 6.8, 5 \times Denhardt's solution (Denhardt, 1966), 0.2% SDS, 40 mM dithiothreitol, 250 μ g/ml salmon sperm DNA, 250 μ g/ml yeast tRNA was applied to each dry slide, and the slides were subsequently incubated for 2–3 h at 45°C for RNA probes, and at room temperature for the oligonucleotide probe. The prehybridization mixture was drained from the slides and replaced with 75 μ l of hybridization mixture per slide. This latter solution consisted of the prehybridization mixture with the addition of 10% dextran sulphate and 0.1–1 ng/ml radiolabeled RNA probe or 30 ng/ml of radiolabeled oligonucleotide probe. Slides were coverslipped, sealed with contact cement, and incubated for 12–14 h at 45°C for RNA probes and room temperature for the oligonucleotide probe. Coverslips were removed in 4 \times SSC containing 20 mM 2-mercaptoethanol, and washed in several changes of 4 \times SSC. Slides hybridized to RNA probes were treated with 20 μ g/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA for 30 min at 37°C and rinsed in the same buffer for 30 min at 37°C, 2 \times SSC for 30 min at room temperature, and 0.1 \times SSC for 30 min at 45°C. Slides hybridized to oligonucleotide

probes were washed in 2 \times SSC twice for 1 h at room temperature, 1 \times SSC for 1 h at 37°C, 0.5 \times SSC for 1 h at 45°C, and 0.5 \times SSC for 1 h at room temperature. After the washes, slides were air dried and apposed to Dupont Cronex 4 film for 24–96 h to obtain x-ray images. Slides that had been hybridized to ³⁵S-labeled probes were then dipped in Kodak NTB-3 emulsion, exposed for 5–10 d, developed, and analyzed using brightfield and darkfield microscopy.

A number of controls were used to ensure specificity of hybridization to the tissue sections: prehybridization or cohybridization of a 100-fold excess of cold anti-sense RNA with the ³⁵S-labeled riboprobe, and hybridization of the tissue sections to a labeled RNA probe transcribed from linearized vector containing no insert. Hybridization of tissue sections to a labeled oligonucleotide of reversed orientation was used as a control for the oligonucleotide probes.

Results

Expression of Two Different α -Tubulin mRNAs in the Developing Rat Nervous System

In previous studies we isolated a clone (pDevo 1) from an embryonic day 16 (E16) rat brain cDNA library that was shown by nucleotide sequence analysis to correspond to *Ta1* α -tubulin mRNA (Miller et al., 1987). This clone was used to define the distribution of α -tubulin mRNAs in the developing rat brain by *in situ* hybridization (Miller et al., 1987). To define the expression of α -tubulin mRNAs in more detail, the levels of two different α -tubulin mRNAs, *Ta1* and *T26*, were examined in the developing brain by Northern blot analysis of RNA samples from E14 through adulthood (Fig. 2). The two mRNAs were detected specifically by probes directed to their 3' noncoding regions: for *Ta1* this was derived from the cloned cDNA, for *T26* an oligonucleotide complementary to part of the 3' noncoding region was used. The levels of *Ta1* α -tubulin mRNA are highest in the E16 and postnatal day 1 (P1) brain, and decrease dramatically by P23 (Fig. 2). In contrast, levels of the *T26* α -tubulin mRNA in the brain decline only twofold during development. *Ta1* is enriched in the E16 brain, whereas *T26* mRNA is present at approximately equal levels in the E16 brain and body. At E14 both *Ta1* and *T26* mRNAs appear to have similar distributions between head and body (Fig. 2), although *in situ* hybridization experiments (not shown) demonstrate that *Ta1* mRNA is also enriched in the nervous system at E14.

The relative abundances of the two α -tubulin mRNAs were determined by hybridizing radiolabeled RNA populations to fragments of the clone pDevo 1 on Southern blots. This experiment was designed so that both *Ta1* and *T26* mRNA se-

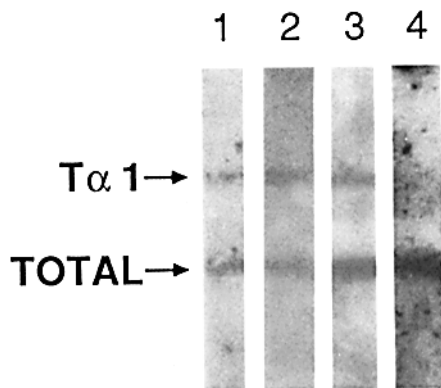


Figure 3. Relative abundances of T α 1 and total α -tubulin mRNAs. Southern blots of fragments of T α 1-tubulin cDNA containing sequences unique to T α 1 mRNA (*upper band*) or common to T α 1 and T26 mRNAs (*lower band*) were hybridized with 32 P-labeled poly (A) $^{+}$ RNA from E16 body (lane 1), E16 brain (lane 2), P5 brain (lane 3), and P23 brain (lane 4).

quences would hybridize with similar efficiency to a 400-nucleotide fragment containing coding region sequences, while the unique 3' noncoding region of T α 1 would hybridize to a 1,500-nucleotide fragment. The relative intensity of hybridization to these fragments gives a measure of the relative abundance of total (T α 1 and T26) α -tubulin mRNAs to T α 1 mRNA. Samples of E16 brain RNA, for example, hybridized with similar intensity to both fragments (Fig. 3), indicating that most of the α -tubulin mRNA in this sample is T α 1. We estimate, from these measurements and RNase-protection experiments (data not shown), that T α 1 tubulin mRNA must account for >95% of the total α -tubulin mRNA at this age.

The relative hybridization to the T α 1 specific fragment is lower with E16 body and P5 brain RNA samples indicating that other α -tubulin mRNAs, including presumably T26, are present. With P23 brain samples, hybridization to the T α 1 fragment is considerably reduced, indicating that other α -tubulin mRNAs comprise a significant fraction of the total α -tubulin mRNA in the brain at this age.

To examine the spatial patterns of expression of the two α -tubulin mRNAs in the developing embryo, a total α -tubulin probe and the T α 1- and T26-specific probes were hybridized in situ to sagittal sections of whole E16 embryos (Fig. 4). Total α -tubulin mRNA is highly enriched in the embryonic nervous system, with a low relative level of expression in other developing organ systems of the embryo, as previously described (Miller et al., 1987) (Fig. 4 c). A similar pattern of hybridization is seen with a probe specific to T α 1 mRNA, demonstrating that this mRNA is virtually specific to the nervous system (Fig. 4 a). In contrast, T26 mRNA appears to be expressed throughout the developing embryo (Fig. 4 b).

Within the developing nervous system at E16, T α 1 mRNA is present at the highest levels in the developing cortical plate, the retinal layer of the optic cup, and the spinal ganglia (Fig. 4 a). In contrast, at E16, T26 mRNA appears to be expressed in a wide variety of developing neural cell types, with somewhat higher levels being present in the ventricular zones of the developing brain (Fig. 4 b). By P3, when levels of T α 1 mRNA in the brain are still high, this α -tubulin mRNA is enriched in the cortical plate, thalamic and hypothalamic nuclei, and deep cerebellar nuclei (Fig. 5, e-f). T26 mRNA is distributed relatively homogeneously throughout the P3 brain, with some enrichment in proliferative zones such as the subventricular zone of the lateral ventricles and the external germinal layer of the cerebellum (Fig. 5, a-c).

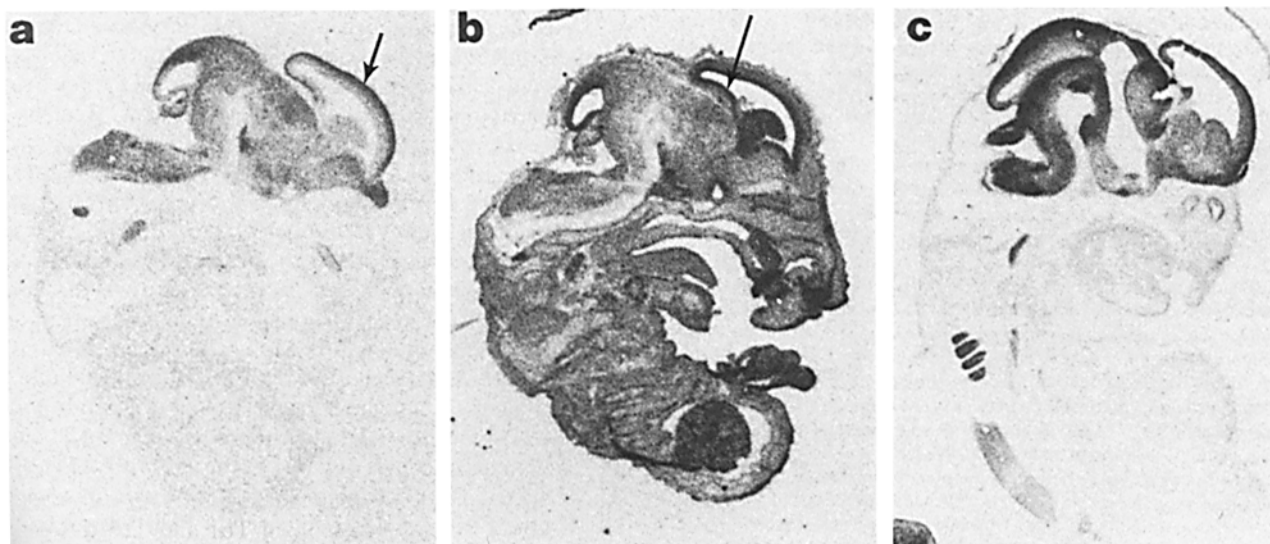


Figure 4. Distribution of T α 1 and T26 α -tubulin mRNAs in E16 embryos. The mRNAs were detected by in situ hybridization to sagittal sections of E16 embryos: representative autoradiographs are shown. (a) Hybridization with a 3' untranslated region RNA probe specific to T α 1 mRNA. Note the intense hybridization to the developing neuroaxis and the lack of hybridization to the rest of the body. The T α 1 mRNA is particularly high in neural structures such as the cortical plate (*arrow*). (b) Hybridization with a 3' untranslated region oligonucleotide probe specific to T26 mRNA. The in situ hybridization demonstrates a widespread pattern of hybridization to a variety of tissues, with a somewhat higher degree of hybridization to ventricular zones of the developing brain, such as that seen around the lateral ventricle (*arrow*). (c) Hybridization with an α -tubulin coding region RNA probe. Total α -tubulin is highly enriched in the developing nervous system, although there is a low level of hybridization to the rest of the embryonic body.

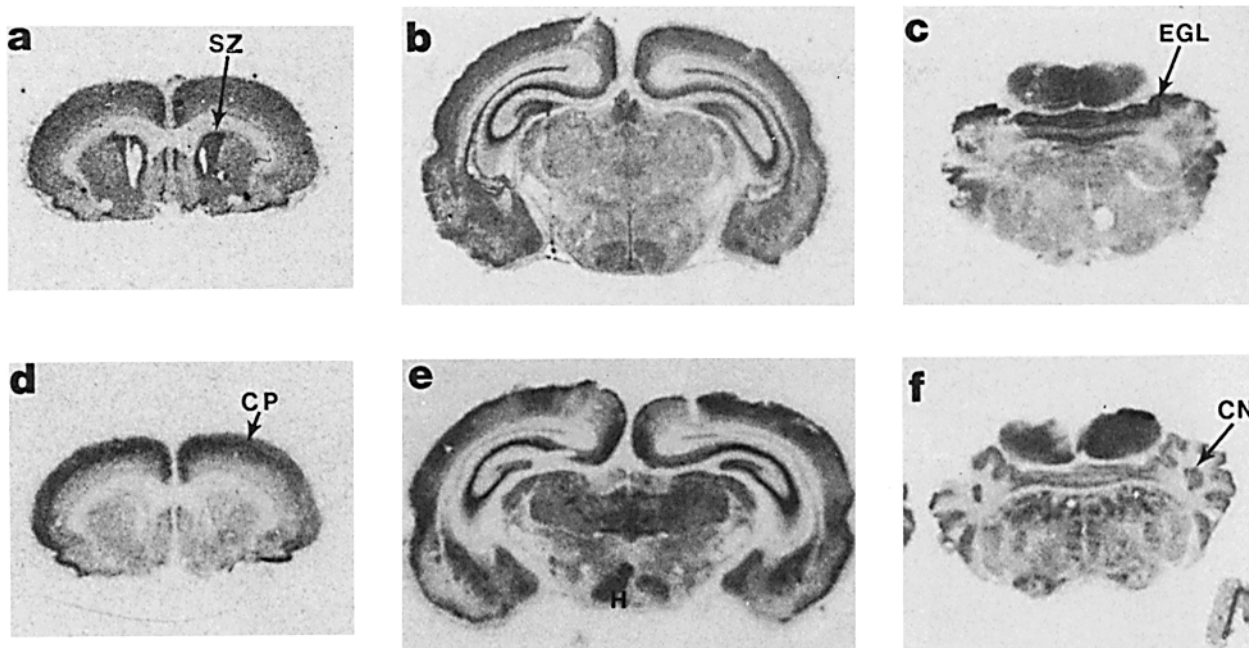


Figure 5. Distribution of *Ta1* (lower panels) and *T26* (upper panels) α -tubulin mRNAs in the early postnatal brain. The autoradiographs show representative in situ hybridization patterns to coronal sections taken at three different rostrocaudal levels of P3 brain. The RNA probe for *Ta1* mRNA (d, e, and f) hybridizes to various regions, including the cortical plate (CP), hypothalamus (HY), and deep cerebellar nuclei (CN). The oligonucleotide probe for *T26* (a, b, and c) demonstrates a much more homogeneous distribution for this mRNA in the brain, with some degree of enrichment in the subventricular zone (SZ) of the lateral ventricles and the external germinal layer (EGL) of the cerebellum.

Expression of *Ta1* and *T26* α -Tubulin mRNAs in the Developing Cerebral Cortex

The expression of α -tubulin mRNAs was examined in more detail in the developing cerebral cortex, where developmental events have been extensively defined, as described below. Northern blot analysis of RNA samples from the postnatal cerebral cortex revealed that the abundance of *Ta1* mRNA in cerebral cortex decreases approximately twentyfold between P3 and P23 (Fig. 6). In contrast, the level of *T26*

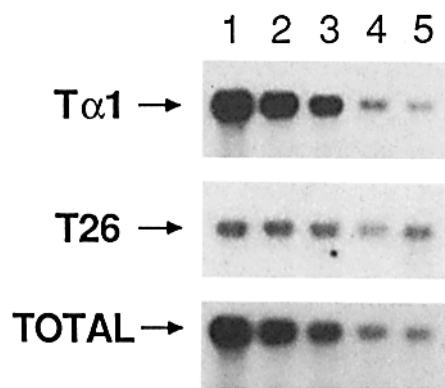


Figure 6. Expression of *Ta1* and *T26* α -tubulin mRNAs during postnatal development of the cerebral cortex. Northern blots of poly (A)⁺ RNA (1 μ g per lane) from P3 (lane 1), P9 (lane 2), P15 (lane 3), P23 (lane 4), and adult (lane 5) cerebral cortex were hybridized with an RNA probe specific to the 3' untranslated region of *Ta1* mRNA, an oligonucleotide probe specific for the 3' untranslated region of *T26* mRNA, or an α -tubulin coding region probe.

mRNA remains fairly constant over the same developmental time period.

The particular advantage of this brain region for our analysis is that the developing cortex presents a laminated pattern of cells in different stages of maturation and differentiation. Cortical neurons are born in the ventricular zone of the lateral ventricles between embryonic days 13 and 21 (Raedler and Raedler, 1978). Around E15, the first neurons migrate away from the ventricle and form the cortical plate. The cortical plate increases in thickness by addition of further recently-born neurons, and forms layers VIa to II of the mature cortex during the first postnatal week, following an inside-out gradient (Angevine and Sidman, 1961; Berry and Rogers, 1965). Axons start to elongate concomitant with neuronal migration, as the migrating neurons approach the cortical plate (Shoukimas and Hinds, 1978). Further maturation of neurons within the cortical plate involves dendritic elongation, branching, and spine development, which continues actively during the second postnatal week (Wise et al., 1979). The developing cortex therefore consists of three laminated zones: a ventricular proliferative zone, an intermediate zone containing largely migrating cells, and the cortical plate, which contains differentiating neurons.

In situ hybridization analysis of *Ta1* and *T26* α -tubulin mRNAs in developing cerebral cortex showed distinct differences in the cellular expression of these mRNAs. In the E16 brain, when the cortical plate has first formed, *Ta1* mRNA is highly enriched in this structure but is largely absent from the intermediate and ventricular zones (Fig. 7a). In contrast, *T26* mRNA is expressed homogeneously throughout the cortical mantle, with some enrichment in the proliferative ventricular zones (Fig. 7b). By P3, when the cortical plate is

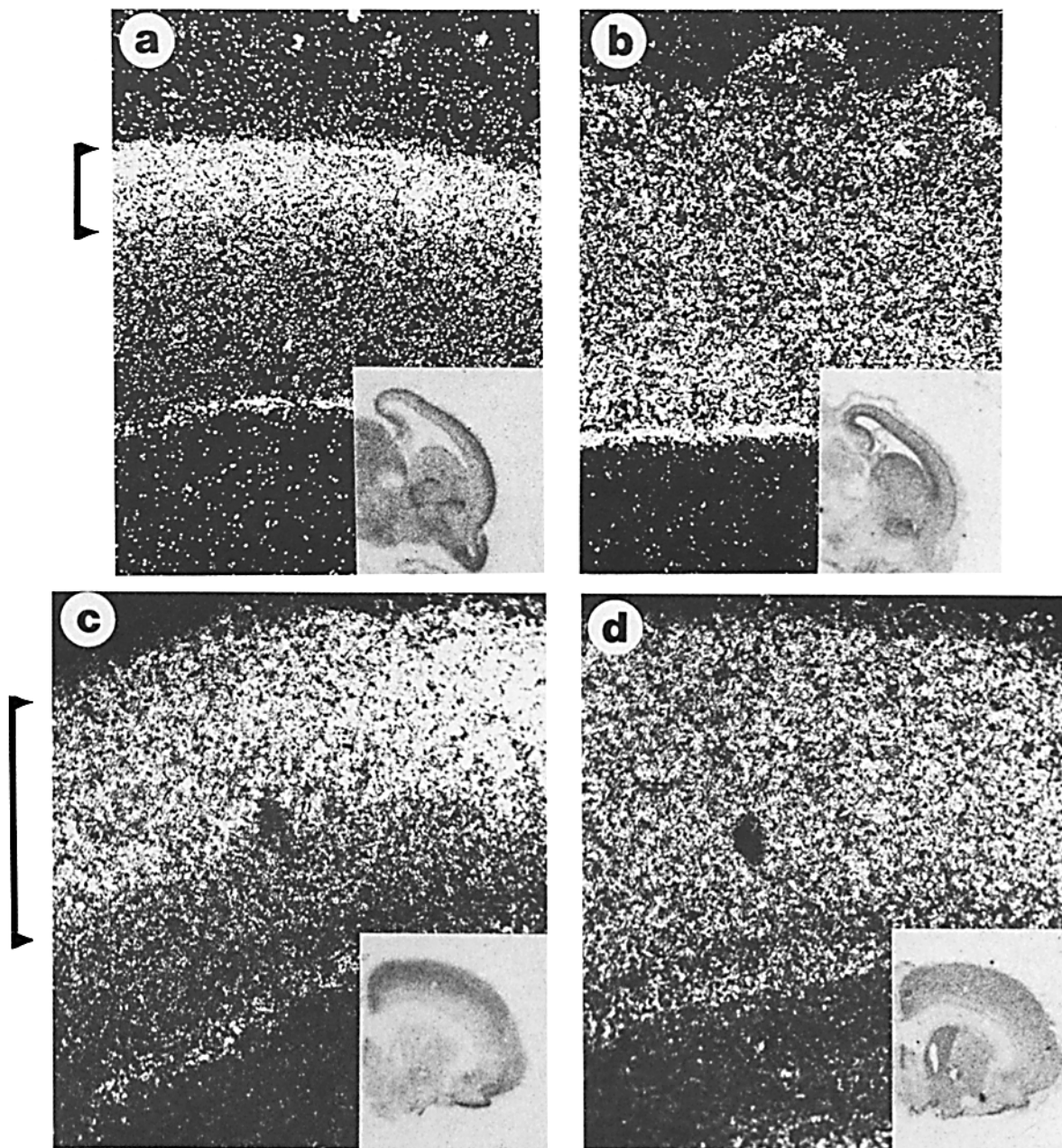


Figure 7. Expression of Ta1 (left panels) and T26 (right panels) α -tubulin mRNAs in the developing cerebral cortex. (a and b) Sagittal sections of E16 brain and (c and d) coronal sections of P3 brains were hybridized with a ^{35}S -labeled anti-sense RNA probe specific for the 3' noncoding region of Ta1 mRNA (a and c) or a ^{35}S -labeled oligonucleotide specific for the 3' untranslated region of T26 mRNA (b and d). The sections were coated with emulsion for autoradiography, developed, and photographed under darkfield illumination to show the cortical mantle. The insets represent x-ray film images of the telencephalic regions from the same sections. The Ta1 mRNA is particularly abundant in the cortical plate region (brackets) at E16 and P3, while T26 mRNA has a relatively homogeneous distribution across the cortex at both ages.

significantly wider and the earlier maturing layers of the cortex (layers V and VI) are just starting to form (Berry and Rogers, 1967), there is considerable heterogeneity in the levels of Ta1 mRNA across the cortical mantle (Fig. 7 c). There are high levels of Ta1 in the cortical plate, reduced levels in the intermediate zone, and somewhat higher levels in the newly formed neurons of layer VI. Again, the levels of T26 mRNA are homogeneous throughout the developing cortex at P3 (Fig. 7 d). At P23, when cortical development is com-

plete, Ta1 mRNA still displays a heterogeneous pattern of expression, with the highest enrichment in the cells of layer V, as we have described previously for total α -tubulin mRNA (Miller et al., 1987), whereas T26 mRNA is homogeneously distributed across the cortex at P23.

Induction of Ta1-Tubulin mRNA in PC12 Cells

The studies in situ indicated that Ta1 tubulin mRNA was expressed abundantly in brain regions, such as the cortical

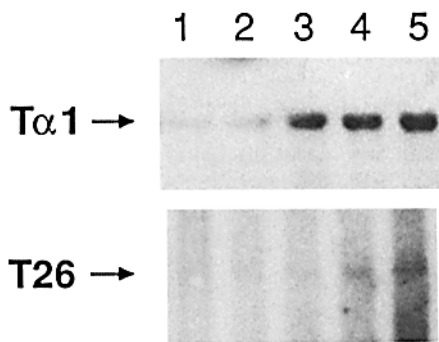


Figure 8. Expression of α -tubulin mRNAs in PC12 cells. Aliquots (10 μ g) of total cytoplasmic RNA extracted from PC12 cells at 0 (lane 1), 6 (lane 2), 12 (lane 3), 24 (lane 4), and 48 (lane 5) h after addition of NGF were hybridized on Northern blots with probes specific for T α 1 and T26 α -tubulin mRNAs.

plate, containing neurons actively undergoing neurite extension. These data suggested that T α 1 mRNA might be expressed specifically during neurite extension. To test this hypothesis, the induction of differentiation in PC12 pheochromocytoma cells by NGF was chosen as a model system to examine the expression of different α -tubulin mRNAs during neurite extension. In the absence of NGF, PC12 cells are rounded and lack cytoplasmic extensions (Tischler and Greene, 1975). When these cells are cultured with NGF for several days, they extend long branched neurites which contain bundles of microtubules extending from the cell body to the tips of the neurites (Luckenbill-Edds et al., 1979).

PC12 cells were induced to undergo differentiation and process extension with NGF: extensions appeared between 12 and 24 h and were well established by 48 h after induction. Total cytoplasmic RNA was prepared from cells at different times after addition of NGF and T α 1 and T26 mRNAs were examined by Northern blotting (Fig. 8). The level of T α 1 mRNA showed a slight increase at 6 h after induction and was severalfold higher in abundance by 12 h. The levels continued to increase up to 48 h. The abundance of T26 mRNA was in general lower and showed only small increases after induction with NGF.

Discussion

In this article we report the differential regulation of the two defined rat α -tubulin isotypes, T α 1 and T26, during neuronal maturation in the developing rat nervous system. This study demonstrates that T α 1 α -tubulin mRNA is expressed at high levels in neuronal cell populations at a time when they are extending processes, whereas T26 α -tubulin mRNA is expressed constitutively at a low level in most or all neural cell types throughout development with some enrichment in proliferative zones. The different temporal and spatial patterns of expression of these two α -tubulin mRNAs suggest either that the different isotypes play different roles in the assembly and function of microtubules, or that at least two α -tubulin genes are required for appropriate transcriptional regulation of α -tubulin mRNA levels during neural development.

The genes encoding T α 1 and T26 are expressed spatially in a completely divergent manner during embryogenesis in

the rat. The T α 1 mRNA is virtually specific to the embryonic nervous system, as indicated by *in situ* hybridization. The apparent expression of T α 1 in samples of RNA prepared from the bodies of E14 and E16 embryos, as detected by Northern blot analysis, can be attributed to the fact that the body dissections included the developing spinal cord and peripheral nervous system. In contrast to T α 1, the T26 α -tubulin mRNA is expressed throughout the embryo, with no significant degree of enrichment in the developing nervous system. Levels of T α 1 mRNA decrease between birth and postnatal day 23 in the developing brain, whereas levels of T26 mRNA remain approximately the same. The temporal and spatial patterns of expression of T α 1 and T26 in the embryo and neonate are similar to those displayed by the mouse M α 1 and M α 2 mRNAs, respectively (Lewis et al., 1985).

Within the embryonic and neonatal brain, T α 1 mRNA comprises greater than 95% of the total population of α -tubulin mRNA. During this developmental time period, T α 1 mRNA is expressed at high levels in those regions of the nervous system where neurons are elaborating processes. This is seen most clearly in the developing cerebral cortex where T α 1 mRNA is most abundant in the cortical plate, the site of neuronal differentiation and neurite extension (Shoukimas and Hinds, 1978; Wise et al., 1979). These data suggest that the expression of T α 1 mRNA in neurons during development is coupled to neuronal process extension. In addition, in the mature brain, at postnatal day 23 or later times, the distribution of T α 1 mRNA is still heterogeneous: in the cortex it is most enriched in layer V pyramidal cells, which possess long axonal projections (Jones, 1984), probably reflecting a further requirement for T α 1-tubulin mRNA for maintenance or remodelling of the neuronal cytoskeleton, particularly that of the axon.

The relationship between T α 1 mRNA expression and neurite extension is strongly supported by the demonstration that there is an increased expression of T α 1, but not T26, mRNA in PC12 cells that had been induced to differentiate and extend neurites by NGF treatment. The increase in T α 1 mRNA, which begins 6–12 h after NGF addition, is consistent with a reported increase in the levels of total α -tubulin protein in PC12 cells at 3–5 d after NGF treatment (Drubin et al., 1985). These studies also demonstrate that microtubule polymerization and neurite outgrowth are probably regulated directly by the expression of microtubule-associated proteins (MAPs), such as Tau and MAP1 (Drubin et al., 1985). The increased expression of T α 1 mRNA is therefore a necessary but insufficient precondition for neurite extension during neural development. We have also detected increases in the expression of T α 1 but not T26 mRNAs in facial nerve nuclei and spinal cord motor neurons that are undergoing axonal regeneration: in this system the level of T α 1 mRNA remains elevated throughout the regeneration process (F. D. Miller, manuscript in preparation).

In contrast to T α 1 α -tubulin mRNA, expression of T26 α -tubulin mRNA appears to be constitutive. There is little temporal or spatial regulation of expression of T26 in the developing nervous system, although there is some degree of enrichment of this mRNA in the proliferative zones of the embryonic and neonatal brains. The relative levels of T26 and T α 1 mRNAs during neural development indicate that the T α 1 protein product is the major, if not the only, α -tubulin isotype that is involved in elaboration of neuronal morphol-

ogy during development. The only other α -tubulin mRNA that is known to be expressed in the developing mammalian brain, the mouse $\text{Ma}4$ mRNA, is of postnatal onset, and is of low relative abundance (Villasante et al., 1986).

The differential regulation of T α 1 and T26 α -tubulin mRNAs during neuronal differentiation and maturation as demonstrated in this article raises several important questions. The rat T α 1, human $\text{ba}1$, and mouse $\text{Ma}1$ α -tubulin mRNAs encode identical proteins and share 82% homology in their 3' noncoding regions (Lemischka et al., 1981; Cowan et al., 1983; Lewis et al., 1985). Although the rat T26 mRNA sequence (Ginzburg et al., 1986) has not been completed, the defined amino acid sequence is identical to that shared by human $\text{ka}1$ (Cowan et al., 1983) and mouse $\text{Ma}1$ (Lewis et al., 1985), and the 3' noncoding regions of the three mRNAs share 72% homology. The $\text{ka}1$ and $\text{Ma}2$ isotypes differ from the T α 1, $\text{ba}1$, and $\text{Ma}1$ isotypes by virtue of a single amino acid difference (serine to glycine) at residue 232. If the T26 isotype is identical in primary sequence to the $\text{Ma}2$ and $\text{ka}1$ isotypes, then the proteins encoded by the T α 1 and T26 α -tubulin mRNAs differ by only one amino acid. This single substitution could alter the conformation of the protein, or provide a new site for posttranslational modification, conferring different structural and/or functional properties on the microtubules formed from these tubulins. The expression of T α 1 mRNA would therefore represent a demand specifically for T α 1 tubulin for a particular process, such as the formation of axonal microtubules. Alternatively, this single amino acid substitution may have no, or minimal, effects on α -tubulin structure, and the genes encoding the T α 1 and T26 mRNAs could thus encode functionally equivalent proteins. Expression of T α 1 mRNA at high abundance could then be a simple mechanism to increase the available pool of α -tubulin when this is in high demand for neurite extension. It may simply be easier to regulate two or more equivalent genes in a tissue-specific manner than a single gene, particularly when the protein serves both ubiquitous and cell type-specific functions. The divergent regulatory patterns displayed by the tubulin genes is therefore a consequence of the wide variety of cellular roles that microtubules, and hence their components, must fulfill.

The mechanisms determining the differential regulation of the levels of T α 1 and T26 α -tubulin mRNAs during neural development are unknown. The large difference in relative abundance of the two mRNAs in the embryonic brain suggests that at least some of the regulation is occurring at a transcriptional level. However, it has recently been demonstrated (Lau et al., 1986) that the pool size of tubulin monomers directly effects the stability of α - and β -tubulin mRNAs in cultured Chinese hamster ovary cells, thus raising the possibility that posttranscriptional regulation of tubulin mRNA stability could play an important role in the differential regulation of T α 1 vs. T26 α -tubulin mRNA levels. Differential mRNA stability could possibly be conferred by the completely divergent 3' noncoding regions of these two mRNAs, perhaps explaining the high degree of interspecies conservation of these regions of the α -tubulin mRNA molecules. Alternatively, the 3' regions might function to direct different α -tubulin mRNAs to particular sites of synthesis within the cell, suggesting that the differential regulation of α -tubulin genes might be a reflection, not of the different structural properties of the proteins or the regulatory proper-

ties of the genes, but of intrinsic functional differences among the mRNAs.

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