

# Induction of Neuron-specific Tropomyosin mRNAs by Nerve Growth Factor Is Dependent on Morphological Differentiation

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**Abstract.** We have examined the expression of brain-specific tropomyosins during neuronal differentiation. Both TmBr-1 and TmBr-3 were shown to be neuron specific. TmBr-1 and TmBr-3 mRNA levels increased during the most active phase of neurite outgrowth in the developing rat cerebellum. In PC12 cells stimulated by nerve growth factor (NGF) to differentiate to the neuronal phenotype, TmBr-1 and TmBr-3 levels increased with an increasing degree of morphological differentiation. Induction of TmBr-1 and TmBr-3 expression only occurred under conditions where PC12

cells were permitted to extend neurites. NGF was unable to maintain levels of TmBr-1 and TmBr-3 with the loss of neuronal phenotype by resuspension of differentiated PC12 cells. The unique cellular expression and regulation in vivo and in vitro of TmBr-1 and TmBr-3 strongly suggests a critical role of these tropomyosins in neuronal microfilament function. The findings reveal that the induction and maintenance of the neuronal tropomyosins is dependent on morphological differentiation and the maintenance of the neuronal phenotype.

**T**HE unique morphological characteristic of neurons, and the major involvement of microfilaments in cytoarchitecture and neurite outgrowth, (Smith, 1988) suggests that neuronal microfilaments possess characteristics different from other cell types. The major components of nonmuscle microfilaments,  $\gamma$ - and  $\beta$ -actin, differ in amino acid sequence by only four residues (Vandekerckhove and Weber, 1978, 1984) and have an apparently similar distribution within neurons (Choo and Bray, 1978). Therefore, the regulation of microfilament function is thought to be controlled by a diverse group of actin-binding proteins (ABPs)<sup>1</sup> that interact directly or indirectly with microfilaments (Stosel, 1990). Tropomyosins (Tm) are integral components of microfilaments (Bailey, 1948; Mooseker et al., 1981). They are rod like proteins that dimerize in a head-to-tail fashion by an overlapping of the NH<sub>2</sub> and COOH amino acids of adjacent molecules (Ueno et al., 1976; Mak and Smillie, 1981). The Tm filament binds in the groove between the paired helical actin filaments (Smillie, 1979). In muscle cells Tms are involved in mediating Ca<sup>2+</sup> regulation of the actin-myosin interaction, through binding of troponin to specific sites on the Tm peptide (Smillie, 1979; Taylor, 1979). The function of Tms in nonmuscle cells is not well understood, but is thought to be involved in stabilizing actin filaments. In addition, tropomyosins show a great deal of isoform diversity, characterized at either the mRNA or protein level, that is achieved largely by alternative splicing but also by differential gene expression (Lin et al., 1985; Macleod et al.,

1985; Helfman et al., 1986; Ruiz-Opazo and Nadal-Ginard, 1987; Wieczorek et al., 1988; Lees-Miller et al., 1990; Goodwin et al., 1991; Forrey-Schaudies and Hughes, 1991). The expression of these Tm isoforms in a highly tissue-specific manner, via alternative promoter and alternative RNA processing (Ruiz-Opazo and Nadal-Ginard, 1987; Wieczorek et al., 1988; Lees-Miller et al., 1990; Goodwin et al., 1991; Forrey-Schaudies and Hughes, 1991) strongly suggests that each isoform is required to carry out specific functions in the microfilaments of muscle and nonmuscle cells.

Evidence that tropomyosins may contain unique regulatory properties in specialized regions of cells has been obtained from both in vitro actin binding (Dabrowska et al., 1980; Keiser and Wagner, 1985), and cell localization studies (Lin et al., 1988). Immunocytochemical studies have shown that lower molecular weight tropomyosins (248 amino acids [aa]) are concentrated at the ruffling motile edges of fibroblasts, whereas higher molecular weight tropomyosins (284 aa) are found exclusively associated with stress fibers (Lin et al., 1988). As some small Tms bind F-actin weaker than their large counterparts in vitro (Lin et al., 1985; Matsumura et al., 1985), this property may make them more appropriate to be localized to regions of actin filament instability such as the motile edges of fibroblasts.

In the developing neuron, tropomyosin has been shown to be present in the growing neurite and in particular in the leading edges of the growth cone (Letourneau and Shattuck, 1989). However, the localization of specific isoform(s) has not been determined. The colocalization of Tm and actin in the growth cone and the unique in vitro actin-binding and biochemical properties of brain Tm (Dabrowska et al., 1980;

1. *Abbreviations used in this paper:* aa, amino acid; ABP, actin-binding protein; NGF, nerve growth factor; Tm, tropomyosins.

Sobieszek and Small, 1981; Sobieszek, 1982), suggests that they have specialized properties and functions in the nervous system. The importance of microfilament function in neurite outgrowth (Smith, 1988) would suggest that brain Tms are likely to be involved in neuronal differentiation. The three tropomyosins thought to be localized in the brain, designated TmBr-1, TmBr-2, and TmBr-3, are derived from the  $\alpha$ -Tm gene by alternative RNA processing (Lees-Miller et al., 1990). These isoforms have only been characterized at the RNA level, but the predicted proteins are 281, 251, and 245 aa in length, respectively. Not only do the predicted proteins differ significantly in size but the NH<sub>2</sub>- and COOH-terminal regions of these molecules are also vastly different (Lees-Miller et al., 1990). TmBr-1 and TmBr-3 have COOH termini that are unique among all the known Tms, whereas TmBr-2 has an alternate NH<sub>2</sub>-terminal region comprising 39 amino acids that differs from TmBr-1 and TmBr-3 (see Fig. 1). Nevertheless the internal region of these molecules is highly conserved and identical to the  $\alpha$ -Tm skeletal muscle isoform. It has been suggested that the individual primary amino acid sequence may impact on the secondary structure of these isoforms and may confer unique F-actin binding properties (Lees-Miller et al., 1990). As a result it is possible that the brain Tms are involved in different aspects of neuronal differentiation. Another interesting feature of the  $\alpha$ -Tm gene is the initiation of transcription from two promoter regions associated with exons 1a and 1b of this gene. TmBr-1 transcription originates from the exon 1a promoter while TmBr-2 and TmBr-3 use the exon 1b promoter (Lees-Miller et al., 1990). This raises the possibility of differential regulation of these isoforms at the level of transcription during neuronal development.

In the present study we have investigated the developmental expression of  $\alpha$ -Tm isoforms in the nervous system, with particular attention to TmBr-1 and TmBr-3 which we have determined to be neuronal specific. Using the PC12 system we were able to show that expression of these isoforms was temporally closely related to neurite outgrowth, strongly suggesting a role in this process. Most importantly, the induction of the neuronal  $\alpha$ -Tm transcripts only occurred when PC12 cells stimulated by nerve growth factor (NGF) were permitted to undergo morphological differentiation.

## Materials and Methods

### Materials

Radionucleotides, Hybond N, and antibodies were obtained from Amersham Australia (North Ryde, N.S.W., Australia). DNA labeling and terminal kinasing kits (TKK-1) for end labeling DNA were obtained from Bresatec Australia (Adelaide, South Australia). All chemicals used for oligonucleotide synthesis were from Applied Biosystems (Australia) (Burwood, Victoria, Australia). Taq polymerase ("Amplitaq") for polymerase chain reactions was obtained from the Perkin-Elmer Corp. (Glen Waverley, Victoria, Australia).

### Tissue Culture

PC12 cells (number CRL1721; purchased from American Type Culture Collection, Rockville, MD) grown on collagen-coated tissue culture plates in the absence or presence of NGF were cultured as previously described (Henke et al., 1991). NGF was purified from mouse salivary glands and was a generous gift from Dr. Ian Hendry of the John Curtin School of Medical Research (Canberra, Australia). In addition, some PC12 cells were grown in 50-ml siliconized glass flasks containing 15 ml medium. The cells were

unable to adhere to the siliconized surface and are referred to in this paper as suspension cultures. Plating density was  $5 \times 10^5$  cells/100-mm dish. PC12 cell lots were passaged no more than ten times and were regularly checked for responsiveness to NGF. Rat2 fibroblast cell line (number CRL 1764; ATCC) was initially plated at a density of  $10^6$  cells/100-mm collagen-coated plates in DME supplemented with 10% FBS (Pacific Bioindustries, Sydney, Australia). Fibroblasts were harvested at confluence. Rat astrocytes were prepared from 2–3 d postnatal cerebral cortices as described by Bartlett and Banker (1984) and plated on collagen-coated plastic plates. Astrocytes were harvested for RNA analysis 7 d after dissociation, at which time they reached ~80% confluence. Using a monoclonal antibody raised against the 200-kD subunit of neurofilaments we were able to detect <3% neuronal contamination in the glial cultures. Rat cerebellar granule neurons were prepared according to Schousbe et al. (1989) with modifications. The modifications included the use of PBS during the dissociation protocol in place of Krebs' solution and 10% FBS in DME as the culturing medium. Based on morphological criteria and by the determination of the presence of glial fibrillary acidic protein by a mAb, we determined that glial contamination was ~7% in this system.

### RNA Isolation

Total cytoplasmic RNA was isolated from cultured cells using the urea protein denaturation method of Gough (1988). Total RNA from brain, skeletal muscle, stomach, and kidney was isolated using the phenol-chloroform-sodium acetate procedure of Chomczynski and Sacchi (1987).

### Gel Electrophoresis of RNA and Hybridization

Samples of total RNA were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and transferred to Hybond N nylon membrane exactly as described (Maniatis et al., 1982). Random primed DNA probes,  $10^9$  dpm/ $\mu$ g were hybridized to RNA blot transfers at  $1-3 \times 10^6$  dpm/ml in a solution containing  $4 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0,  $5 \times$  Denhardt's solution (Denhardt, 1966), and 10% (wt/vol) dextran sulfate at 65°C for 12–18 h. The blots were then washed twice quickly in  $1 \times$  SSC at room temperature, two times for 30 min each in  $1 \times$  SSC, 0.1% SDS at 65°C, and finally for 30 min in  $0.5 \times$  SSC, 0.1% SDS at 65°C. This washing procedure was followed for all blots. After densitometry all blots were treated with boiling Tris-EDTA, pH 8.0, to remove bound radioactivity. To correct for any discrepancies in the loading of total RNA samples, the oligomer 5'-GCATATGCT-CTGGCAGGATCAACCAGGRA-3' which was antisense to the 5' end of 18S rRNA, was end labeled (Bresatec, terminal kinasing kit TKK-1) and hybridized to each filter at 55°C, under conditions of probe excess. Two washes were performed at 25°C followed by one wash at 55°C in  $4 \times$  SSC/0.1% SDS for 20 min each. Filters were then exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY) with intensifying screen at -80°C for all DNA probes except in the case of the 18S rRNA probe for which no intensifying screen was used.

### Western Transfer and Immunoblotting of PC12 Cellular Protein

PC12 cells were harvested and pelleted at 500 g to remove the medium. The cells were washed once with 25 ml PBS and repelleted after which they were lysed in 10 vols of 50 mM Tris-HCl, pH 7.5. Protein estimations were performed according to the method of Lowry et al. (1951). Cellular protein samples were then solubilized for SDS-PAGE by the addition of "sample" solubilizing buffer according to the method of Laemmli (1970). 100  $\mu$ g of protein per lane was separated in 10% SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes as previously described (French and Jeffrey, 1986). Immunoblotting was performed essentially as described by French and Jeffrey (1986). Transferred membranes were blocked, incubated with the primary antibody (mAb to the 68-kD subunit of neurofilaments [NF-68]) and subsequently incubated with a HRP-linked rabbit anti-mouse Ig (1:500). After washing, substrate solution was added and color development of a 68-kD protein band was monitored.

### Densitometry

Autoradiographs and immunoblots were scanned with a Molecular Dynamics computing densitometer Molecular Dynamics, Sunnyvale, CA). Bands were quantitated by scanning and integrating volume of the entire band.

## Oligonucleotide Synthesis

Oligonucleotides were synthesized on a 380B Applied Biosystems DNA synthesizer (Applied Biosystems Australia, Burwood, Victoria, Australia) using cyanoethyl phosphoamidite chemistry. The oligonucleotides were cleaved from the column, deprotected, desalted as per manufacturers recommendations, and directly used for polymerase chain reaction DNA amplification.

## Polymerase Chain Reaction DNA Amplification and cDNA Cloning

Polymerase chain reactions were performed using a Hybaid heating block (Integrated Sciences, Crows Nest, N.S.W., Australia). Each reaction contained 5  $\mu$ g rat genomic DNA, 1  $\mu$ g each of the two oligonucleotides, 2 mM for each of the deoxynucleotides, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 8.3, 0.01% gelatin, and 2.5 U Amplitaq. After incubation of the reaction mix at 94°C for 2 min the reaction was run through 30 cycles of denaturation, annealing, and polymerization. Annealing temperatures varied depending on the specific rat  $\alpha$ -Tm exon being amplified (Lees-Miller et al., 1990). For amplification of exons 2a and 9c a 60°C annealing temperature was used and for exons 1a and 1b, 64° and 55°C, respectively, were used. Each cycle consisted of 1 min at 92°C, 2 min at the specific annealing temperature and 2 min at 70°C. The reaction was finished with a 10-min incubation at 70°C and the amplified fragment recovered after electrophoresis on a 2% agarose gel. Exon 1a of the rat  $\alpha$ -Tm gene containing 79 bp of 5'UTR plus amino acids (aa) 1-38 was amplified using the oligonucleotides 5'-GCTCGCACTCCGGCTCTGCCAC and 3'-GGTAGACGCAGACCTGCTTGC (Ruiz-Opazo and Nadal-Ginard, 1987). Exon 2a containing amino acids 39-80 was amplified using the oligonucleotides 5'-GTCTCCGTGTCCAGTCGAG and 3'-GGGTACCTTGGCGGGTCTC (Ruiz-Opazo and Nadal-Ginard, 1987). Exon 1b containing 78 bp of 5'UTR plus aa 1-44 was amplified using oligonucleotides 5'-GGCTCCGTAAGTGGAGCC and 3'-TGATGGGTGGATCCCTAACGG (Lees-Miller et al., 1990). Exon 9c containing 248 bp of the 3'UTR immediately after the termination codon was amplified by oligonucleotides 5'-GGATTAACCCCTGGGCCAAG and 3'-CTGCTGATGCAGGAAGAGCTG (Lees-Miller et al., 1990). A BamHI site was also included at the 5' end of one of each pair of oligonucleotides to facilitate cloning of the amplified product. The BamHI/blunt fragments were cloned into BamHI/HincII of pGEM3 as previously described (Gunning et al., 1990). Each fragment was released from the plasmid by digestion with HindIII and EcoRI.

## Preparation of Plasmid DNA and DNA Fragments

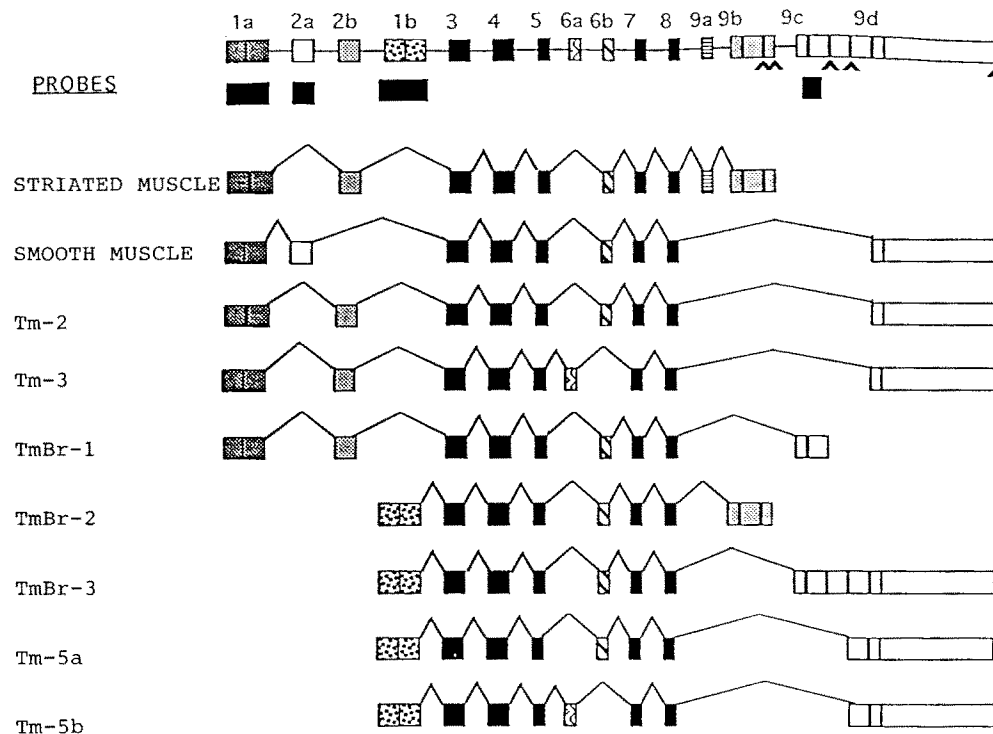
Plasmid DNA was prepared from overnight cultures by lysis and banding in CsCl gradients (Maniatis et al., 1982). Enzyme digestions of plasmid DNA were performed to manufacturer's recommendations and DNA fragments were separated by agarose gel electrophoresis and electroeluted into dialysis bags (Maniatis et al., 1982).

## Results

The rat  $\alpha$ -tropomyosin gene generates nine different mRNAs each coding for a unique protein (Lees-Miller et al., 1990) (Fig. 1). This is accomplished via the use of two promoters and alternative splicing. To identify these tropomyosin transcripts we have cloned four probes corresponding to the four diagnostic exons 1a, 1b, 2a, 9c of the rat  $\alpha$ -Tm gene. The strategy for the generation of isoform-specific tropomyosin probes is shown in Table I. The utility of these probes was then tested by hybridization to RNA isolated from different rat tissue and cell types.

The RP- $\alpha$ 1a probe allows us to follow the expression of the fibroblast Tm2 + Tm3 transcripts in neural tissue. The 2-kb transcript observed using RP- $\alpha$ 1a in all RNA samples (Fig. 2 a) potentially contains Tm-2, Tm-3 and the smooth muscle Tm (Table I). However, hybridization with the RP- $\alpha$ 2a probe reveals that the smooth muscle transcript (Table I) is essentially restricted to the smooth muscle sample (Fig. 2 c, lane 2). In addition, the RP- $\alpha$ 1a probe detects transcripts below 2 kb (Fig. 2 a) as predicted (Table I). Finally, we could barely detect any products from the 1a promoter in PC12 cells either after (Fig. 2 a, lane 5) or before differentiation (not shown).

The RP- $\alpha$ 1b probe discriminates between the combination of Tms 5a plus 5b (Tm5a + 5b), TmBr-2 and TmBr-3 transcripts by virtue of their size differences (Table I). The 3-kb



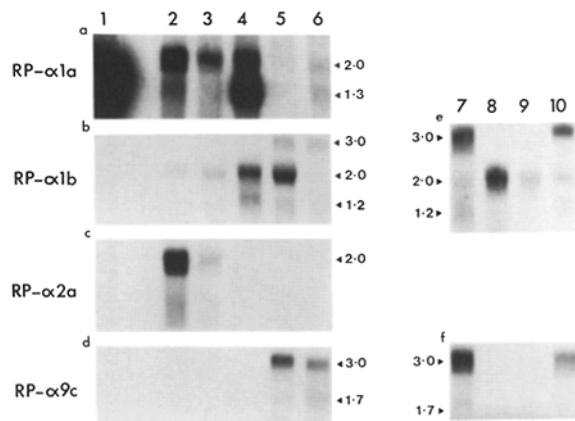
**Figure 1.** Diagram representing the organization of the  $\alpha$ -tropomyosin gene and its mRNA products. Exons are indicated by boxes and introns by horizontal lines. Polyadenylation signals are indicated by arrowheads. The black boxes beneath the gene structure represent the regions of the  $\alpha$ -tropomyosin gene on which the DNA probes were modeled. The figure has been adapted from a similar representation in Lees-Miller et al. (1990). For the sake of uniformity the same nomenclature for the exons as described in Lees-Miller et al. (1990) has been maintained.

**Table I. List of Tropomyosin Probes and Their Specificities**

Probe	$\alpha$ -Tm specificity	mRNA size kb
RP- $\alpha$ 1a	striated muscle	1.3
	smooth muscle	2.0
	Tm-2	2.0
	Tm-3	2.0
	TmBr-1	1.7
RP- $\alpha$ 2a	smooth muscle	2.0
RP- $\alpha$ 1b	TmBr-2	1.2
	TmBr-3	3.0
	Tm-5a	2.0
	Tm-5b	2.0
RP- $\alpha$ 9c	TmBr-1	1.7
	TmBr-3	3.0

TmBr-3 mRNA is only observed in differentiated PC12 cells and brain (Fig. 2 *b*, lanes 5 and 6). In contrast, the 1.2-kb TmBr-2 mRNA is observed in fibroblasts in addition to the two neural samples (Fig. 2 *b*, lanes 4–6). Forrey-Schaudies and Hughes (1990) have observed that a chicken Tm isoform similar to TmBr-2 (denoted BRT-2) is also expressed in both chicken brain and fibroblasts. The 2-kb transcript containing Tm5a plus Tm5b is detected in all samples except skeletal muscle and is expressed in fibroblasts but more highly in PC12 cells (Fig. 2 *b*).

The RP- $\alpha$ 9c probe detects the brain specific TmBr-1 and TmBr-3 transcripts (Table I) as shown in Fig. 2 *d*. The 3-kb TmBr-3 transcript is only detected in differentiated PC12 cells and brain (Fig. 2 *d*, lanes 5 and 6) and shows the same distribution as that visualized using the RP- $\alpha$ 1b probe (Fig.



**Figure 2.** Expression of  $\alpha$ -tropomyosin mRNAs in various adult rat tissues and cultured cells. 10  $\mu$ g of RNA from (lane 1) skeletal muscle, (lane 2) stomach, (lane 3) kidney, (lane 4) Rat 2 fibroblasts, (lane 5) PC12 cells stimulated to differentiate in the presence of NGF for 6 d, (lane 6) forebrain, (lane 7) cerebellar granule neurons, (lane 8) Rat 2 fibroblasts, (lane 9) astrocytes, and (lane 10) cerebellum, was size fractionated on denaturing 1% agarose gels. RNA panels were hybridized to radiolabeled probes RP- $\alpha$ 1a (*a*), RP- $\alpha$ 1b (*b* and *e*), RP- $\alpha$ 2a (*c*) and RP- $\alpha$ 9c (*d* and *f*). Transcript size is indicated at the right hand side (*a-d*) or left hand side (*e* and *f*) of the panels. Lane 1 in *a* is over-exposed to visualize clearly transcripts in lanes 2–4, and in particular to highlight the very low levels of expression observed in lanes 5 and 6.

2 *b*). The 1.7-kb TmBr-1 transcript is also only detected in the neural samples (Fig. 2 *d*). Thus, using the three probes  $\alpha$ 1a,  $\alpha$ 1b, and  $\alpha$ 9c, it is possible to follow the mRNA levels of Tm2 + 3, Tm5a + 5b, TmBr-1, TmBr-2, and TmBr-3 in neural tissue.

### Cell Type Specific Expression of TmBr-1 and TmBr-3

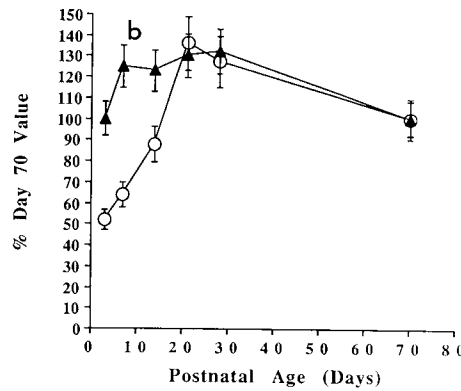
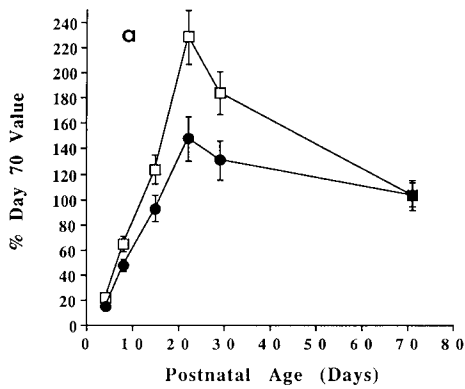
The TmBr-1 and TmBr-3 isoforms are preferentially expressed in neurons. Fig. 2 *f* shows the result of hybridization of the RP- $\alpha$ 9c probe to RNA isolated from cerebellum, cerebellar granule neuron, astrocyte, and fibroblast cultures. TmBr-3 and TmBr-1 are expressed in neuron cultures but were undetectable in astrocytes and fibroblasts (Fig. 2, *e* and *f*). In addition, the relative expression of TmBr-3 to TmBr-1 is similar between the total cerebellum (12:1) and granule neuron (18:1) samples (Fig. 2 *f*, lanes 7 and 10). Although TmBr-1 is not clearly visible in lane 10, the ratio of TmBr-3 to TmBr-1 in cerebellum was obtained from the autoradiographs used to provide the data represented in Fig. 3. The neuronal culture therefore reflects the *in vivo* situation. Notably, both these transcripts were absent from cultured astrocytes which is the other major cell type in the nervous system. Although we can not rule out entirely the possibility of expression of these transcripts in oligodendrocytes, or astrocytes from other regions, these results indicate that both TmBr-1 and TmBr-3 are most likely neuron specific.

### Expression of the $\alpha$ -Tm Transcripts in the Developing Cerebellum

Induction of TmBr-1 and TmBr-3 during cerebellar development parallels neuronal differentiation. Total RNA was isolated from rat cerebellum at stages before, during and after neuronal differentiation. The levels of TmBr-1, TmBr-2, and Tm5a + 5b mRNA were measured and the results are shown graphically in Fig. 3. Fig. 3 *a* shows a dramatic increase in both TmBr-1 and TmBr-3 expression from day 3 to day 21 postnatal that was  $\sim$ 11-fold and 14-fold, respectively. This coincides with the most active period of neurite outgrowth and synaptogenesis in the rat cerebellum (Altman, 1969, 1971, 1972). From the third week to adult levels there was a decline in expression of 130% for TmBr-1 and 40% for TmBr-3. Nevertheless, expression of both transcripts is maintained at relatively high levels in the adult rat cerebellum, suggesting a function for these isoforms in both the developing and mature nervous system. TmBr-2 showed a similar developmental profile except that day 3 levels are considerably higher than for TmBr-3 and TmBr-1, there was a slightly delayed rate of increase within the first 2 wk, and the peak of day 21 was only a  $\sim$  threefold increase above day 3 levels (Fig. 3 *b*). By contrast Tm5a + 5b show a relatively unchanged expression, with a 29% increase from day 3 to day 7 which is maintained till the fifth week with a decline to adult levels (Fig. 3 *b*). These results are consistent with a role for TmBr-1 and TmBr-3 in neuronal differentiation and maintenance of structure.

### $\alpha$ -Tm Gene Expression in PC12 Cells Stimulated by NGF to Differentiate to the Neuronal Phenotype

TmBr-1 and TmBr-3 mRNA expression is induced during NGF promoted differentiation to the neuronal phenotype in



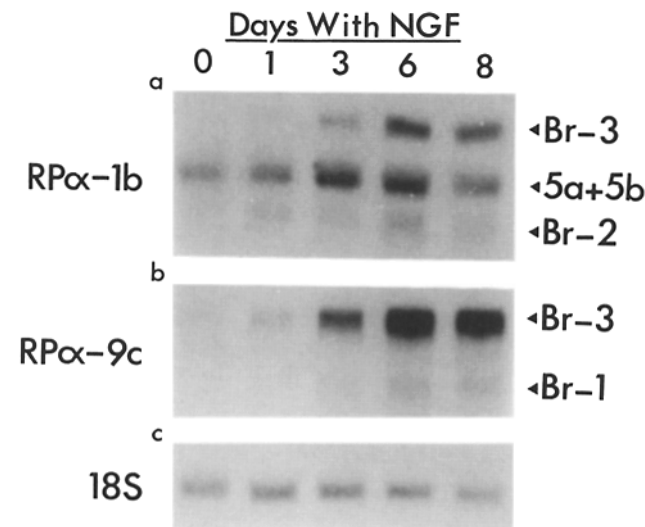
**Figure 3.** Developmental expression of  $\alpha$ -tropomyosin mRNAs in rat cerebellum. 20  $\mu$ g total RNA was size fractionated on denaturing 1% agarose gels. RNA panels were then hybridized to radiolabeled probes. Autoradiographs were quantitated by densitometry and the results presented as graphs. Results are expressed as a percentage of the day 70 values of each individual transcript. TmBr-3 (a,  $\square$ ) and TmBr-1 (a,  $\bullet$ ) were quantitated

by densitometry from hybridizations with the probe RP- $\alpha$ 9c. TmBr-2 (b,  $\circ$ ) and Tm5a + 5b (b,  $\blacktriangle$ ) were quantitated from hybridizations using probe RP- $\alpha$ 1b. Two RNA extractions were performed at each time point and values represent the mean  $\pm$  SEM of three separate hybridizations.

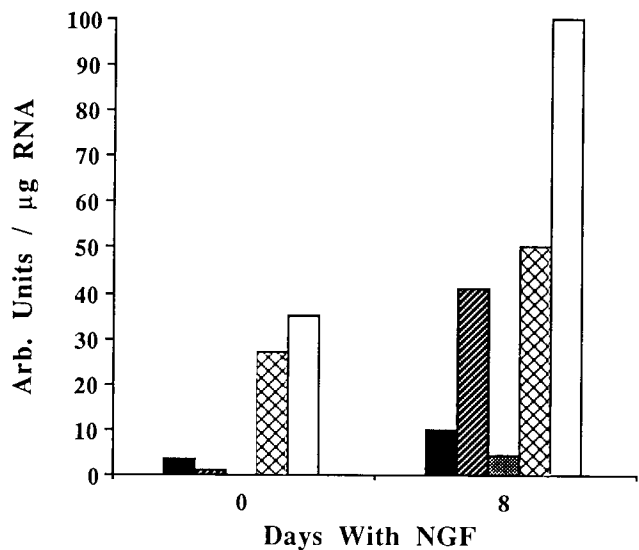
PC12 cells. RNA was isolated from PC12 cells at different times of exposure to NGF and hybridized to the RP- $\alpha$ 1b and RP- $\alpha$ 9c probes. The resulting autoradiographs are shown in Fig. 4. Visually, the most dramatic change with increasing exposure to NGF, is the appearance of the 3-kb TmBr-3 transcript which is present at very low levels in PC12 cells not exposed to NGF, but becomes a major transcript after six days in the presence of NGF (Fig. 4, a and b). Both probes reveal an almost identical NGF-stimulated increase in TmBr-3. TmBr-1 mRNA shows a pattern of accumulation that parallels TmBr-3 whereas TmBr-2 shows a reduced level of induction.

Total  $\alpha$ -Tm gene expression, relative to the individual transcripts present in PC12 cells, is shown quantitatively in Fig. 5. There is very low level expression initiated from the exon 1a promoter in PC12 cells that have received NGF for 6 d (Fig. 2 a) or not at all (not shown) as visualized by the

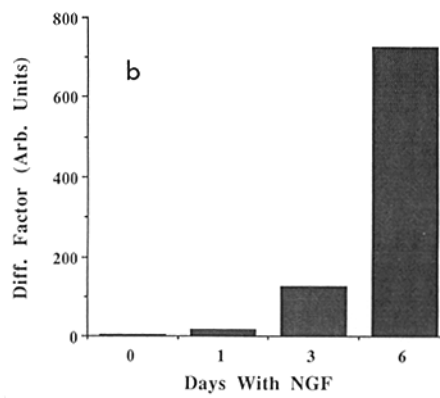
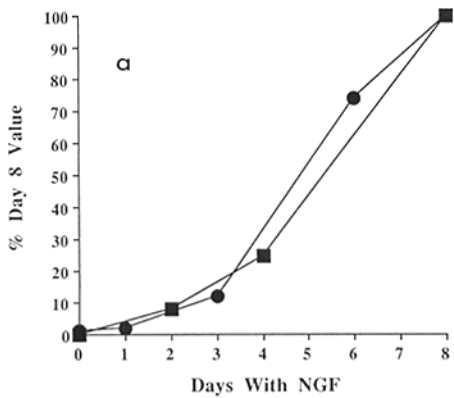
RP- $\alpha$ 1a probe. Therefore, total  $\alpha$ -Tm expression was quantitated using only the RP- $\alpha$ 1b and RP- $\alpha$ 9c probes. There is a threefold increase in total  $\alpha$ -Tm gene expression from PC12 cells grown in the presence of NGF for 8 d (Fig. 5). The combination of Tm5a + 5b increased by twofold whereas TmBr-3 levels increased 125-fold from day 0 to 8, at which point it accounts for 42% of total  $\alpha$ -Tm expression. TmBr-1 expression is not detected at day 0, but even after 8-d exposure to NGF is still the most minor transcript in PC12 cells accounting for only 4.4% of total expression. TmBr-2 mRNA levels in day 0 PC12 cells grown without NGF increased by threefold to day 8 and represents 7.2% of total expression at this time point. The fibroblast Tm5a + 5b are by far the major transcripts at day 0. However, by day 8 the



**Figure 4.** Expression of  $\alpha$ -tropomyosin mRNAs in adherent PC12 cells stimulated to differentiate to the neuronal phenotype by NGF. Cytosolic RNA (5  $\mu$ g) from PC12 cells treated with NGF for 0, 1, 3, 6, and 8 d was size fractionated as discussed in Fig. 2 and panels hybridized to radioactive probes RP- $\alpha$ 1b (a) and RP- $\alpha$ 9c (b). c shows the result of hybridization of the 18S oligomer to the blot in panel a to indicate the minor loading differences amongst the lanes.



**Figure 5.** Expression of individual  $\alpha$ -tropomyosin gene transcripts and total  $\alpha$ -tropomyosin gene expression in PC12 cells exposed to NGF for 0 or 8 d. The autoradiographs in Fig. 3 were quantitated by densitometry to obtain this histogram. Results are expressed as arbitrary OD units per microgram RNA relative to the total gene expression at day 8. Total gene expression was quantitated by summing the expression levels of the individual transcripts. (■) TmBr-2 levels; (▨) TmBr-3 levels; (▩) TmBr-1 levels; (▧) Tm5a + 5b levels; and (□) total  $\alpha$ -tropomyosin gene expression.



**Figure 6.** Relationship of TmBr-3 mRNA expression to PC12 differentiation induced by NGF. (a) TmBr-3 mRNA (●) and NF-68 protein levels (■) in PC12 cells exposed to NGF for up to 8 d. Cytosolic RNA (5 μg) and 100 μg protein per time point was size fractionated as discussed in Materials and Methods and legend to Fig. 2. Autoradiograms and immunoblots were quantitated by densitometry as described in Materials and Methods. Values are expressed

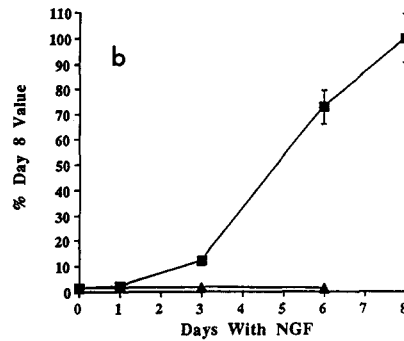
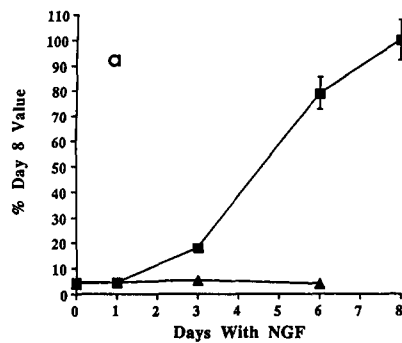
relative to the day 8 value. (b) Morphological differentiation of PC12 cells grown on collagen coated plastic and exposed to NGF for 0, 1, 3, and 6 d. Cells were grown in two separate wells for each time point, fixed with 2% paraformaldehyde and stained with toluidine blue. Stained cells were photographed and measurements made directly from the prints. The number of cells quantitated ranged from 50 (day 1) to 117 (day 3). The "Differentiation Factor" expressed on the y-axis takes into account both the length of the neurites and the number of neurites per cell (neurite length x number of neurites/cell). Processes greater in length than one cell body diameter were classified as a neurite and included for quantitation. The length of branched neurites is defined to be the sum of the lengths of all of the branches of one neurite.

TmBr-1 and TmBr-3 transcripts account for half the output from this gene.

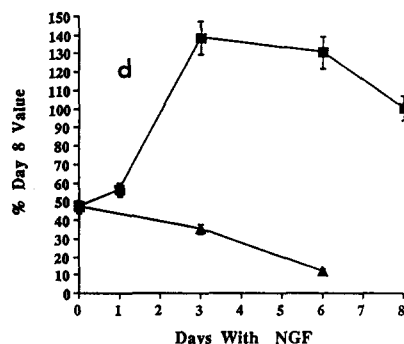
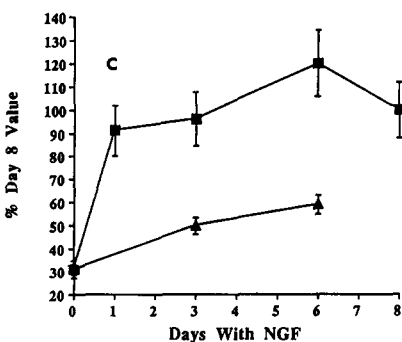
#### Relationship of Brain Isoform Expression to Neurite Outgrowth

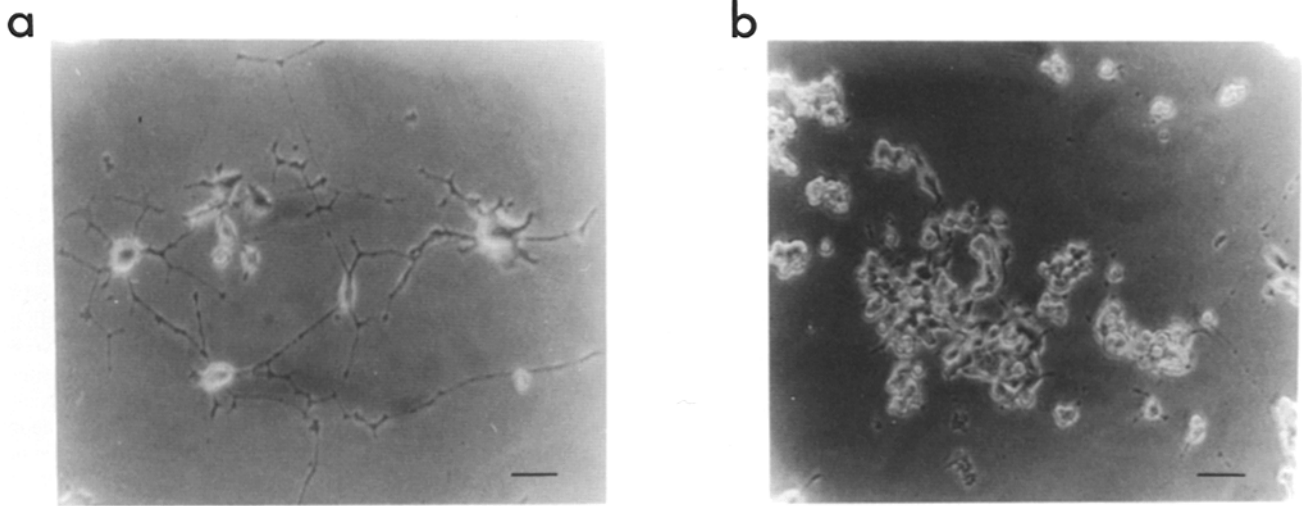
The kinetics of induction of TmBr-3 mRNA during PC12 differentiation closely parallels the induction of neuronal processes as measured by neurofilament NF-68 protein. Fig. 6 a shows a time course of TmBr-3 expression of PC12 cells grown in the presence of NGF for various periods on

collagen-coated plastic plates. The most dramatic increase in TmBr-3 expression occurs between days 3 and 6, with sixfold increase over this time interval. An almost identical expression pattern is observed for TmBr-1 (Fig. 7 b). The expression of the NF-68 protein, a well characterized cytoskeletal marker for neuronal differentiation and neurite outgrowth (Lee and Page, 1984; Dickson et al., 1986) has been plotted in Fig. 6 a. For comparison, an evaluation of the differentiation state of the cells based on neurite outgrowth has also been plotted (Fig. 6 b). The expression of NF-68 correlated



**Figure 7.** Expression of α-tropomyosin mRNAs in PC12 cells exposed to NGF for 0, 1, 3, 6, and 8 days and grown on collagen coated plastic (■) or as nonadherent suspension cultures (▲). Cytosolic RNA (5 μg) was size fractionated on denaturing 1% agarose gels. RNA blots were hybridized with the DNA probes used to visualize specific α-tropomyosin mRNAs as described in Fig. 2 and Results. (a) TmBr-3; (b) TmBr-1; (c) TmBr-2; and (d) Tm5a + 5b. Values are expressed relative to day 8 values, n = 3 separate hybridizations from two RNA extractions ± SEM.





**Figure 8.** PC12 cells primed with NGF in suspension cultures for 6 d and subsequently plated on collagen-coated plastic for 2 d (a) and PC12 cells grown normally with NGF on collagen-coated plastic for 2 d (b). Bar, 20  $\mu$ m.

very closely to TmBr-3 and TmBr-1 mRNA levels which also parallels neurite outgrowth, suggesting that the expression of these  $\alpha$ -Tm transcripts is related to, and possibly involved in, neurite outgrowth.

#### ***Is NGF Sufficient to Stimulate Brain Isoform Expression or is Morphological Differentiation Required?***

The induction of TmBr-3 and TmBr-1 transcripts in PC12 cells by NGF is dependent on the morphological differentiation of PC12 cells. It has been shown that exposure of PC12 cells to NGF in suspension prevents morphological differentiation but promotes the ability of these cells to differentiate when transferred to a permissive substratum. This promotion of differentiating ability steadily increases with exposure to NGF and is called "priming" (Green, 1977; Green et al., 1982). It is therefore possible to address the question of whether the expression of  $\alpha$ -Tm transcripts requires morphological differentiation. Fig. 7 shows the expression of  $\alpha$ -Tm transcripts in PC12 cells grown in the presence of NGF in suspension or attached to collagen coated plastic. Fig. 7, A and B clearly show no stimulation of TmBr-1 and TmBr-3 in suspension cultures, in direct contrast to the dramatic increases seen with adherent cultures. This indicates that NGF alone is insufficient to stimulate TmBr-1 and TmBr-3 expression. However, NGF presented to PC12 cells in suspension increases TmBr-2 expression but down-regulated Tm5a + 5b expression (Fig. 7, c and d). These experiments reveal a complex regulation of  $\alpha$ -Tm isoforms during PC12 differentiation. Whereas the neuron-specific isoforms require morphological differentiation to permit their expression, TmBr-2 and Tm5a + 5b levels can be regulated by NGF in the absence of a substrate that allows neurite outgrowth.

#### ***Induction of $\alpha$ -Tm Transcripts Is Accelerated in PC12 Cells Undergoing an Increased Rate of Morphological Differentiation***

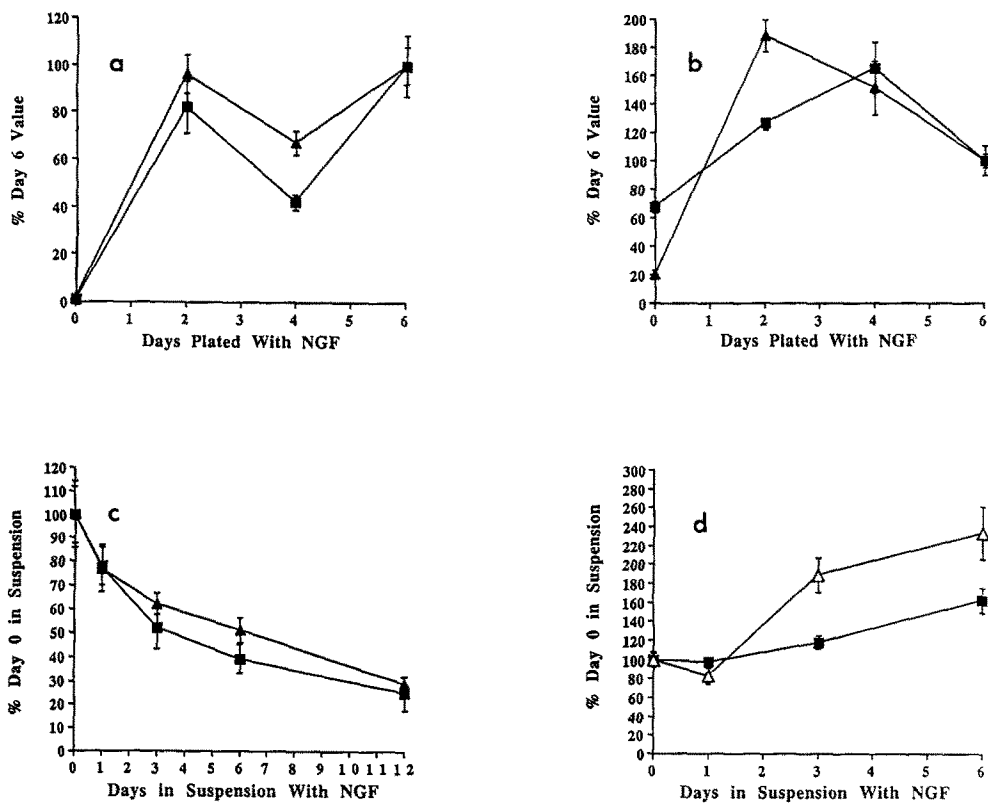
The kinetics of induction of TmBr-3 and TmBr-1 mRNAs are increased when the rate of PC12 morphological differentiation is increased. PC12 cells were grown in suspension in the

presence of NGF for 6 d ("priming") and then replated on collagen-coated dishes in the presence of NGF. One to two days after replating, morphological differentiation of the cells is considerably accelerated when compared with unprimed cells (Fig. 8) (Green et al., 1982). The levels of transcripts from the  $\alpha$ -Tm gene were measured at 0, 2, 4, and 6 d after replating and the results are shown graphically in Fig. 9. Within 2 d of replating, TmBr-3 and TmBr-1 transcript levels are induced to the same level as that seen with 6 d after replating (Fig. 9 a). This is in dramatic contrast to the usually slow induction observed with unprimed cells (Fig. 7, a and b). Thus, there is a close temporal relationship between the rate of TmBr-1 and TmBr-3 mRNA accumulation and the rate of morphological differentiation.

The priming of PC12 cells also influences the response of the Tm5a + 5b and TmBr-2 transcripts to PC12 differentiation. Differentiation of unprimed PC12 cells results in three- and fourfold increases in Tm5a + 5b and TmBr-2 mRNA levels, respectively (Fig. 7, c and d). In contrast, 10- and 2.5-fold increases in Tm5a + 5b and TmBr-2 mRNA levels, respectively, accompany the differentiation of primed PC12 cells (Fig. 9 b). This can be readily explained by the finding that cells primed for 6 d in suspension reduce Tm5a + 5b mRNA by fourfold and increase TmBr-2 mRNA by twofold (Fig. 7, c and d). Thus, the starting transcript levels differ between unprimed and primed cells but the peak levels attained during differentiation are very similar. This suggests that absolute transcript levels of Tm5a + 5b and TmBr-2 are under tight regulation during PC12 cell differentiation independent of their levels before the induction of differentiation.

#### ***Disruption of Neuronal Morphology Alters $\alpha$ -Tm Gene Regulation***

NGF cannot maintain high level expression of brain specific Tms in the absence of morphological differentiation. PC12 cells which had been differentiated in the presence of NGF on a collagen substratum for 6 d were transferred to suspension culture in the continued presence of NGF. This results in a rapid loss of neuronal morphological characteristics (Green et al., 1982). Fig. 9 c shows that the levels of TmBr-3



**Figure 9.** Expression of  $\alpha$ -tropomyosin mRNAs in response to the regulation of PC12 morphology. Cytosolic RNA (5–10  $\mu$ g) was size fractionated, hybridized to the appropriate radiolabeled probes indicated in the text. Autoradiographs were quantitated by densitometry and the results presented as graphs. (a and b) Expression of  $\alpha$ -tropomyosin mRNAs in PC12 cells that have been primed with NGF in suspension for 6 d and subsequently plated on collagen-coated plastic. PC12 cells were grown in the presence of NGF for 0, 2, 4, and 6 d after plating. (a) TmBr-1 (▲); TmBr-3 (■). (b) Tm5a + 5b (▲); TmBr-2 (■). (c and d) Expression of  $\alpha$ -tropomyosin mRNAs in PC12 cells that have been grown on collagen-coated plastic in the presence of NGF for 6 d and then transferred to siliconized flasks in the presence of NGF. Time in days refers to the time since transfer to suspension. (c) TmBr-1 (▲); TmBr-3 (■). (d) Tm5a + 5b (▲); TmBr-2 (■).  $n = 3 \pm$  SEM (as described in Fig. 6) in a–d.

and TmBr-1 mRNAs are progressively lost upon transfer to suspension culture. This suggests that the maintenance of high level expression of brain-specific tropomyosin mRNAs is dependent on the maintenance of morphological differentiation.

Both Tm5a + 5b and TmBr-2 mRNA levels are both elevated after the transfer to suspension culture. This elevation of Tm5a + 5b contrasts with the decrease observed when untreated cells are exposed to NGF in suspension (compare Figs. 7 d and 9 d). This cannot be explained by different starting levels of Tm5a + 5b because the level of these mRNAs in 6-d differentiated cells is, in fact, already substantially elevated over that seen in untreated cells. Thus, in the case of Tm5a + 5b, the “environmental history” of the cells appears to impact on the regulation of these transcripts. TmBr-2 transcript levels show essentially identical regulation in suspension culture independent of their previous history (compare Figs. 6 c and 8 d). This further dissociates the regulation of TmBr-2 from that observed with TmBr-1 and TmBr-3.

## Discussion

### TmBr-1 and TmBr-3 Are Neuron-Specific Tms

The present study strongly suggests that TmBr-1 and TmBr-3 are neuron-specific ABPs. Although unlikely, it is still possible that some nonneuronal populations of cells may express these tropomyosins in the nervous system. To date the only

other neuron specific ABP that has been identified is synapsin I. Synapsin I is a neuron-specific phosphoprotein that is concentrated in the presynaptic nerve terminal in association with the cytoplasmic surface of synaptic vesicles (DeCamilli and Greengard, 1986). It has been demonstrated to bundle F-actin in vitro in a phosphorylation-dependent manner (Bähler and Greengard, 1987; Bähler et al., 1989); a property consistent with its proposed role in linking synaptic vesicles to the cytoskeleton and its involvement in the regulation of neurotransmitter release (Llinas et al., 1985). EM has supported the in vitro evidence by showing that synapsin I appears to form linkages between synaptic vesicles and microfilaments as well as synaptic vesicles and microtubules (Hirokawa et al., 1989). By analogy with synapsin I, it is to be expected that the neuronal Tms will also have specialized roles in the regulation of neuronal microfilament function.

### TmBr-1 and TmBr-3 May Confer Unique Functional Properties to Specialized Microfilaments within Neurons

The two major actins present in the brain,  $\gamma$ - and  $\beta$ -actin (Choo and Bray, 1978; Pardee and Bamburg, 1979) are highly similar proteins differing in sequence by only four amino acids (Vandekerckhove and Weber, 1978, 1984). Relatively crude subcellular fractionation studies have shown no regional differences in concentration of either  $\gamma$ - or  $\beta$ -actin within the neuron (Choo and Bray, 1978). In an earlier study from this laboratory, the developmental expression of  $\beta$ - and



$\gamma$ -actin mRNA in PC12 cells stimulated with NGF was shown to be almost identical, reaching a peak well before the major period of neurite outgrowth (Henke et al., 1992). Therefore it appears unlikely that the actin proteins will contain sufficient information to regulate microfilament diversity in highly specialized structures within the nervous system. The diversity of microfilament function is likely to be largely controlled by such molecules as Tms and other ABPs (Stossel, 1990). This would of course be dependent on the regional localization of  $\alpha$ -Tm isoforms within neurons and in the case of TmBr-1 and TmBr-3, localization to the growing neurite. By comparison, regional specificity of neuronal microtubules is thought to be regulated by microtubule associated proteins (MAPs). The best studied of the neuron-specific MAPs are the tau and MAP2 families. MAP2 reveals a dendritic localization in mature neuronal populations (Caceres et al., 1984; Peng et al., 1986; Kosik and Finch, 1987; Ferreira et al., 1989) while tau proteins have been shown to segregate to the axon in cultured neurons (Peng et al., 1986; Kosik and Finch, 1987; Ferreira et al., 1989) and the brain (Binder et al., 1985). These proteins have been shown to regulate microtubule polymerization in vitro. Tau protein is thought to function primarily by stabilizing microtubules; a function similar to that postulated for tropomyosins in relation to microfilaments. Constitutive expression studies of antisense MAP2 RNA demonstrated a requirement for MAP2 in normal neurite development (Dinsmore and Soloman, 1991). Application of tau antisense oligonucleotides to cerebellar macroneurons in vitro prevented the formation of an asymmetric axon while not affecting the development of short exploratory neurites, or the size of the cell soma (Caceres et al., 1991). However, in contrast to the microfilament system another level of specificity may be conferred by  $\beta$ -tubulin isoforms which show a differential distribution within neurons (Joshi and Cleveland, 1991). It is quite probable that neuronal microfilaments will also possess diversity similar to that found in the microtubule system.

The molecular mechanism whereby TmBr-1 and TmBr-3 may contribute to regional microfilament specificity is likely to be contained within the NH<sub>2</sub>- and COOH-terminal regions of these molecules. These terminal regions show the greatest diversity among the  $\alpha$ -Tm isoforms and have been implicated in the ability of Tms to bind F-actin and in head to tail Tm dimerization (Ueno et al., 1976; Mak and Smillie, 1981). The 19.66 residue pattern thought to be important for F-actin binding is not conserved in the COOH-terminal region shared by both TmBr-1 and TmBr-3, and is also not conserved in the NH<sub>2</sub> terminus of TmBr-3 (Lees-Miller et al., 1990). As a result it is possible that these molecules will differ in their interaction with actin filaments, even though TmBr-1 and TmBr-3 mRNA appeared to be identically regulated under all conditions investigated in this study. Based on the apparent lack of conservation of the actin binding repeat sequence, Lees-Miller et al. (1990) suggested that these proteins may have weaker actin binding properties than muscle Tm. This correlates well with early studies showing the weaker F-actin binding properties of whole brain Tm compared with skeletal muscle Tm (Dabrowska et al., 1983; Keiser and Wegner, 1985). Exon 9c, which encodes the COOH-terminal region of TmBr-1 and TmBr-3 is conserved in the major brain transcript (BRT-1) of the Tm-1 gene of

chickens (Forrey-Schaudies and Hughes, 1990) and indicates a strong selective advantage for the retention of this COOH sequence. This finding suggests that a unique property of BRT-1, TmBr-3 and TmBr-1 is maintained across evolution from birds to mammals.

#### *Expression of TmBr-1 and TmBr-3 Is Closely Linked to Neuronal Morphology and May Reflect a Process of Feedback Regulation by $\alpha$ -Tm Products*

The finding that most sets apart TmBr-1 and TmBr-3 mRNA expression in PC12 cells from other  $\alpha$ -Tm products and Tm genes (results not shown) is the observation that TmBr-1 and TmBr-3 induction can only occur under conditions where PC12 cells are allowed to differentiate and extend neurites. This inability of NGF to induce TmBr-1 and TmBr-3 mRNA expression unless PC12 cells were permitted to extend neurites, is in direct contrast to the situation observed with NF-68, an intermediate filament structural component that is associated with the acquisition of the neuronal phenotype (Dickson et al., 1986). In PC12 cells unable to extend neurites, NF-68 mRNA levels could still be induced by NGF (Dickson et al., 1986). Expression of TmBr-1 and TmBr-3 is not merely associated with the presence of NGF, but is dependent on the acquisition of the neuronal phenotype which is directly under environmental control. Removal of neurites of "dedifferentiation" of the PC12 cells results in a large decrease in TmBr-1 and TmBr-3 mRNA levels.

These phenomena may involve a process of feedback regulation of Tm mRNA levels in association with competition of Tms for inclusion into specific neuronal structures during differentiation. (a) As the neuritic microfilament system develops in response to the process of cellular differentiation, Tms most appropriate for incorporation into these specialized microfilaments may be synthesized. As the complexity of the neurite network increases, so too may the demand for the neuronal Tms. (b) Conversely, removal of neuronal morphology results in a major disruption of the cytoskeletal system within PC12 cells. As a result it is possible that the neuronal Tms are unable to be incorporated stably into microfilaments. The presence of a large pool of unincorporated neuronal Tms may feedback to inhibit neuronal Tm mRNA levels. Such a process of feedback regulation has been shown to operate for the tubulin subunits which are thought to bind to the nascent amino-terminal tetrapeptide of  $\beta$ -tubulin (Cleveland, 1989). This binding is transduced through the adjacent ribosome to activate an RNase which degrades the ribosome-bound mRNA (Cleveland, 1989). Whether a similar mechanism is involved in neuronal Tm mRNA regulation remains to be determined. Interestingly, TmBr-1 and TmBr-3 were the only  $\alpha$ -Tm mRNAs identically regulated under all conditions studied, even though transcription is controlled from two different promoters. It is clear from these studies that the environmental demand for expression of these isoforms is very similar and that cytoarchitecture may be a major regulator of  $\alpha$ -Tm gene expression.

In the undifferentiated PC12 cell, nonneuronal isoforms account for >95% of  $\alpha$ -Tm gene output. However, after differentiation has proceeded for 8 d in culture, TmBr-1 and TmBr-3 comprise ~50% of  $\alpha$ -Tm gene expression. This pattern of Tm isoform regulation to the differentiation state of

the cell has also been observed in skeletal muscle development in vivo and myotube formation in vitro (Gunning et al., 1990). In this instance, nonmuscle Tm and actin isoforms are down regulated as the expression of skeletal muscle isoforms predominate. This phenomenon is closely associated with the development of the mature skeletal muscle phenotype or myotube formation in vitro. The pattern of Tm isoform expression was also highly variable and unique for each isoform. These results are analogous to the situation observed in PC12 cells as they differentiate from a round chromaffin-like cell into the neuronal phenotype. Gunning et al. (1990) proposed a model suggesting that the variable regulation of nonmuscle isoforms during myogenesis may reflect a process of feedback regulation coupled to competition for inclusion into specific cellular structures. The proportional decrease of the nonneuronal  $\alpha$ -Tm mRNAs relative to total  $\alpha$ -Tm output may reflect their poorer ability to compete for inclusion into neuritic microfilaments as compared with TmBr-3 and TmBr-1. Although the net levels of Tm5a + 5b and TmBr-2 increased with neuronal differentiation, their pattern of expression did not correlate well with neurite outgrowth. The major increase in these isoforms was observed within the first 3 d after NGF addition when TmBr-1 and TmBr-3 expression was very low. However, during this period, the cell body enlarges and increases in adhesiveness as the first stage in neuronal differentiation. The net increases in the nonneuronal  $\alpha$ -Tm mRNAs may be related to these events.

### ***A Role for TmBr-1 TmBr-3 in the Mature Nervous System?***

TmBr-1 and TmBr-3 are expressed at relatively high levels in the mature cerebellum, indicating that these isoforms are not only involved in the dynamic process of neurite outgrowth but also in the maintenance of mature neuronal structures. The maintenance of significant levels of the neuronal Tms in the mature system suggests that the presence of the highly specialized neurites determines neuronal Tm expression rather than these isoforms being expressed as a response to the cellular differentiation process per se. This is in agreement with the PC12 studies presented in this paper which indicated a stringent regulation of neuronal Tm mRNA levels linked to neuronal morphology.

Actin is present at high levels at the synapse in particular in dendritic spines and the subcellular specialization, the postsynaptic density (Matus et al., 1982; Fikova, 1985; Sedman et al., 1986) which have been shown to alter morphology in response to various stimuli (reviewed by Siekevitz, 1985). It has been suggested that actin may play a major role in regulating morphological changes at the synapse (Fikova and Van Harreveld, 1977; Matus et al., 1982; Bradley and Horn, 1979). The involvement of actin in neuronal cyto-mechanics (Smith, 1988), the motility of cells (Theriot and Mitchison, 1991) and subcellular organelles (Satterwhite and Pollard, 1992), make this molecule an ideal candidate for a role in molecular events associated with synaptic plasticity. Molecules involved in microfilament structure and function during development such as Tms will probably also be involved in the response of microfilaments to environmental changes in the mature nervous system. It is reasonable to assume that the neuronal Tms will colocalize within specific mature structures enriched in actin. Evidence supporting the role of neuronal Tms in neurite outgrowth described in this

study, their potential localization at dendritic spines and synapses in general and their ability to respond to altered environmental conditions, indicate that the neuronal Tms will probably be involved in the response of microfilaments to changes in morphology induced at the adult synapse.

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