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Regulation of Tubulin and Actin mRNA Production in Rat Brain: Expression of a New β-Tubulin mRNA with Development

JULIAN F. BOND AND STEPHEN R. FARMER*

Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

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The expression of α-tubulin, β-tubulin, and actin mRNA during rat brain development has been examined by using specific cDNA clones and in vitro translation techniques. During brain maturation (0 to 80 days postnatal), these mRNA species undergo a significant decrease in abundance. The kinetics of this decrease varies between the cerebrum and the cerebellum. These mRNAs are most abundant in both tissues during week 1 postnatal, each representing 10 to 15% of total mRNA activity. Both α- and β-tubulin mRNA content decreases by 90 to 95% in the cerebrum after day 11 postnatal, and 70 to 80% decreases in the cerebellum after day 16. Actin sequences also decrease but to a lesser extent in both tissues (i.e., 50%). These decreases coincide with the major developmental morphological changes (i.e., neurite extension) occurring during this postnatal period. These studies have also identified the appearance of a new 2.5-kilobase β-tubulin mRNA species, which is more predominant in the cerebellar cytoplasm. The appearance of this form occurs at a time when the major 1.8-kilobase β-tubulin mRNA levels are declining. The possibility that the tubulin multigene family is phenotypically expressed and then this expression responds to the morphological state of the nerve cells is discussed.

The growth and differentiation of a variety of cell types both in vivo and in vitro require elaborate changes in cell morphology. Extracellular matrices are intimately involved in such processes (for review see references 16 and 18); as well as facilitating cell migration, they enhance the adhesion of cells to surfaces, resulting in overall cell shape changes which are required for growth (10, 11) and phenotypic expression (16, 18).

Several researchers are now beginning to ask: what are the signals to which the cell responds during such shape changes and how are these signals transmitted to the genome? In recent years, the cytoskeleton has been implicated in playing a role in these mechanisms. For instance, several studies have suggested that microtubules and microfilaments both act to transmit serum growth factor signals that stimulate the initiation of DNA synthesis in fibroblasts (6, 7, 12, 24). We have recently proposed that the expression of cytoskeletal protein genes may respond to the configuration state of the cytoskeleton. In these studies, we demonstrated that the regulation of tubulin mRNA production responds to the polymerization state of microtubules (2) and that actin mRNA expression changes dramatically during the cell configuration changes accompanying cell growth (9). Other studies suggest that this actin regulation is probably responsive to alterations in the organization of the microfilaments (36). We have further shown that tubulin, actin, and vimentin mRNA synthesis decreases with the morphological differentiation of adipocytes in vitro (34).

To investigate the regulation of genes in response to morphological differentiation of cells in vivo, we have initiated a study of the expression of tubulin genes during rat cerebellum development. This is an ideal system to carry out such a study, since a characteristic feature of the differentiation of neurons present is an extensive neurite outgrowth involving the mobilization of large pools of cytoskeletal proteins into the various structural elements of the axons and dendrites. Furthermore, this system is particularly suited to the study of tubulin gene expression, since previous investigations have shown that tubulin synthesis represents a large percentage of total protein synthesis in neonatal brains and then it declines to very low levels during later development (30). Studies by these and other investigators have also demonstrated an extensive tubulin microheterogeneity in the whole brain and in the cerebellum compared to other organs such as spleen and liver (14, 35). This microheterogeneity also appears to change during development (14).

These previous studies were performed on whole rat brain, an organ containing many different nerve cell types and segregated into several regions undergoing asynchronous development. The observations made may therefore reflect changes in cell populations and not absolute changes in specific gene expression in a particular nerve cell type. Furthermore, the absence of specific probes for tubulin and actin mRNAs in these initial studies prevented the investigators from defining levels of regulation and also determining whether the microheterogeneity they observed is due to differential expression of the tubulin multigene family.

In the present study, we have analyzed the expression of tubulin and actin mRNA production, using specific cDNA clones, during rat brain development. We have compared two morphologically distinct regions, the cerebrum and cerebellum, in maturing rats. The cerebellar cortex is a particularly suitable tissue to study, since it is comprised of a few well-defined cell types, and the granular neuronal cells are by far the most abundant cells present, appearing shortly after birth (17).

The results presented here reveal that both tubulin and actin mRNA production is regulated during cerebral and cerebellar development. The kinetics of the regulation differs between the two tissues. However, the overall result is a dramatic decline in mRNA levels, and this accompanies the morphological differentiation of the nerve cells present. Coincident with this response is the expression of a new β-tubulin mRNA species. The possibility that the tubulin genes in particular are phenotypically expressed in brain nerve cells and that this expression responds directly to changes in the morphological state of the cells is discussed.

(Portions of this paper are derived from the thesis of J.F.B. presented in 1983 in partial fulfilment of the requirements of the Ph.D. degree, Boston University, Boston, Mass.)

MATERIALS AND METHODS

Tissue preparation and RNA extraction. Sprague-Dawley rats, obtained from Charles River Breeding Laboratories, Inc., were used as the source of brain tissue. In the whole brain study, rats were sacrificed at various ages from 4 days prenatal to 11 months postnatal. Whole brain tissue was removed, twice washed in ice cold phosphate-buffered saline, minced, and then homogenized on ice in RSB (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris [pH 8.3], 10 mM vanadyl-adenosine, 1% Nonidet P-40) with 10 strokes of a Potter-Elverheim homogenizer. Nuclei were pelleted by centrifugation at 4°C. Total RNA extraction from the supernatant was performed by the modified phenol-chloroform procedure described recently (23). Separated cerebral and cerebellar tissue were removed from rats at several different times from birth through 80 days. These tissues were homogenized in 6 M guanidine-hydrochloride in an RNA extraction procedure outlined elsewhere (25) with the only modification being the use of guanidine-hydrochloride instead of guanidine-isothiocyanate. Cerebellar tissue that was used for preparation of separated nuclear and cytoplasmic RNA was homogenized in sterile STKM (0.32 M sucrose, 50 mM Tris-hydrochloride [pH 7.6], 50 mM KCl, 1 mM $MgCl_2$) with 10 mM vanadyl-adenosine. Nuclei were spun down, separated from the supernatant, and extracted as above in phenol-chloroform. Human cerebellar RNA was extracted with 6 M guanidine-hydrochloride from frozen tissue received from Edward Bird (McLean Hospital, Belmont, Mass.), obtained from postmortem victims of Huntingtons chorea. All extracted aqueous RNA preparations were brought to 0.2 M sodium acetate, and the RNA was precipitated at −80°C in 70% ethanol.

Selection, quantitation, and in vitro translation of poly(A)$^+$ mRNA. Isolation of polyadenylic acid-containing [poly(A)$^+$] RNA was performed by using oligodeoxythymidylic acid [oligo(dT)] cellulose (Collaborative Research, Inc.) (1). Poly(A)$^+$ RNA was quantitated by hybridization to [^{3}H]polyuridylic acid [poly(U)] as outlined elsewhere (3). Translations of poly(A)$^+$ RNA by using reticulocyte lysate with [^{35}S]methionine (New England Nuclear Corp.) were carried out in 25-μl reaction mixtures containing 0.5 μg of poly(A)$^+$ RNA (28). Radiolabeled amino acid incorporation into polypeptides was assayed as trichloroacetic acid-precipitable radioactivity in 2 μl of reaction mixture.

Protein gel electrophoresis, staining, and fluorography. One-dimensional sodium dodecyl sulfate slab gels were performed with 10% polyacrylamide by the method of Laemmli (20). Two-dimensional gel electrophoresis was run by the method of O'Farrell (26), using a mixture of ampholytes (Ampholines; LKB Instruments, Inc.); 1.6%, pH 3 to 10; 0.4%, pH 5 to 7. Gels were stained with Coomassie blue and destained with 20% methanol–7% acetic acid. Gels were fluorographed as outlined previously (21).

RNA gel electrophoresis and filter hybridization. Poly(A)$^+$ mRNA (1 μg per lane) was electrophoresed on a 1% agarose gel containing 6% formaldehyde and 0.5 μg of ethidium bromide per ml as described previously (9). The gel running buffer was 20 mM MOPS (morpholinopropanesulfonic acid)–50 mM sodium acetate–1 mM EDTA (pH 7.0). RNA samples were denatured at 67°C for 15 min in deionized formamide, 6% formaldehyde, and running buffer containing bromophenol blue. Electrophoresis was carried out overnight at constant voltage (50 V). After electrophoresis, the gel was washed in 10× SSC (1× SSC is 1.5 M NaCl plus 0.15 M sodium citrate) for 30 min. Stained RNA bands were viewed under UV light after washing. RNA was transferred to nitrocellulose paper (Schleicher & Schuell Co.) in 20× SSC for 4 h. After the transfer, the blots were checked under UV light for efficiency of transfer, washed in 3× SSC, and air dried overnight. Drying was completed in a vacuum oven at 80°C for 2 h. Hybridization with 0.5×10^6 cpm per lane of ^{32}P-labeled cDNA was performed in a plastic Seal-n'-Save bag (Sears Roebuck and Co.) as described recently (5). Before exposure to film, all blots were washed at high stringency; 20 min in 2× SSC at room temperature, 15 min in 0.4× SSC at

50°C, and finally 30 min in 0.2× SSC with 0.1% sodium dodecyl sulfate at 65°C. For reuse, the RNA blots were washed free of radioactive probe in 50% deionized formamide plus 10 mM Tris-hydrochloride–1 mM EDTA for 1 h at 60°C with agitation. Before reprobing, the blots were exposed to film as a check for the efficiency of probe removal. Acquisition and characterization of the rat α-tubulin cDNA clone are described elsewhere (23). A manuscript describing the rat β-tubulin and actin cDNA characterization is in preparation. Radiolabeling of cDNA was performed as previously described (34). Densitometric tracing of film exposed to radiolabeled RNA blots was performed on a Transidyne 2955 scanning densitometer equipped with an integrator for the quantitation of signals.

RESULTS

Quantitation of tubulin and actin mRNA levels. (i) Whole brain. Before analyzing the different regions of the brain we decided, first of all, to quantitate the changes in tubulin and actin mRNA production in the whole brain. This was done so that we could eventually relate any differences observed between the cerebellum and the cerebrum with the pattern in the whole brain and also to confirm previous observations made without the use of specific cDNA clones (14, 30).

The following studies involved both a quantitative and qualitative analysis of α- and β-tubulin and actin mRNAs by using in vitro translation and specific cDNA hybridization techniques. Total RNA was isolated from whole rat brain at the various ages shown; poly(A)$^+$ mRNA was separated and quantitated, and equal amounts were translated in the cell-free reticulocyte lysate system (Fig. 1A). It is apparent from this fluorograph of the translation products that there is a significant decrease in the in vitro synthesis of α- and β-tubulin and actin proteins. The pattern of shutoff appears to be more dramatic than observed previously (30). At birth, both tubulin and actin each represent 20 to 25% of total in vitro protein synthesis. This value declines rapidly reaching 2 to 5% by 33 days. Also evident in this cell-free translation analysis are other interesting changes in mRNA species. Several high-molecular-weight proteins disappear, whereas a number of smaller species accumulate. This is in agreement with other studies in mRNA translation during rate cerebellar development (33).

This decrease in mRNA activity was shown to reflect a relative decrease in mRNA concentration for both the tubulins and actin as evidenced by Northern blot hybridization, using specific cloned cDNAs. Equivalent amounts of poly(A)$^+$ RNA {quantitated by [^{3}H]poly(U) hybridization} were electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose. Filters were hybridized to plasmid ^{32}P-labeled DNA containing cDNA sequences complementary to rat β-tubulin or actin. The blot shown in Fig. 1B

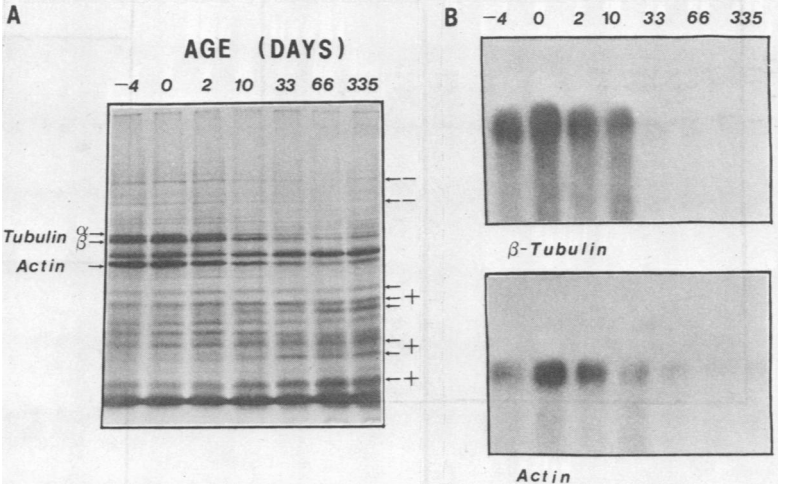

FIG. 1. Analysis of in vitro translation products from developing whole rat brain. Each lane contains equal amounts of [^{35}S]methionine-labeled proteins (5×10^4 cpm) from the translation of 0.5 μg of poly(A)$^+$ RNA extracted at the times indicated. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) as outlined in the text. Arrows on the right hand margin indicate the position of unknown proteins which change in abundance during rat brain maturation (+, for those which increase; − for proteins that decrease with aging). (B) Northern blot hybridization analysis of poly(A)$^+$ RNA (1 μg per lane) was electrophoresed through 1% agarose–6% formaldehyde as described in the text. The lanes correspond to the same time points and RNA preparations as in (A). Two separate blots were prepared from duplicate loadings on the same gel. The relative sizes for actin and β-tubulin mRNAs are 2.2 and 1.8 kb, respectively.

supports the in vitro translation data, showing that β-tubulin sequences begin to decrease gradually in quantity after birth until 10 days and then decline dramatically to the very low levels seen at 33 days. The decline in actin sequences is also evident but not so dramatic. It also appears that there is an induction in both β-tubulin and actin mRNA sequences occuring during the last 4 days of gestation reaching maximum levels at birth.

(ii) Cerebrum versus cerebellum. The one problem encountered with studies such as these involving whole brain tissue is the heterogeneity of both neuronal and glial cell populations. A significant change in proportions of these various cells during development could possibly account for the observations seen in Fig. 1. In an attempt to clarify this issue, we have analyzed two major regions of the brain, the cerebrum and cerebellum. These tissues are known to have significantly different morphological features comprised of different neuronal and glial cells. The cerebellum in particular is an ideal tissue to study since it is less heterogeneous, containing a few well-defined cell types, with a high abundance of granular neurons (17). mRNA was isolated from these regions as described above and translated in vitro (Fig. 2A). Surprisingly, we observed very similar developmental patterns in translation products. Again, both α- and β-tubulin and actin mRNA activity decreased during the time period analyzed. In the cerebral cortex, α- and β-tubulin mRNA translation products increased after birth, peaked at day 8, and then declined thereafter. The cerebellum pattern was similar but not as pronounced. Actin mRNA activity appeared similar to both tubulins. As seen in whole brain, there were changes in several other mRNAs.

To unequivocally demonstrate that the major decreases in mRNA activity in these regions were accounted for by tubulins and actin, we analyzed selected translation products on two-dimensional gels. Figure 2B shows such an

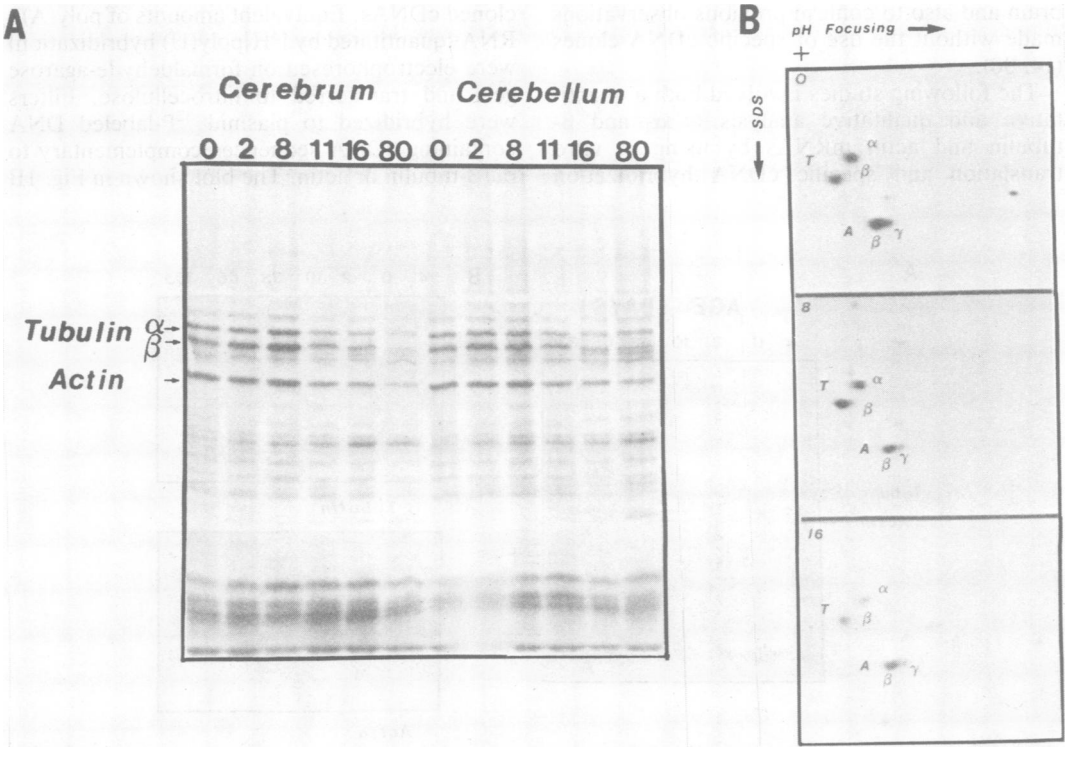

FIG. 2. (A) Analysis of proteins produced by in vitro translation of poly(A)$^+$ mRNA from rat cerebrum and cerebellum. RNA isolated from cerebral and cerebellar tissue was extracted at the times indicated. Protein gel analysis was the same as in Fig. 1A. (B) Two-dimensional gel electrophoretic analysis of cerebral cell-free translation products. Equal amounts of reaction mixtures, the same ones used in (A), were analyzed by isoelectric focusing followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see text). Three time points, as indicated on each gel, from cerebral cortex were chosen to demonstrate the degree of translation decrease for α-tubulin, β-tubulin, and actin during this short developmental period. The gels were deliberately underexposed to emphasize these cytoskeletal proteins.

analysis of cerebrum; similar results were obtained for the cerebellum (data not shown). It is quite apparent that tubulin and actin mRNAs are the sequences affected and that the major shutoff occurs between days 8 and 16 after birth in the cerebrum.

To perform a definitive quantitative analysis of the decrease in these three mRNA sequences during the development of both brain regions, Northern blot hybridizations (Fig. 1) were carried out. Figure 3 represents the same filter sequentially hybridized to the three cDNAs: α-tubulin, β-tubulin, and actin with removal of the previous radioactive DNA by special washing conditions (see above). This procedure allowed us to compare relative quantities of the different mRNA sequences in the same RNA preparations. Each lane contained 1 μg of poly $(A)^+$ RNA isolated from the tissues. Both nuclear and cytoplasmic RNA were analyzed since the total tissue was extracted by using 6 M guanidine-hydrochloride–cesium chloride in the procedure outlined above. A decrease in hybridization signal with age is seen with all three cDNA clones. However, there are significant time course and quantitative differences between the tissues for the different mRNAs. This is better represented in Fig. 4 (see below). A possible precursor form is seen at day 0 in cerebellum for all three probes and in cerebrum for α-tubulin. This higher-molecular-weight signal is ca. 4 kilobases (kb) long and is the expected size for a primary transcript of α-tubulin genes (22). The sizes indicated in the figure were calculated from known mRNA lengths and ethidium bromide staining of 18S and 28S rRNAs.

The relative decrease in mRNA sequence quantity was estimated by densitometric scanning of the respective autoradiographs shown in Fig. 3. These data are represented in Fig. 4 as a graph of the signal intensity at each time point relative to the cerebral value at birth (i.e., the first lane of each blot). Since the different ^{32}P-labeled cDNA plasmids were at different specific activities and hybridized at different times, there is no attempt made to compare absolute abundance of each of the three sequences in the same mRNA sample. The data presented only represent a relative change in the separate mRNAs for the two brain regions, since all these RNA samples are on the same filter. The data shown suggest an earlier decrease in the cerebrum than in the cerebellum. In the cerebrum, there is a slight increase in α- and β-tubulin mRNA content peaking at 8 days after birth. Immediately after this time, there is a dramatic decrease in both sequences declining rapidly to 5 to 10% of the peak values by 80 days after birth. The pattern observed for the cerebellum shows that there is no increase in tubulin sequences after birth. Instead, there is a slight decrease up until 16 days after birth and then a more significant drop between 16 and 80 days. The decrease in α-tubulin sequences is 70% and in β-tubulin is 80%. Interestingly, it appears that α- and β-tubulin mRNA production may not be coordinately controlled in the cerebellum. The signal intensity for β-tubulin in the cerebellum is much

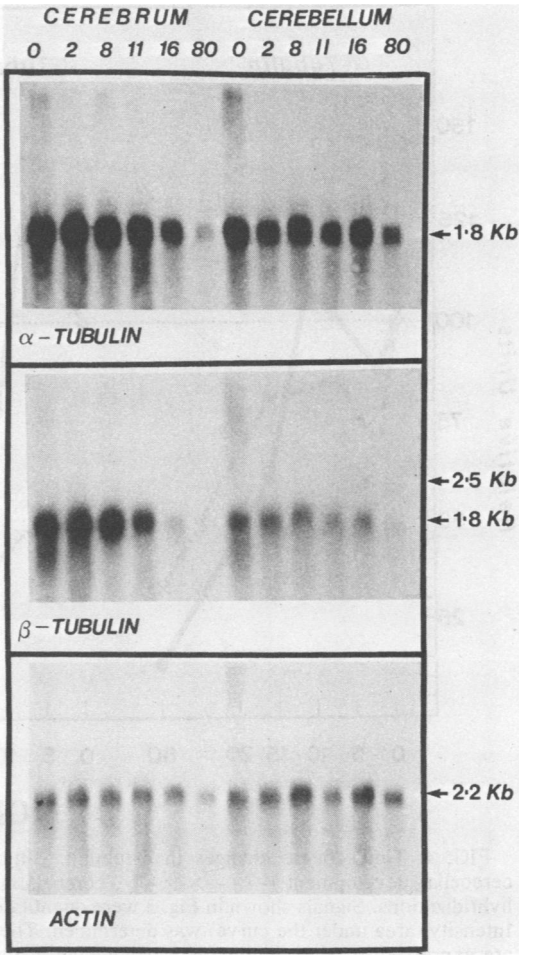

FIG. 3. Hybridization analysis of poly$(A)^+$ RNA (1 μg per lane) from rat cerebrum and cerebellum. This figure represents the same blot hybridized to the three separate ^{32}P-labeled cDNA clones indicated. RNA preparations were the same as those used for the in vitro translations seen in Fig. 2A. Removal of each probe for reuse was accomplished by a wash procedure with 50% deionized formamide in Tris-EDTA (see text). Labeled arrows indicate relative message size in poly$(A)^+$ RNA from total tissue preparations. The 2.5-kb arrow points to a faint band of RNA that hybridizes to β-tubulin cDNA and migrates at a slower rate upon formaldehyde-agarose electrophoresis. This form is most prevalent in cerebellum and seen only at 16- and 80-day time points (see Fig. 5).

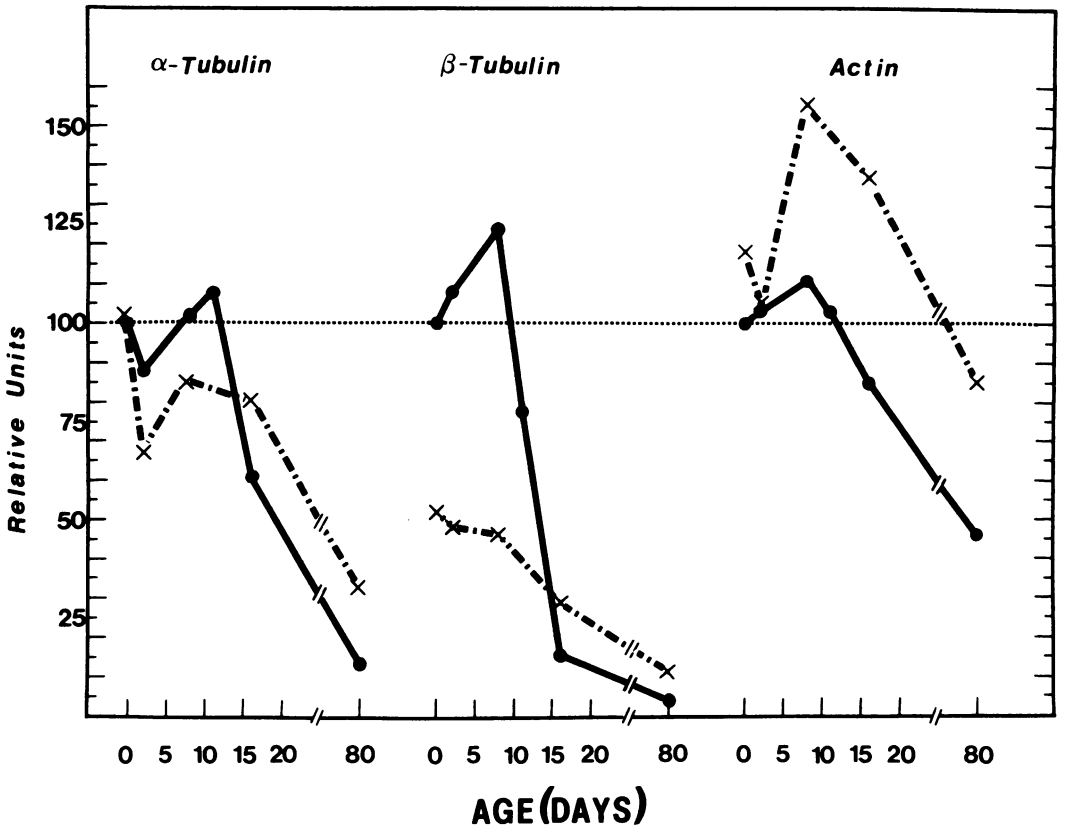

FIG. 4. Time course changes in α-tubulin, β-tubulin, and actin mRNA quantities during cerebral and cerebellar development (- - - - ×- - - - -, cerebellum; ——●——, cerebrum) by relative quantitation of blot hybridizations. Signals shown in Fig. 3 were quantitated by densitometric scanning using a Transidyne scanner. Intensity (area under the curve) was determined. These values were expressed in the graph above. These data are expressed in relative units as a percentage of the first signal (day 0 cerebrum) of each probing.

less than that for the cerebrum, whereas the α-tubulin signal is comparable in both tissues. This suggests there is significantly less β-tubulin mRNA species present.

The developmental pattern observed for actin mRNA content in both tissues is very similar. There is an increase in sequences after birth peaking at day 8. This is followed by a decline that is not as dramatic as seen for the tubulins down to 50% of peak values by day 80.

These densitometric data were confirmed by serial dilution of day 0 mRNA from each tissue on a single filter until a signal equivalent to a fixed amount of day 80 RNA was obtained (data not shown). The dilution factor then represents the relative decrease during development, and this corresponded to the data seen in Fig. 4.

Due to the relatively high abundance of these sequences in young rat brains, the intensity of hybridization signal was very high; thus, only short exposures to film were necessary to generate Fig. 3. However, during a longer exposure, we noticed the presence of a new β-tubulin mRNA species accumulating during development, and this species was particularly predominant in the cerebellum. Figure 5 shows this longer exposure of the cerebellar RNA blot probed with β-tubulin cDNA (Fig. 3, middle panel). This new form appears at day 16, the time when overall β-tubulin sequence abundance starts to decline in the cerebellum; it is not detectable at day 11. The relative abundance of this 2.5-kb mRNA remains high and is detectable at 80 days, whereas the amount of 1.8-kb β-tubulin sequence decreases significantly during this time period. This longer exposure also reveals a larger 2.9-kb β-tubulin mRNA faintly visible at the earlier time points. The 4-kb arrow indicates the location of discrete bands seen at shorter exposure times. Again, this may represent the nuclear precursor to β-tubulin mRNA.

To ascertain whether or not this 2.5-kb mRNA

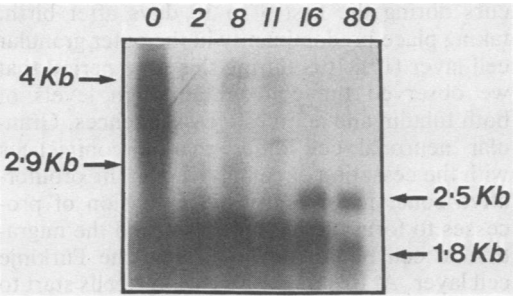

FIG. 5. Expression of a unique 2.5-kb β-tubulin mRNA species during rat cerebellar development. The blot corresponding to cerebellum β-tubulin in Fig. 3 was exposed to film three times longer. The larger sizes of β-tubulin poly(A)$^+$ RNA is clearly visible, 2.5 kb at 16 and 80 days and 2.9 kb at 2 and 8 days. Sizes were estimated from known message sizes and ethidium bromide stained 28S and 18S rRNAs.

species was a stable nuclear precursor, we analyzed, separately, nuclear and cytoplasmic RNA from 80-day rat cerebellum. Figure 6 is a Northern blot hybridization analysis of various RNA preparations probed first with β-tubulin cDNA and then with actin as an internal control. Nuclear and cytoplasmic RNA was isolated from the 80-day-old cerebellum as outlined above. The amount of nuclear RNA recovered was 10% of the total cellular RNA. Respectively, 5 μg of total nuclear RNA and 15 μg of total cytoplasmic RNA were electrophoresed in lanes A and B. Thus, 3.3 times more nuclear RNA than cytoplasmic RNA per cell was analyzed. Figure 6 shows that the 2.5-kb form is exclusively located in the cytoplasm and is therefore not a stable nuclear precursor. It is interesting that we can detect an actin nuclear signal but very little β-tubulin signal.

It is conceivable that this new species was the 2.9-kb species observed by others in CHO cells, chick fibroblasts, *Xenopus* cells, and chick brain (4, 5). We have also identified such a 2.9-kb species in 3T3 mouse fibroblasts and rat epithelial cells (unpublished data). Thus, to confirm that this new brain species was different to these other known forms, we compared the relative migration of mRNA species from 80-day rat cerebellum (Fig. 6, lane D), rat epithelial cells (lane E), and also from adult human cerebellum (lane C). The β-tubulin mRNA species from rat epithelial cells migrate at 2.9 kb and 1.8 kb, whereas the larger species in rat cerebellum are definitely migrating faster than the 2.9-kb species, with an apparent size of 2.5 kb. It is also interesting that the larger β-tubulin species in human cerebellum is 2.9 kb and that there may be two smaller forms migrating at 1.8 kb.

DISCUSSION

The data presented here show that the production of tubulin and actin mRNAs changes during the development of the whole brain and the different regions. The overall pattern observed is characterized by a decrease in sequence level for all three mRNAs: α-tubulin, β-tubulin, and actin. However, the time course and extent of change differs among the three mRNAs and the regions analyzed. The decreases in the whole brain appear to be more dramatic and occur a few days sooner than in either of the separated tissues (cf. Fig. 1 with Fig. 3). It is quite likely that this is to some extent due to changes in

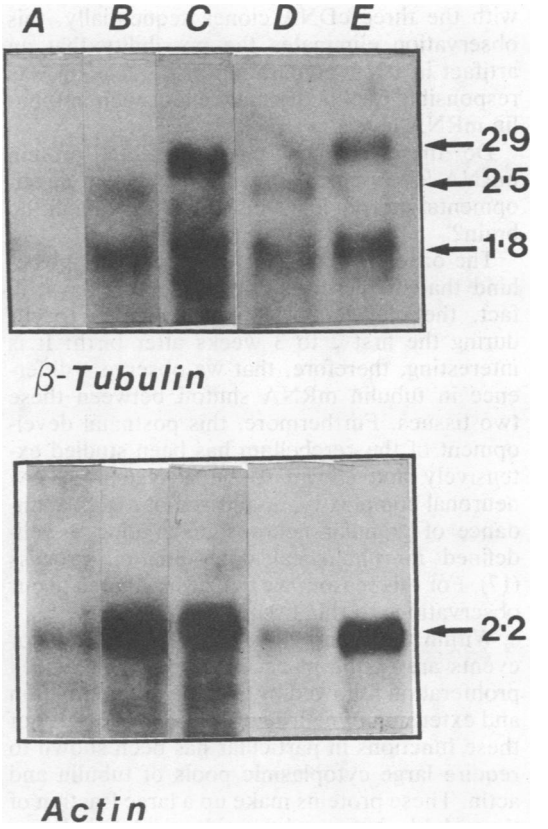

FIG. 6. Northern blot analysis of 2.9- and 2.5-kb species in RNA from different sources. RNA was electrophoresed on formaldehyde-agarose gel followed by blot hybridization (see text). The blots were probed first with β-tubulin cDNA followed by exhaustive washing and reprobing with actin. Lane A, 5 μg of nuclear RNA from 80-day rat cerebellum; Lane B, total cytoplasmic RNA (15 μg) from 80-day rat cerebellum; Lane C, total cellular RNA (15 μg) from human adult cerebellum; Lane D, cellular poly(A)$^+$ RNA (1 μg) from 80-day rat cerebellum; Lane E, poly(A)$^+$ RNA (1 μg) from rat epithelial cells. Kilobase sizes are given in the righthand margin.

overall cell populations. As stated earlier, analysis of the separated tissues and the cerebellum in particular is much more meaningful. The kinetics of the response for α- and β-tubulin mRNA levels is dramatic in the cerebrum, with a 90% decrease in α-tubulin mRNA levels and a 95% decrease in β-tubulin mRNA levels over the time period analyzed. The response in the cerebellum is slightly less dramatic: α-tubulin decreases 75%, whereas β-tubulin decreases 80% of the peak values.

Actin mRNA levels decrease to a lesser extent, declining to 50% of day 0 levels in both tissues. The fact that actin changes to a lesser degree than either of the tubulins reinforces the observations made from Fig. 3 for the tubulins. That is, since the same RNA blot was probed with the three cDNA clones sequentially, this observation eliminates the possibility that an artifact in RNA preparation or gel loading was responsible for the dramatic effect seen in tubulin mRNA levels.

Do these changes in cytoskeletal protein mRNA levels correlate with any known developmental morphological events occurring in the brain?

The onset of cerebellar development lags behind that of the cerebrum by several days; in fact, the major morphological changes occur during the first 2 to 3 weeks after birth. It is interesting, therefore, that we observe a difference in tubulin mRNA shutoff between these two tissues. Furthermore, this postnatal development of the cerebellum has been studied extensively and shown to be a system of low neuronal complexity, comprised of a high abundance of granular neurons undergoing a well-defined morphological differentiation process (17). For this reason, we will primarily relate our observations to this tissue.

Within the time points presented, two major events are taking place in the cerebellum: cell proliferation followed by terminal differentiation and extension of neurite processes. The latter of these functions in particular has been shown to require large cytoplasmic pools of tubulin and actin. These proteins make up a large fraction of the soluble brain polypeptides isolated during early development (30). It is not surprising, therefore, that this abundance is reflected in their respective mRNA levels (Fig. 2 [i.e., 40 to 50% of the total in vitro translatable mRNA]). However, it is worth noting that these levels of tubulin mRNA are much higher than those recorded in any other tissue or cell type in vitro. This implies that the genes coding for these proteins are phenotypically expressed at these high levels to accommodate the later developmental processes (see below).

The proliferative stage of differentiation occurs during the first 5 to 10 days after birth, taking place predominantly in the outer granular cell layer (17). It is during this time period that we observed the concomitant high levels of both tubulin and actin mRNA sequences. Granular neuronal cell differentiation commences with the cessation of cell division in the proliferative zone, followed by the extension of processes to form the parallel fibers and the migration of cell bodies down through the Purkinje cell layer. At this time, the Purkinje cells start to differentiate by extending an elaborate dendritic tree which synapses with the parallel fibers. The result of this whole process, occurring between 10 and 21 days after birth, is the reduction in the outer proliferative granular layer and the formation of the well-differentiated molecular and inner granular cell layers. It is interesting that the shutoff in both tubulin and actin mRNA levels coincides exactly with this morphological process. It therefore appears that mobilization of tubulin and actin proteins into axons and dendrites occurs at a time when their respective mRNAs are declining. This would imply a stockpiling of these subunits in the rapidly proliferating neurons followed by their incorporation into the microtubules and microfilaments of the neurites. This hypothesis is supported by studies showing that differentiating neuroblastoma cells and nerve growth factor-stimulated embryonic chick neurons can produce extensive neurites, in vitro, after the inhibition of protein synthesis (27, 31).

The expression of tubulin and actin genes is required by all eukaryotic cells for proliferation. However, it is the phenotypic morphological functions of these cytoskeletal proteins in developing neurons that requires the magnitude of the levels observed. This is also reflected in the high abundance of mRNAs coding for these subunits in early postnatal cerebellar development. The rapid decrease in these specific RNA sequences at the onset of neurite extension implies a strict regulation correlated with terminal differentiation. Several mechanisms could be operating to effect such a response. Regulatory signals could be generated as a result of the cessation of cell division or the extension of the neurites or both. We believe that the cessation of cell division alone does not initiate the decline. This is supported by previous studies which have shown that growth arrest of cells does not result in the dramatic decreases in tubulin mRNA levels observed here (34). The mechanism is more likely related to the significant changes in the polymerization state of the cytoskeletal proteins during this time period. It has been demonstrated in many systems that the level of cytoplasmic tubulin mRNA is responsive to the amount of unpolymerized tubulin protein present (2, 5). An

increase in unpolymerized tubulin leads to a specific decrease in their respective mRNAs. What role this response phenomenon plays in developing cerebella is not known. However, it is conceivable that with the cessation of cell division, the neurons become sensitive to the high levels of tubulin subunits and respond by a decrease in their mRNAs.

The data presented here do not distinguish among the possible levels of regulation (i.e., decreased transcription, altered nuclear RNA processing, or increased turnover rates). The same decrease in specific mRNA sequences observed in the poly(A)+ RNA were also seen on blots of total RNA using the same time points (data not shown). This rules out the possibility that the sequence decrease is caused by the selective loss of the poly(A)+ tail with maturation. It is interesting that we observe a nuclear precursor for all three mRNAs at birth, but this signal disappears rapidly several days before the decline in mRNA content. It is tempting to speculate that the nuclear activity may decrease with the cessation of cell division, and the mRNA levels decline only after extensive neurite projection has begun. We are at present analyzing the time course of nuclear RNA production with specific intervening sequence probes. The differences in kinetics between actin and tubulin may be due to known differences in their respective message half-lives. The half-life for actin mRNA has been reported to be greater than 12 h, whereas that for α- and β-tubulin is ca. 2 h (2, 5).

Expression of a new 2.5-kb β-tubulin mRNA species with brain development. This new β-tubulin species is present both in the cerebrum and cerebellum but is particularly enriched in the latter tissue. This species appears in the cytoplasm at day 16 and remains at the same abundance until 80 days. The data in Fig. 6 clearly demonstrate that it does not arise due to the accumulation of a stable nuclear precursor and that it is also a different form from the previously observed 2.9-kb species.

At present, we are not able to comment on the possible function of this species. Several investigations have suggested that tubulin microheterogeneity exists in the brain and increases with development (14). Furthermore, it has been suggested that some of this microheterogeneity could be due to the presence of multiple mRNAs generated from different genes (13). There are at least 10 α-tubulin genes and 10 β-tubulin genes in the rat genome; how many of these are pseudogenes is not known. This 2.5-kb species may therefore be the product of a different gene which is under developmental control. The tubulin protein product may vary from the 1.8-kb product and serve a different function in neuronal cells. There is already direct proof of primary structure variation in different isotypes of tubulin from porcine brain (19). Single neurons appear to have multiple isotypes of tubulin (15), and it is possible that these different forms have different roles at specific subcellular sites. It has recently been suggested that there is a differential distribution of the α- and β-tubulin subunits (8). These researchers demonstrate that there is a selective deficiency of α-tubulin compared with β-tubulin in parallel fibers in the cerebellum.

This 2.5-kb form could also be generated from the same gene as the 1.8-kb species simply by direct readthrough at the 3′ terminus, adding an extra 3′ untranslated region. This phenomenon has been observed for other mRNA sequences (29, 32, 37) and in one particular case to be under developmental control (29). These questions are now being addressed by DNA sequencing of genomic and cDNA clones.

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