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Differential regulation of β_{III} and other tubulin genes during peripheral and central neuron development

YUAN QING JIANG and MONICA M. OBLINGER*

Department of Cell Biology and Anatomy, The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064, USA

*Author for correspondence

Summary

Mammalian peripheral and central neurons differ considerably in the composition and properties of their axonal cytoskeletons. Recent reports of the selective expression of a high molecular weight (HMW) tau protein in neurons with peripherally projecting axons have furthered the idea that the microtubules in central and peripheral neurons are disparate. In the present study, we examined the possibility that the various tubulin genes are differentially expressed in central versus peripheral neurons. To examine this, we compared the expression of four of the β-tubulin mRNAs (classes β_I, β_{II}, β_{III}, β_{IV}) and the α_1-tubulin mRNA in rat dorsal root ganglion (DRG) neurons with their expression in cerebral cortex during postnatal development (P5-90), using northern blots and in situ hybridization. We document both similarities and differences in tubulin gene expression in these two regions of the neuraxis during postnatal development. In both DRG and cortex, the expression of the class β_I- and β_{II}-tubulin mRNAs and the α_1-tubulin mRNA was higher at earlier stages of postnatal development than in the adult. However, class β_{IV}-tubulin mRNA levels increased during cortical development but decreased during DRG postnatal development. The opposite pattern was found for the neuron-specific class β_{III}-tubulin gene, the mRNA levels of which were high in cortex, at birth and then decreased with increasing postnatal development. In DRG, the β_{III}-tubulin mRNA levels generally increased during postnatal development. β_{III}-tubulin protein levels were examined qualitatively at different developmental stages (5-90 days) by immunoblotting and immunocytochemistry. These studies showed a robust reduction in β_{III}-tubulin levels from P5 to adult stages in cortex, but a more complex pattern of change in the DRG. The results suggest that differential utilization of the tubulin gene products exists in central and peripheral nervous tissue and that this may play a role in specifying differences in the functional properties of neuronal cytoskeletons in these regions.

Key words: microtubules, microtubule-associated proteins (MAPs), neuronal development, cytoskeleton, axons.

Introduction

Microtubules are major cytoskeletal components involved in diverse cytosolic functions, such as the generation and maintenance of cell morphology and the transport of membranous organelles (Brady, 1987; Dustin, 1984). Microtubules are assembled from dimers of α- and β-tubulin which are products of two small multigene families. In vertebrates, 6 functional genes for α-tubulin and 7 for β-tubulin have been identified (Cleveland, 1987; Sullivan, 1988). While no tissues in mammals appear to express all of these genes, 5 distinct α-tubulin isotypes (Villasante et al., 1986) and 5 different β-tubulin isotypes appear to be expressed in the mammalian brain (Sullivan, 1988). Two of these mRNAs (β_{IVa} and β_{IVb}) have identical coding regions and differ only in 3′ untranslated sequence. All of the β-tubulins are highly homologous, differing from each other primarily in the 15 or so amino acids at the carboxy-terminal region of the protein (Burgoyne et al., 1988; Cleveland, 1987; Lewis et al., 1985; Sullivan, 1988; Sullivan and Cleveland, 1986; Wang et al., 1986). β_{III}-tubulin is perhaps the most divergent since its amino acid sequence differs by about 10% from other β-tubulins in brain (Sullivan, 1988), and because it is the only phosphorylated tubulin (Edde et al., 1989; Gard and Kirschner, 1985; Luduena et al., 1988). The expression of β_{III}-tubulin is limited to neurons in avian species and is nearly neuron-specific in rat where limited expression of unmodified β_{III} is also seen in the testis (Burgoyne et al., 1988; Lee et al., 1990a,b; Sullivan, 1988; Sullivan and Cleveland, 1986).

The significance of the many different tubulin isotypes is not fully understood. For example, it is not clear if the different tubulin isotypes are always completely functionally interchangeable, as has been shown in some cases (Lewis et al., 1987; Lopata and Cleveland, 1987), or if unique properties are endowed to microtubules by different tubulin gene products in some cells, either directly or through selective interactions of some tubulins with specific

microtubule-associated proteins (MAPs). Observations that the various tubulin genes are differentially regulated during brain development support the argument that certain tubulin gene products are better suited for the requirements of growing neurons while others have properties that are more suited to the requirements of mature neurons (Bond et al., 1984; Lewis et al., 1985; Miller et al., 1987). In addition, observations that the expression of some tubulin isotypes change differentially when adult neurons are injured and undergo axonal regeneration (Hoffman and Cleveland, 1988; Miller et al., 1989; Wong and Oblinger, 1990) provide further support to the argument that certain tubulin gene products may be better matched to meeting the specific functional requirements of regenerating neurons.

Tubulin isotype expression has not been well studied in peripheral neurons during development but it is commonly assumed that the patterns of expression of major structural genes will follow similar patterns to those observed in brain. This may not be a valid assumption, however, because mammalian PNS neurons differ considerably from CNS neurons in a variety of ways. For example, peripherally-projecting neurons (such as DRG cells, and spinal or brainstem motor neurons) typically under histogenesis and develop at earlier stages than do cerebral cortical and other CNS neurons in the mammal (Lund, 1978). By morphological criteria, it is well established that peripheral neurons and their axons are typically much larger than most CNS neurons in adult mammals. With respect to the cytoskeleton, the rate of slow component "a" of axonal transport (SCa) that conveys microtubules and intermediate filaments is nearly 10-fold greater in PNS axons than in CNS axons, and tubulin is virtually excluded from SCb, the faster of the two slow transport components, in CNS axons (Black and Lasek, 1980; Brady and Lasek, 1982; McQuarrie et al., 1986; Oblinger, 1988; Oblinger et al., 1987). Other differences include the segregation of some cytoskeletal proteins to either PNS or CNS neurons. These include peripherin, an IF protein expressed in PNS neurons and typically excluded from most CNS neurons (Brody et al., 1989; Parysek and Goldman, 1988; Portier et al., 1984), α-internexin, an IF protein with the opposite pattern of distribution (Fliegner et al., 1990; Pachter and Liem, 1985), and HMW tau, a MAP expressed in many peripheral neurons but generally absent from CNS neurons (Georgieff et al., 1991; Oblinger et al., 1991; Taleghany and Oblinger, 1992).

On the issue of functional differences related to the cytoskeleton, it is well known that the regenerative ability of axotomized CNS and PNS axons in adult mammals differs. The failure of long tract axon regeneration in the CNS has been linked to differences in the response of tubulin genes to injury in CNS versus PNS. For example, in corticospinal neurons α1-tubulin and β_{III}-tubulin mRNA levels are downregulated following axotomy (Kost and Oblinger, 1992; Mikucki and Oblinger, 1991; Oblinger and Kost, 1992), while in DRG neurons, the levels of α_1 and other-tubulin mRNAs such as β_{II} and β_{III} increase substantially following axotomy (Hoffman and Cleveland, 1988; Miller et al., 1989; Oblinger et al., 1990; Oblinger and Wong, 1990; Wong and Oblinger, 1990). Since, for several major cytoskeletal genes, axonal regeneration after injury in the adult appears to reinitiate patterns of gene expression found at earlier points in development, these findings raise the question of whether the various tubulin genes are also regulated differently in CNS and PNS neurons during early development.

At present there is little comparative information about the tubulin isotype composition and its developmental regulation in PNS neurons versus those in CNS. In this study, we compared the expression of α_1-tubulin and 4 of the β-tubulin isotypes during rat postnatal development. The focus was on the neuron-specific β_{III}-tubulin isotype (Sullivan, 1988; Sullivan and Cleveland, 1986). The expression of this tubulin gene has not been previously examined at the mRNA level during postnatal development of peripheral or central neurons. Using northern blotting, in situ hybridization and immunochemical studies, we document in this report that the β_{III}, as well as the β_{IV}-tubulin, genes are regulated quite differently during postnatal development in PNS tissues compared to brain, but that similar developmental patterns of expression are present for β_I-, β_{II}- and α_1-tubulin mRNAs in both CNS and PNS.

Materials and methods

Animals

Postnatal Sprague-Dawley rats aged 5, 7, 10 and 21 days postnatal (P) and adult (~90 days) were used in the experiments. For harvesting of tissue samples from cerebral cortex and DRG, the animals were deeply anesthetized with ether and then decapitated.

cDNA probes

The probes used included cDNAs specific for the 3′ untranslated region (UT) of the following tubulin isotypes: mouse α_1-tubulin (Mα1, from Dr. Nick Cowan; (Lewis et al., 1985)); rat class β_I-tubulin (RBT3,3′UT, this subclone was a generous gift from Dr. Don Cleveland and was derived from the RBT3 clone (Bond et al., 1984); rat class β_{II}-tubulin (RBT1,3′UT, from Dr. Steve Farmer (Bond et al., 1984); rat class β_{III}-tubulin (KS-5, 3′UT; from Dr. Tony Frankfurter); rat class β_{IVa}-tubulin (RBT2, 3′UT, from Dr. Steve Farmer (Bond et al., 1984)). Mα1 is an ~800 kb mouse cDNA consisting of the subcloned 3′UT region of α_1-tubulin sequence that is highly homologous to the rat-derived Tα1 (Miller et al., 1987). The 3′UT sequences of the α-tubulin mRNAs are unusually highly conserved between species (Lewis et al., 1985) and thus the mouse probe Mα1 hybridizes very well in rat. The β_{III}-tubulin probe we used was derived from the entire KS-5 clone which encodes amino acids 71-448 of rat β_{III}-tubulin and also contains the 3′UT nucleic acid sequence. Sequence analysis of KS-5 cDNA has revealed complete homology with the carboxy-terminal sequence of mouse class β_{III}-tubulin (mβ6 clone; (Burgoyne et al., 1988) and very high homology in the remaining coding region (A. Frankfurter, unpublished observations; see also Oblinger et al., 1990). For hybridizations specific to β_{III}-tubulin mRNA, we used an *Apa*I fragment of KS-5 which consisted of the terminal lysine and ~750 bp of 3′UT sequence.

For hybridizations, the cDNA inserts were isolated from the various plasmids using appropriate restriction enzymes and electrophoresed on 1% agarose gels; the insert band was purified using GeneClean (Bio 101). For northern blots, the cDNA inserts were labeled with ^{32}P-dCTP using the Prime-It kit (Stratagene) and unincorporated nucleotides were removed using NucTrap Push Columns (Stratagene). For in situ hybridizations, cDNA inserts were labeled with ^{35}S-dCTP by nick translation and the unincorporated nucleotides removed by centrifugation in Centricon 10 units (Amicon).

RNA isolation and northern blotting

RNA was isolated from samples of cerebral cortex and DRG using the methods described previously (Chomcyznski and Sacchi, 1987). 5 μg of total RNA from the different samples were separated by electrophoresis on 1% agarose gels in the presence of formaldehyde (Ausubel et al., 1987). The RNA was blotted onto Nytran membranes (Schleicher and Schuell) using a Posiblot positive pressure blotter (Stratagene) and crosslinked to the nylon membranes by exposure to 0.12 J of u.v. light using an Ultra Lum ultraviolet multilinker (VWR Scientific). The blots were hybridized with ^{32}P-labeled cDNA tubulin isotype-specific probes, according to conditions previously described (Oblinger et al., 1991). After hybridization, blots were washed to a final stringency of 0.1 × SSC with 0.1% SDS at 50ºC. Typically, blots were reprobed sequentially with the different tubulin probes after stripping the blots by immersion in boiling water for 5 min. Autoradiographs were made by exposing the blot to Kodak Xomat AR film for 1-4 days. A total of 6 different blots were used in this study.

In situ hybridization

The fourth and fifth lumbar DRGs were harvested from rats, fixed by immersion in 4% paraformaldehyde for 2 h and embedded in paraffin. Histological sections were cut at 10 μm and mounted on gelatin-chrome-alum subbed slides. After deparaffinization, the sections were hybridized with ^{35}S-labeled tubulin cDNAs, with modifications of the method of Wong and Oblinger (1987). Briefly, sections were first permeabilized with 0.3% Triton X-100 in 0.1M Tris, pH 7.4 for 15 min, rinsed in 0.01 M Tris, and then digested with 0.25 μg/ml proteinase K in 0.01 M Tris/5 mM EDTA at 37ºC for 15 min and then rinsed extensively with 2 × SSC. Sections were next incubated with prehybridization solution for 2 h at 37ºC in a box humidified with paper towels soaked in 4 × SSC and 50% formamide. The prehybridization solution contained 1.2 M NaCl, 20 mM Tris pH 7.6, 2 mM EDTA, 0.2 mg/ml bovine serum albumin, 0.5 mg/ml sodium pyrophosphate, 0.4 mg/ml Ficoll, 0.4 mg/ml polyvinylpyrrolidine, 100 μg/ml yeast total RNA, 100 μg/ml yeast tRNA, and 0.5 mg/ml salmon sperm DNA. Just before use, this solution was boiled for 5 min, placed on ice, and then diluted with an equal volume of deionized formamide and 20 μg/ml dithiothreitol (DTT) was added. After incubation, the prehybridization solution was aspirated from the sections and replaced with hybridization solution containing an appropriate amount of labeled cDNA. The composition of the hybridization solution was identical to the prehybridization mixture with the exception that it also contained 20% dextran sulfate (5×10^5 average molecular mass) and 0.1 mg/ml polyadenylic acid. Just prior to use, the labeled cDNA insert was added and the hybridization solution was boiled for 5 min, placed on ice and diluted with an equal volume of deionized formamide and 20 μg/ml DTT was added. Hybridization reactions were done using 1×10^5 counts/min of ^{35}S-labeled cDNA in 10 μl per individual DRG section for 18 h at 37ºC in humidified chambers (see above). After hybridization, the sections were washed to a final stringency of 0.1 × SSC with 0.1% SDS at 50ºC for 15 min and then dehydrated through an increasing series of ethanols/300 mM ammonium acetate to xylene. The sections were then air dried and dipped into 37ºC Kodak NTB2 emulsion (diluted 1:1 with 600 mM ammonium acetate) and stored at 4ºC in the dark for 7-14 days after which they were developed and lightly counterstained with cresyl violet.

Antibodies

The monoclonal antibodies used included: a β_{III}-isotype specific tubulin monoclonal antibody, TuJ1 (Geisert and Frankfurter, 1989; Lee et al., 1990a), a generous gift from Dr. Tony Frankfurter, used at 1:10,000 dilution and RMDO17 monoclonal antibody specific for the middle molecular weight neurofilament protein, NF-M, (provided by Dr. Virginia Lee), used at 1:1000 dilution.

Immunoblotting

Immunoblotting was as described previously (Oblinger, 1987). Proteins were obtained from rat cerebral cortex and DRG at different postnatal stages by homogenization in cold 100 mM sodium phosphate buffer, pH 7.4. An aliquot of each sample was used to determine protein concentration using a standard Coomassie dye binding assay. The samples were then diluted 1:1 in BUST (2% beta-mercaptoethanol, 8 M urea, 1% sodium dodecyl sulfate, 1 M Tris, pH 6.8) and equal amounts of total protein (5 μg) were loaded onto 7.5% acrylamide mini-slab gels, electrophoresed and transferred to nitrocellulose. Blots were incubated with primary antibody overnight at 4ºC, rinsed extensively, and then incubated in peroxidase-labeled goat anti-mouse secondary antibody (1:300, Boehringer Mannheim) for 2-3 h at 4ºC. After rinsing, the antigen-antibody complex on the blots was visualized by catalysis of 3,3′-diaminobenzidine (DAB) by horseradish peroxidase in the presence of 0.03% hydrogen peroxide.

Immunocytochemistry

For immunocytochemical studies, the histological sections of rat DRGs described above (in situ hybridization methods) were used. Immunostaining was essentially the same as described previously (Oblinger et al., 1989). Briefly, deparaffinized sections were incubated in TuJ1 antibody (diluted 1:10,000 in 1% normal goat serum/100 mM PBS) at room temperature overnight. The antigen-antibody complex was subsequently visualized using the mouse ABC Vectastain Elite peroxidase Kit (Vector Labs). The sections were then dehydrated and coverslipped with Permount and examined using a Nikon Optiphot microscope.

Results

A major goal of the present study was to determine if the developmental patterns of tubulin gene expression differ in central and peripheral neuronal tissue. Towards this end, we compared the expression of various tubulin mRNAs in cerebral cortex and DRG at early postnatal (7 day) and adult stages using northern blotting. Total RNA was obtained from 7 day postnatal and adult DRG and cerebral cortex and equal amounts of RNA were blotted and probed with various tubulin isotype-specific cDNAs. Fig. 1 illustrates that both similarities and major differences in tubulin isotype expression exist in cortex and DRG during development. β_I-tubulin and β_{II}-tubulin mRNA expression were quite similar in both regions. The levels of both of these mRNAs were relatively high at postnatal day 7 and dropped to very low levels in the adult. A similar, but somewhat less dramatic, change occurred with α_1-tubulin mRNA levels in cortex during development. However, in the DRG, only a modest decline in α_1-tubulin mRNA levels was observed between postnatal day 7 and the adult stage (Fig. 1).

The developmental patterns of β_{IV}- and β_{III}-tubulin mRNA expression were opposite in cerebral cortex compared to DRG (Fig. 1). In cortex, the β_{IV}-tubulin mRNA levels were substantially higher in the adult stage compared to 7 day postnatal. However, in the DRG, the levels of β_{IV}-tubulin mRNA were actually higher during the first post-

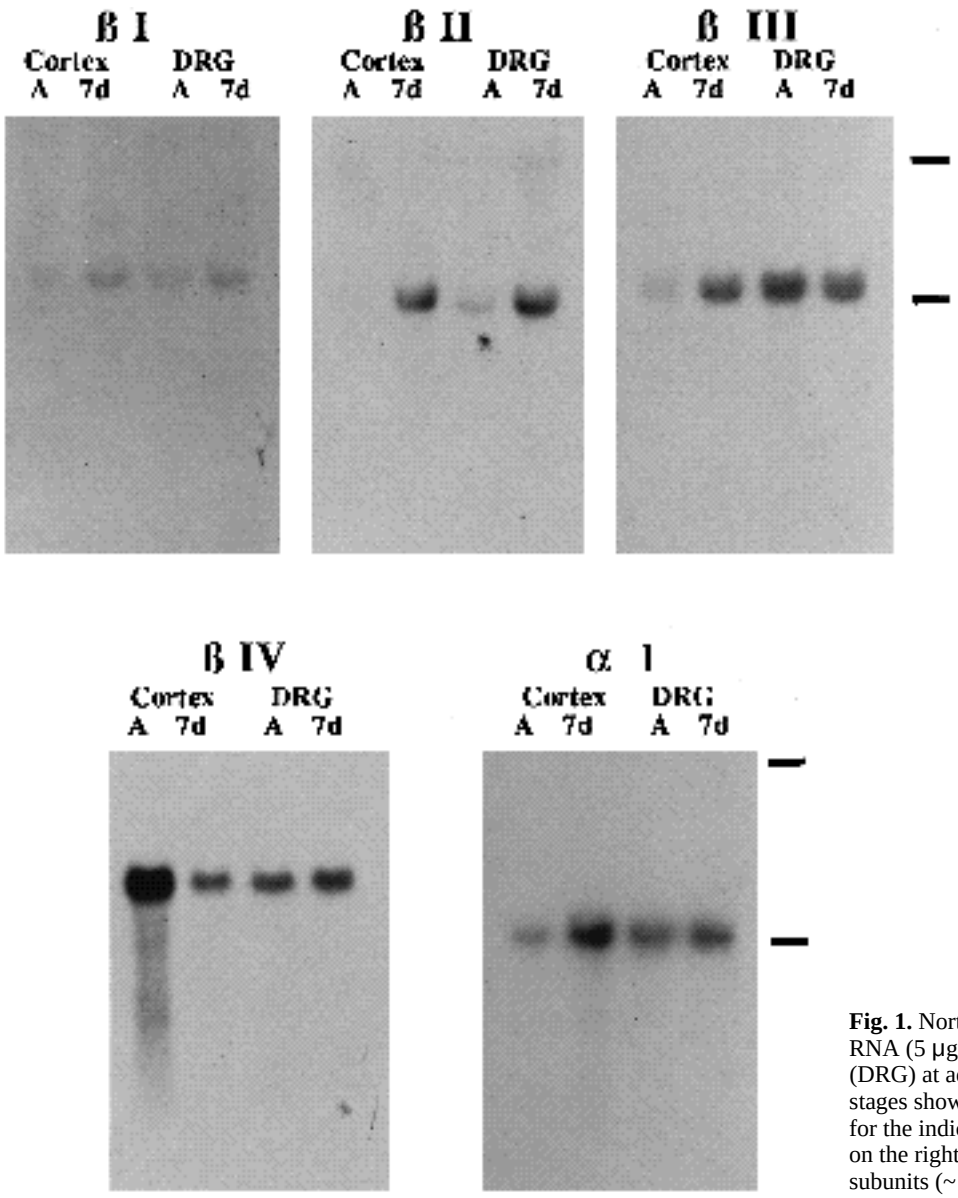

Fig. 1. Northern blots of equal amounts of total RNA (5 μg) from cortex and dorsal root ganglia (DRG) at adult (A) and postnatal day 7 (7d) stages showing changes in the levels of mRNA for the indicated tubulin isotypes. Horizontal bars on the right side indicate position of ribosomal subunits (~ 5 kb and ~ 2 kb).

natal week compared to the adult stage. β_{III}-tubulin mRNA levels were considerably higher in the immature cortex than in the adult (Fig. 1). The opposite was the case in the DRG where the levels of β_{III}-tubulin mRNA appeared somewhat lower at postnatal day 7 than in the adult (Fig. 1). To further explore the developmental changes in β_{III} mRNA in cortex and DRG development, a more expanded time course was examined by northern blotting. Fig. 2 illustrates that a dramatic decrease in β_{III} mRNA levels was found in cerebral cortex during postnatal development, with the major downregulation in β_{III} mRNA expression occurring somewhere between day 10 and day 21 postnatal. In the DRG, the overall pattern was one of higher expression of β_{III} tubulin mRNA at later developmental stages than at earlier stages. However, additional complexity was observed for expression of the neuron-specific β_{III} isotype in the developing DRG. That is, β_{III}-tubulin mRNA levels were somewhat biphasic, dipping to lower levels at postnatal day 10 compared to 5 days postnatal and then increasing again between 10 and 21 days postnatal. This pattern was not an artifact in the particular experiment shown since we consistently observed a similar result in 3 other experiments, each utilizing different RNA samples.

Tubulin mRNA expression was further examined in the DRG during postnatal development by in situ hybridization. Fig. 3 illustrates emulsion autoradiograms of DRG sections from animals of different postnatal ages after hybridization with either a ^{35}S-labeled β_{III}-tubulin cDNA probe (Fig. 3 A,C,E,G) or a β_{II}-tubulin cDNA probe (Fig. 3 B,D,F,H). In these experiments the majority of labeling was neuronal, confirming that the predominant cells expressing these tubulin genes in the DRG are neurons. Labeling of the neurons for β_{III}-tubulin mRNA was noticeably biphasic across developmental time. That is, the apparent density of grains over DRG neurons at postnatal day 5 was higher than at day 10 and then the level of β_{III} mRNA increased again to

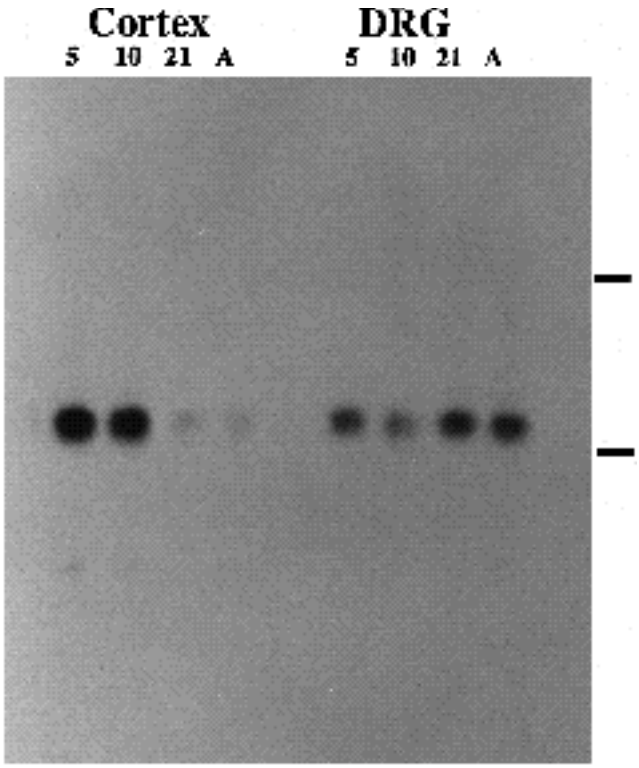

Fig. 2. Northern blot of equal amounts of total RNA (5 μg) from cerebral cortex and DRG at postnatal days 5, 10, 21 and adult (A) showing changes in the levels of β_{III}-tubulin mRNA. Horizontal bars on the right side indicate position of ribosomal subunits (~ 5 kb and ~ 2 kb).

noticeably higher levels on day 21 postnatal. Note that the size of the DRG neurons also increased during postnatal time, with the large neurons continuing to increase substantially in cross-sectional area, even between the 3rd postnatal week and the adult stage (all panels are at the same magnification). In situ hybridization of DRG neurons with the β_{II}-tubulin cDNA probe produced similar results to northern blotting experiments. That is, higher levels of labeling for β_{II}-tubulin mRNA were seen in the autoradiograms after in situ hybridization at earlier postnatal stages (Fig. 3B,D) than at later times (Fig. 3F,H). The in situ hybridization patterns for the other 3 tubulin mRNAs studied in the DRG also faithfully followed the patterns revealed by northern blotting (data not shown).

We next explored whether β_{III}-tubulin protein levels mirrored the changes in β_{III} mRNA levels across postnatal development using western blotting. Equal amounts of total protein obtained from cerebral cortex and DRGs at different developmental stages were electrophoresed, blotted and probed with the monoclonal antibody, TuJ1, which is specific for β_{III}-tubulin. The results of these experiments demonstrated that in cerebral cortex the β_{III} protein levels decreased with increasing maturity (Fig. 4). However, the change in protein levels (on qualitative evaluation) appeared less dramatic than the mRNA changes (compare Figs 2 and 4). A possible explanation for this difference is that β_{III}-protein is stabilized in cortex by some post-translational mechanism in later stages of postnatal development.

The pattern of β_{III}-protein changes in the DRG during development differed from that in cortex. On western blots, it was apparent that β_{III}-tubulin levels were relatively high at postnatal day 5, dropped to lower levels at day 10, increased to an apparent peak at 3 weeks postnatal and then declined some in the adult DRG (Fig. 4A). Similar patterns were observed in 3 replicate experiments using different protein samples. The cortex and DRG samples were also examined by western blotting for NF-M changes using the monoclonal antibody RMDO17 (Fig. 4B). The results indicated that NF-M protein levels increased progressively in both regions of the neuraxis during postnatal development, consistent with many previous reports (Carden et al., 1987; Schlaepfer and Bruce, 1990; Shaw and Weber, 1982). The NF-M blots were done largely to verify that the samples used for the western blots of β_{III}-tubulin were of good quality. Thus, the lower levels of β_{III}-tubulin protein in the adult DRG relative to the 21 day postnatal stage (Fig. 4A) were not due to problems with the adult DRG protein samples used.

Finally, we examined the expression of β_{III}-tubulin in the DRG using immunocytochemistry with the TuJ1 antibody. The immunocytochemical staining results were quite consistent with those of western blotting experiments. DRG neurons were stained relatively heavily for β_{III}-protein at postnatal day 5 (Fig. 5A). At postnatal day 10, the overall level of immunoreactivity was lighter but some larger neurons were darkly stained (Fig. 5B). At postnatal day 21, the level of β_{III} immunoreactivity was high in most neurons in the DRG (Fig. 5C), while in the adult stage lower levels of β_{III} protein were apparent in DRG neurons (Fig. 5D). Axons in the DRG were stained quite heavily with the TuJ1 antibody at all stages of development from postnatal day 5 to adult (Fig. 5).

Discussion

In the present study, we characterized the pattern of expression of specific tubulin mRNAs during postnatal development of both cerebral cortex and DRG, with an emphasis on the neuron-specific β_{III} gene. These studies have revealed that while some of the tubulin mRNAs, including α_1, β_I and β_{II}, exhibit similar developmental patterns of expression in CNS and PNS tissues others, such as β_{III} and β_{IV}, have opposite patterns of expression in these two regions of the neuraxis. In cortex, β_{III} mRNA levels are very high during the initial postnatal interval and then fall to low levels, while in DRG the opposite pattern occurs. In the case of β_{IV}-tubulin, the mRNA levels are low in cortex early in development and then become very substantial in the adult, while in the DRG, the β_{IV} mRNA levels are appreciable at early postnatal stages but decline slightly with maturity. This and previous in situ hybridization studies have shown that neurons are the predominant source of mRNA expression for α_1-tubulin in brain (Kost and Oblinger, 1992; Mikucki and Oblinger, 1991) and DRG (Miller et al., 1989); for β_{II} in DRG (Hoffman and Cleveland, 1988; Wong and Oblinger, 1990) and for β_{III} in DRG and cortex (Kost and Oblinger, 1992; Oblinger et al., 1990). Detailed in situ hybridization analyses of the other β-tubu-

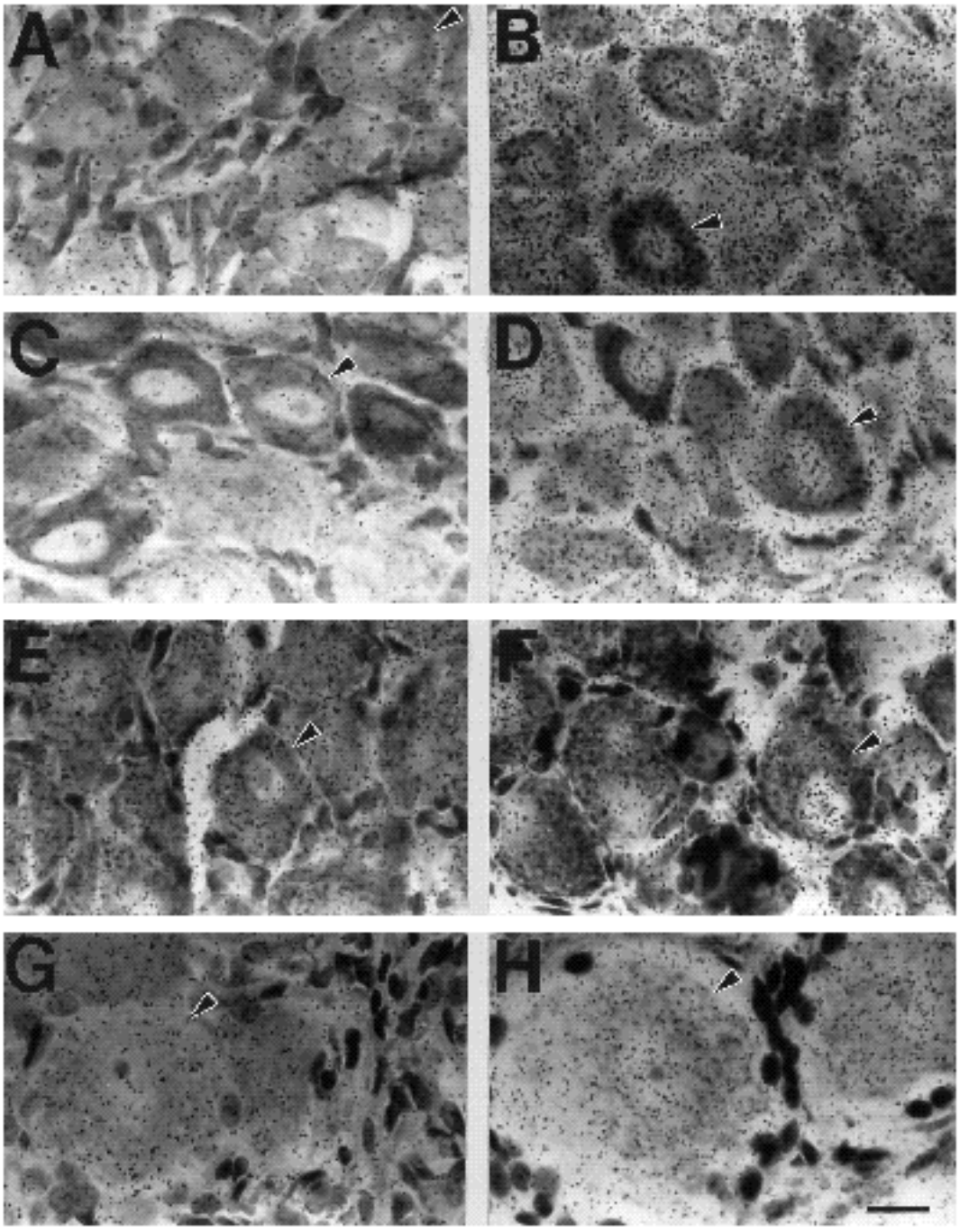

Fig. 3. In situ hybridization of developing DRG neurons with β_{III}-tubulin and β_{II}-tubulin cDNAs. Autoradiograms shown are from DRG neurons obtained at P5 (A,B), P10 (C,D) P21 (E,F) and adult (G,H). ^{35}S-labeled cDNA probes to class β_{III}-tubulin (left column: A,C,E,G) and class β_{II}-tubulin (right column: B,D,F,H) were used. In each panel, the arrowhead indicates the plasmalemma of one DRG neuron. Bar, 23 μm.

lin mRNAs remain to be done in DRG versus brain but will be important in detailing the relative levels of expression in neural versus non-neural cells.

The present study assessed steady-state levels of various tubulin mRNAs but did not examine protein synthesis or protein levels for all of these in a quantitative manner during development. Thus, it is not yet clear if the mRNA changes drive similar protein changes in the nervous system throughout development. The dramatic changes in the levels of various tubulin mRNAs during development indicate that tight regulatory controls are extant but little is known about the level of this regulation. Transcriptional controls as well as regulation at the level of mRNA stability and beyond must be considered likely since these mechanisms are operative in other cell types. In this regard, it is of interest that β_{III}-tubulin protein changes appeared to be much less dra-

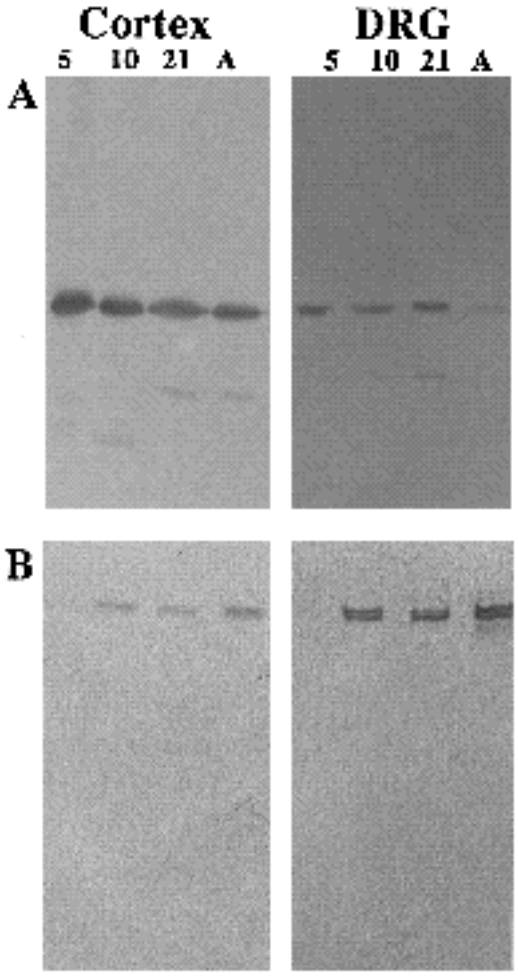

Fig. 4. Western blots of equal amounts of total protein from cerebral cortex and DRG at the indicated postnatal stages of development showing changes in levels of β_{III}-tubulin protein (A) using the TuJ1 antibody, and NF protein (B) using a monoclonal antibody (RMDO17) specific for the middle molecular weight NF protein (NF-M, ~145 kDa).

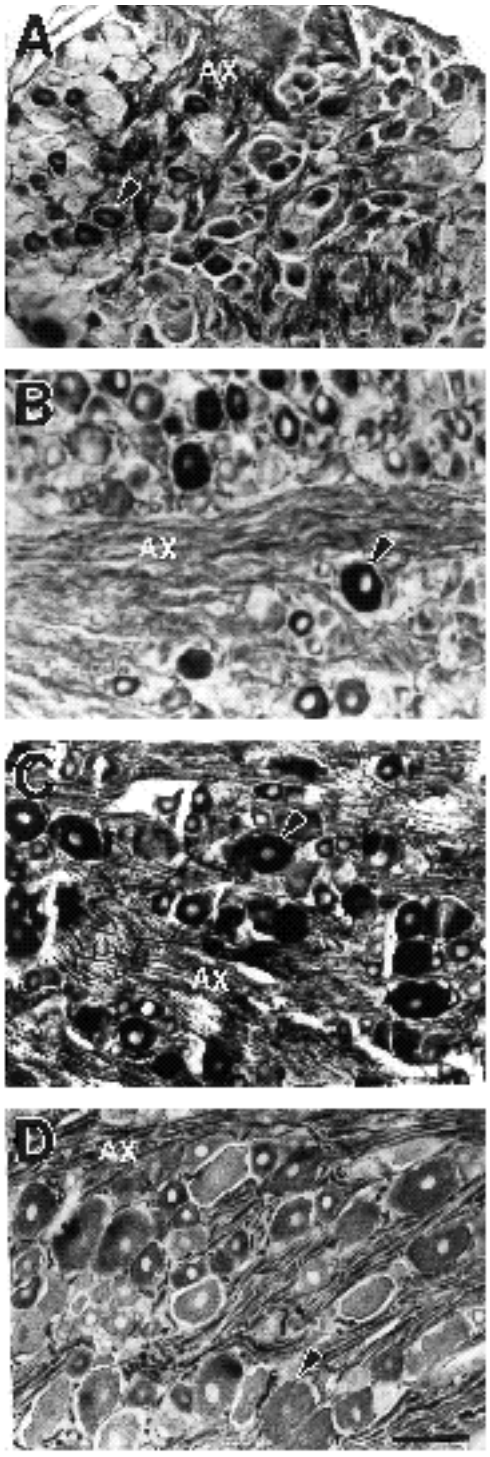

Fig. 5. Immunocytochemistry of DRG neurons at postnatal day 5 (A), day 10 (B), day 21 (C) and adult (D) using the TuJ1 monoclonal antibody specific for β_{III}-tubulin protein. In each panel, a labeled neuron is indicated by the arrowhead and a region of axons (Ax) coursing within the DRG is indicated. Bar, 91 μm.

matic than mRNA changes in both cortex and DRG during development, suggesting that the regulation of β_{III} protein levels are somewhat complex, involving post-transcriptional mechanisms of protein stabilization. Various post-translational modifications of β_{III}-tubulin have been documented, including phosphorylation on a carboxy-terminal serine residue (Diaz-Nido et al., 1990; Edde et al., 1989; Gard and Kirschner, 1985; Luduena et al., 1988). This apparently occurs only after β_{III} is incorporated into microtubules (Gard and Kirschner, 1985) and at increased amounts during development (Lee et al., 1990a,b). The role of phosphorylation or other factors (such as MAP binding) in β_{III} protein stability remain to be defined.

The observations from this study suggest that CNS and PNS neurons may differentiate quite different microtubule networks due to differences in their regulation of tubulin gene expression. If protein levels follow mRNA changes, our data would suggest that microtubules in cortex become somewhat dominated by the β_{IV} isotype, while in adult DRG, a more even composite of β_{II}-, β_{III}- and β_{IV}-tubulin develops with maturity. This conclusion is somewhat at odds with previous work that suggested β_{II} and β_{III} are the predominant β-tubulin isoforms in brain. For example, it has been suggested that 58% of cerebral β-tubulin is β_{II},

the "major" neuronal tubulin (Banerjee et al., 1988), while β_{III}, the "minor" neuronal tubulin, reportedly constitutes 25% of cerebral β-tubulin (Banerjee et al., 1990). If this is the case, there is a clear dissociation between mRNA levels and protein levels for several of the tubulins in adult brain. However, another possibility that must be seriously considered is that some protein estimates are inaccurate, depending on how "total" tubulin was defined. It must be noted that a large fraction of tubulin in brain is not solubilized by cold temperatures or calcium (Brady et al., 1984; Webb and Wilson, 1980) and thus is not present in most typical MT preparations. It is not inconceivable that this insoluble microtubule fraction from adult brain is rich in the β_{IV}-tubulin isotype, but this remains to be carefully evaluated in future studies. Recent studies have suggested that somewhat larger amounts of tubulin are cold/calcium insoluble in CNS regions compared to peripheral nerve (Taleghany and Oblinger, 1992).

The expression of all of the various tubulin isotypes at the protein level has not been studied due to the limited availability of good antibodies that are completely specific for only one isotype. However, previous studies of β_{III}-tubulin protein expression have been reported in the embryonic chick (Lee et al., 1990a) where it was noted that initial expression of β_{III} correlated with the earliest phase of neuronal differentiation. With subsequent embryonic development, the levels of β_{III} increased as more terminally differentiated neurons accumulated. In the postnatal interval, our present studies indicate that β_{III} as a proportion of total protein declines in brain. It will be of interest to determine if this is a result of progressive restriction in expression of β_{III} to fewer cells or to reduced levels within all neurons as they develop.

If development indeed channels the expression of different mixtures of tubulin isotypes in CNS and PNS neurons, what might the functional consequences of this be? This question is a component of the larger issue of whether microtubules containing different relative amounts of the various tubulin isotypes have different properties. The consensus had been that the tubulin isotypes are largely interchangeable, at least for assembly reactions (Lewis et al., 1987; Lopata and Cleveland, 1987). However, this has been somewhat challenged by observations that assembly of brain tubulin is facilitated by removal of β_{III} (Banerjee et al., 1990) and that β_{III} appears to be under-utilized for assembly in PC12 cells (Joshi and Cleveland, 1989). Since the carboxy-terminal regions of tubulin appear to be involved in the binding of MAPs (Serrano et al., 1984) and since these regions are divergent among the β-tubulin isotypes (Sullivan, 1988), it is quite conceivable that differences in the relative amounts of various tubulin isotypes might influence the degree of binding to various MAPs. Thus, the preferential association of particular MAPs with different tubulin isotypes is a potential mechanism by which the property of microtubules, including efficiency of assembly or microtubule stability, could be modulated. Further study of the functional consequences of modifying the expression of the various tubulin isotypes in vivo by molecular techniques may help clarify the actual role the various tubulin isotypes play in endowing neurons and non-neural cells with unique morphological and functional characteristics during development.

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