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Brain-specific tropomyosins TMBr-1 and TMBr-3 have distinct patterns of expression during development and in adult brain

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ABSTRACT **In this study we report on the developmental and regional expression of two brain-specific isoforms of tropomyosin, TMBr-1 and TMBr-3, that are generated from the rat α-tropomyosin gene via the use of alternative promoters and alternative RNA splicing. Western blot analysis using an exon-specific peptide polyclonal antibody revealed that the two isoforms are differentially expressed in development with TMBr-3 appearing in the embryonic brain at 16 days of gestation, followed by the expression of TMBr-1 at 20 days after birth. TMBr-3 was detected in all brain regions examined, whereas TMBr-1 was detected predominantly in brain areas that derive from the prosencephalon. Immunocytochemical studies on mixed primary cultures made from rat embryonic midbrain indicate that expression of the brain-specific epitope is restricted to neurons. The developmental pattern and neuronal localization of these forms of tropomyosin suggest that these isoforms have a specialized role in the development and plasticity of the nervous system.**

Due to their function as conductors of information in a mostly vectorial manner, neurons differ significantly from other cell types in size and geometry. Their size and shape are reflected in the neuronal cytoskeleton (1) composed of a unique set of structural proteins, such as neurofilaments, and a variety of neuron-specific actin binding proteins, such as brain-specific isoforms of myosin (2), spectrin (3), and tropomyosin (TM) (4). The process of modifying, strengthening, or eliminating synaptic connections both during and well after embryonic development has ended is now hypothesized to be the mechanism underlying such dynamic processes as learning and memory (5–8). Thus, there is a potential requirement for a changing repertoire of unique neuronal cytoskeletal elements that confer differential capabilities upon the neuron at various times. Structural proteins unique to neurons are generated by several different mechanisms. Some of these, such as the neurofilament triplet (9), are transcribed from different genes. Other isoforms are created by alternative splicing of a single pre-mRNA, an important mechanism to generate multiple isoforms from one gene (4, 10–16).

TMs are a group of actin-binding proteins that are expressed in most eukaryotic cells. TMs are encoded by multiple genes, but diversity is also generated by alternative RNA splicing (for a review, see ref. 17). Whereas TM functions in skeletal and cardiac muscles in association with the troponin complex to regulate the calcium-sensitive actin and myosin interaction, the biological functions of TM in nonmuscle and smooth muscle cells are not yet understood (18). Since nonmuscle and smooth muscle cells are devoid of a troponin complex, they likely have a distinct function compared to TMs of skeletal and cardiac muscle.

The rat α-TM gene expresses at least nine isoforms (17), including three isoforms in the rat brain, which were named TMBr-1, TMBr-2, and TMBr-3, and are 281, 251, and 245 amino acids long with apparent molecular weights by SDS/PAGE of 36,000, 31,000, and 31,000, respectively (4).

To study the localization and possible function of the brain-specific TM isoforms, we have generated an isoform-specific antibody. Our data indicate that expression of exon 9c in the α-TM gene is limited to neurons and is developmentally regulated.

MATERIALS AND METHODS

Neuronal and Glial Cultures. Cultures from 16-day Sprague–Dawley rat embryos were established from the midbrain as described (19) and in a similar way from the prosencephalon. Glial cultures were made from the primary cultures by adding epidermal growth factor (Collaborative Research, 10 ng/ml), followed by trypsin treatment after 9 days and replating on uncoated dishes in Dulbecco's modified Eagle's medium/15% (vol/vol) fetal calf serum.

Development of Anti-rTM9c, an Antibody Against an Epitope in Exon 9c. The peptide CSH085 (Cys-Tyr-His-Gln-Leu-Glu-Gln-Asn-Arg-Arg-Leu-Thr-Asn-Glu-Leu-Lys-Leu-Ala-Leu-Asn-Glu-Asp) representing the amino acid sequence of brain-specific exon 9c of the α-TM gene (Fig. 1), with the exception of the first Cys that was used for coupling, was coupled to keyhole limpet hemocyanin as described (20). The resulting polyclonal rabbit serum was affinity-purified (21).

Immunoblot Analysis. Protein for immunoblot analysis was prepared by homogenizing 0.25 g of tissue in 1 ml of sample buffer [60 mM Tris·HCl, pH 6.8/2% (wt/vol) SDS/0.1 M dithiothreitol] using a 20-gauge 1½ inch (1 inch = 2.54 cm) syringe needle, followed by boiling and centrifugation. Protein was quantitated in sample buffer using a modification of the Bradford assay (Bio-Rad) (22). Western blot analysis was performed as described (20) using the ECL system (Amersham).

Immunocytochemistry of Primary Neuronal and Glial Cultures. Immunocytochemistry was performed as described (19). Primary antibodies were PHF-1, which recognized tau in rats (a gift from S. Greenberg, Burke Medical Research Institute, White Plains, NY), anti-glial fibrillary acidic protein (GFAP), and anti-neurofilament (heavy, medium, and light chains) from Boehringer Mannheim. They were visualized using fluorescein-conjugated anti-mouse IgG or biotinylated anti-rabbit immunoglobulin (Amersham) and streptavidin-conjugated tetramethylrhodamine (Molecular Probes).

RNA Isolation and PCR Analysis. RNA from tissue was isolated using the hot phenol/guadinium salt method (23). The sequences of the oligonucleotides (5′ → 3′) were as follows: AS1B01, ATGGCGGGTAGCAGCTCGCTGG; AS1A01, AAGAAGATGCAGATGCTGAAGCTC; AA501, CGTCTCGATGATGACCAGCTTAC; AS301, ACAGCTC-

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Abbreviations: TM, tropomyosin; GFAP, glial acid fibrillary protein; E, embryonic day; P, postnatal day.

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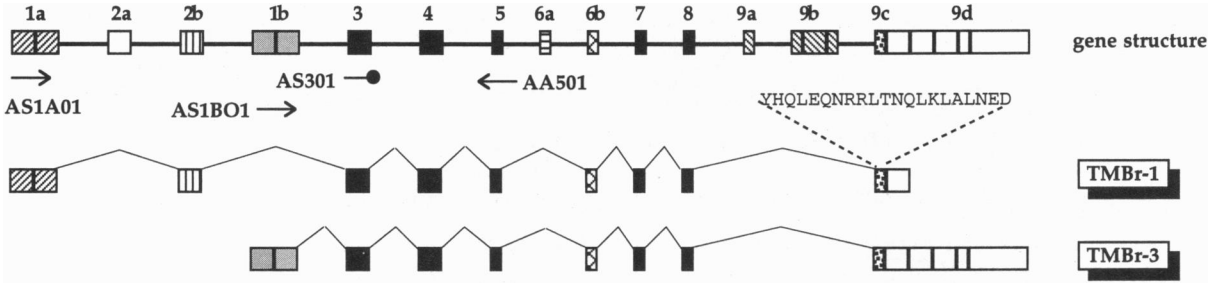

FIG. 1. Schematic representation of the organization of the rat α-TM gene and two (TMBr-1 and TMBr-3) of its nine associated mRNA products. Exons are represented as boxes and introns are represented as lines as described (4). Exon-specific oligodeoxynucleotides AS1B01, AS1A01, AA9C01, and AA501 were used for PCR analyses. Oligodeoxynucleotide AS301 was used for Southern blot analysis. All oligodeoxynucleotides are shown below their corresponding exons. The peptide sequence that was used to raise the rabbit polyclonal antibody is indicated above exon 9c in the TMBr-1 mRNA.

TACAGAAGCTGGAG (Fig. 1). PCRs were essentially performed as described (16).

RESULTS

Anti-rTM9c Specifically Recognizes an Epitope Contained in Exon 9c in TMBr-1 and TMBr-3. To determine the specificity of the anti-rTM9c antibody, we performed Western blot analysis with protein from various brain regions and other tissues (Fig. 2). Two bands corresponding to TMBr-1 and TMBr-3 were detected by anti-rTM9c in protein samples from brain (Fig. 2*A*). The antibody did not exhibit any cross reactivity with TMs in liver, kidney, or skeletal muscle. A parallel blot probed with the TM311 antibody (Fig. 2*B*) showed that the TM311 antibody detected additional TM isoforms in samples that showed no reaction with anti-rTM9c. The TM311 antibody recognized protein sequences encoded by exon 1a and thus TM311 did not react with TMBr-3 but did react with TMBr-1 and other isoforms. These results demonstrate that anti-rTM9c is specific for TMBr-1 and TMBr-3.

TMBr-1 and TMBr-3 Have Different Regional Distributions in Adult Rat Brain. To determine the relative distributions of TMBr-1 and TMBr-3 throughout the brain, we dissected rat brains and analyzed their expression patterns using the Western blot technique. TMBr-3 immunoreactivity was detected in essentially equal amounts throughout the adult rat brain (Fig. 2). In contrast, the signal representing the presence of TMBr-1 was weaker than that for TMBr-3 and, furthermore, the signal for TMBr-1 was relatively stronger in cortex, hippocampus, olfactory bulb, striatum, and thalamus than in the midbrain, where it was hardly detectable. TMBr-1 was detectable in the brainstem and cerebellum only when the filters were exposed ≈10 times longer. TMBr-1 and TMBr-3 differ at their N-terminal regions due to the use of two promoters (Fig. 1). To analyze the distribution of other TM isoforms throughout the brain, we probed the protein samples with the TM311 antibody (Fig. 2*B*). In addition, this antibody also recognizes sequences contained within the β-TM gene and the α-TM gene. The isoforms recognized by the TM311 antibody give rise to three bands on a Western blot, termed TM-A, TM-B, and TM-C. In brain tissue, TM-A corresponded to TMBr-1 and fibroblast TM-2 (apparent M_r = 36,000), whereas TM-B corresponded to smooth muscle α-TM, and TM-C corresponded to smooth muscle β-TM. The three isoforms TM-A, TM-B, and TM-C could be detected in all brain areas, indicating that the promoter associated with the use of exon 1a is active in brain areas that do not strongly express TMBr-1.

TMBr-1 and TMBr-3 Are Expressed at Different Times in Development. The expression of TMBr-1 and TMBr-3 during development was examined by Western blot analysis (Fig. 3). TMBr-3 was detected as early as embryonic day 16 (E16). In contrast, TMBr-1 was detected at postnatal day 20 (P20). A band corresponding to TMBr-1 could not be detected in earlier stages, even when the filters were exposed 10 times longer. The same filter probed with the antibody TM311 revealed that the expression of other TM isoforms decreased during development whereas the relative levels of the brain-specific isoforms increased (Fig. 3*B*). It is worth noting that previous studies of the α-TM gene in rat brain using RNA analysis showed that mRNA expressed from this gene peaked at a time soon after birth (24, 25). Our data demonstrate that the amount of TMBr-1 and TMBr-3 protein increases postnatally and remains constant after 20 days. The observed peak in postnatal RNA expression appears to correspond to the postnatal increase in protein. Since TMBr-1 and TMBr-3 are transcribed from two promoters, the differences in the developmental expression of these isoforms could be due to differential regulation of the promoters (Fig. 1). To address

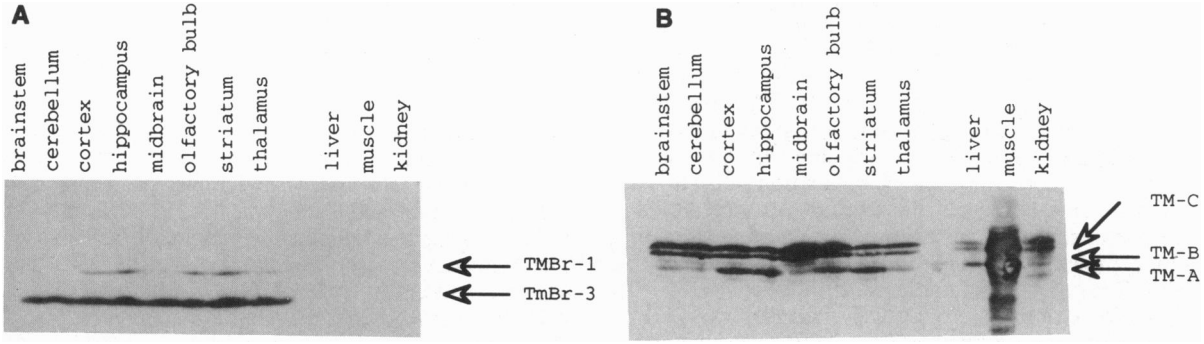

FIG. 2. Distribution of the brain-specific isoforms TMBr-1 and TMBr-3 in rat brain. Equal amounts of protein (15 μg) from various brain regions, liver, skeletal muscle, and kidney were analyzed. (*A*) The blot was probed with anti-rTM9c. The arrows indicate the position of TMBr-1 and TMBr-3. (*B*) A parallel blot was probed with the Sigma TM311 antibody. The arrows indicate the position of three immunoreactive bands indicated TM-A, TM-B, and TM-C.

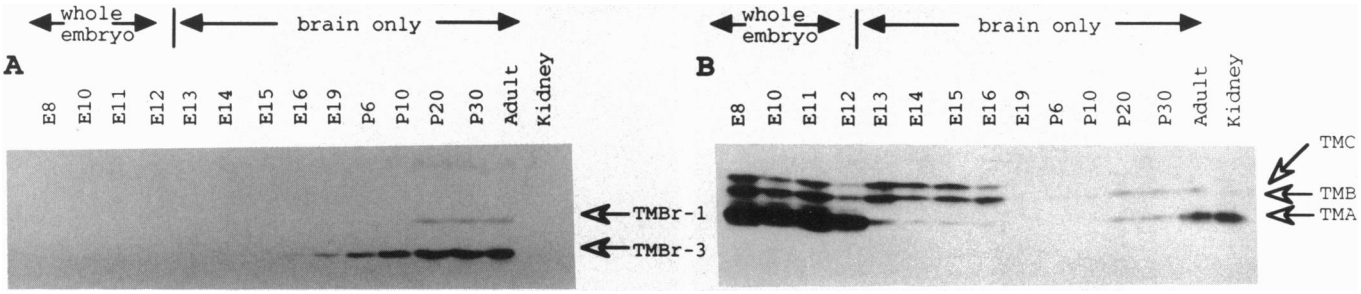

FIG. 3. Expression of the brain-specific α-TM isoforms TMBr-1 and TMBr-3 at different developmental stages. (*A*) Western blot analysis of equal amounts of protein (15 μg) using anti-rTM9c. Protein was prepared from whole embryos at E8–E12 and from whole brains at E13–E19, 6–30 days after birth (P6–P30), and 3 months (adult). (*B*) The filter in *A* was reprobed using the Sigma TM311 antibody. The arrows indicate the position of three immunoreactive bands indicated TM-A, TM-B, and TM-C.

this question, we used PCR to analyze RNA isolated from brain in different developmental stages (Fig. 4). By using this analysis, RNA generated from the 1a and 1b promoter could be detected at E8 and E10, respectively, in total brain. For both promoters, this is earlier than the expression of exon 9c, which occurs at E16 (Fig. 3). This indicates that both promoters are active before the exon 9c of the α-TM gene is expressed and demonstrates that the differences in expression of TMBr-1 and TMBr-3 are likely regulated at the level of alternative RNA splicing.

Expression of Exon 9c Is Restricted to Neurons. We have previously demonstrated (4) by RNase protection analysis that isoforms TMBr-1, TMBr-2, and TMBr-3 are brain-specific. To determine which cell types express TMBr-1 and TMBr-3, we performed immunocytochemistry with the anti-rTM9c antibody on primary neuronal and glial cultures. Although in primary neuronal cultures the presence of glial elements, mostly consisting of astrocytes, was minimal, we distinguished astrocytes from neurons by staining them with an antibody to GFAP, a cytoskeletal protein found only in astrocytes (26). The identity of neurons was confirmed with antibodies to tau and neurofilament, both constituents of the neuronal cytoskeleton. Exon 9c immunoreactivity was detected in all neurons (Fig. 5 *A* and *B*). Double labeling for the neuronal markers tau and neurofilament revealed correspondence with the exon 9c immunoreactive population. Some cells that stained brightly with anti-rTM9c stained poorly with tau and neurofilament, which is probably due to different developmental regulation. The staining pattern obtained with the anti-rTM9c could be blocked by preabsorbing the antibody with recombinant TMBr-1 (Fig. 5*C*). In addition, immunocytochemistry was performed on a mixed glia/neuronal culture using both anti-rTM9c and antibody to GFAP. We did not observe astrocyte-specific GFAP and exon 9c immunoreactivity in the same cell (Fig. 5 *F–H*). The specificity of anti-rTM9c for neurons was further confirmed by Western blot analysis that detected a protein band corresponding to TMBr-3 in cortical and mesoncephalic cultures but not in glial cultures (Fig. 6*A*). In contrast, GFAP staining was readily apparent in lysates prepared from glial and cortical cultures (Fig. 6*B*). Collectively these studies demonstrate expression of exon 9c is restricted to neurons in cultures of embryonic rat brain. Furthermore, the lack of TMBr-1 detected in the primary neuronal cultures derived from embryonic brain (Fig. 6) is in agreement with the late developmental expression pattern of this isoform (Fig. 3).

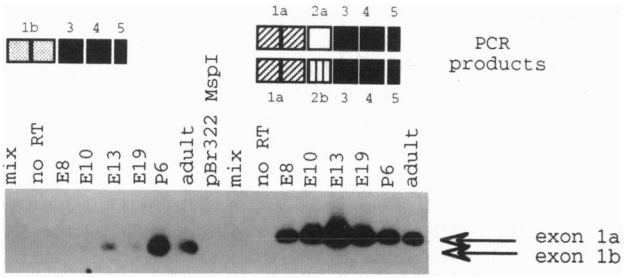

FIG. 4. PCR analysis of the use of the α-TM gene exon 1a and 1b promoters during development in brain. The various PCR products generated by use of the 1a and 1b promoters are indicated. Total RNA (1 μg) was reverse-transcribed using the AA501 primer and amplified using a combination of AS1A01/AA501 or AS1B01/AA501 (Fig. 1). Twenty eight cycles (1 min at 94°C, 45 sec at 56°C, and 2 min at 72°C) were used. The PCR products were analyzed using a Southern blot of the products probed with ^{32}P-end-labeled AS301. Mix, PCR was performed without adding nucleic acid; no RT, PCR was performed using adult RNA without reverse transcription as a template; pBr322 *Msp* I, *Msp* I digest of pBr322 DNA.

Intracellular Localization of TMBr-3. To investigate whether the functional significance of the brain-specific isoforms may be related to differences in their intracellular localization, immunofluorescence microscopy was performed using the anti-rTM9c antibody. Although this antibody would recognize both TMBr-1 and TMBr-3, the cells used in these studies notably only express TMBr-3 (Fig. 6). TMBr-3 was located mainly in the cell body but was also detected in the processes (Fig. 5 *A, E, F,* and *H*). In addition, in agreement with previous studies of the distribution of actin in neurons, the staining pattern of TMBr-3 appeared diffuse and we were unable to observe microfilament bundles or stress fibers using the anti-rTM9c antibody (27, 28).

DISCUSSION

Developmental Regulation of TMBr-1 and TMBr-3. The different regional and temporal patterns of expression of TMBr-1 and TMBr-3 that we demonstrated here raise two important questions. (*i*) What are the mechanisms responsible for the differential expression of these isoforms from the α-TM gene, and (*ii*) what is the functional significance of these isoforms in the nervous system? Since these isoforms both contain exon 9c, it remains to be determined whether these isoforms are coexpressed in the same cell or in distinct cell types. Although the promoters for TMBr-1 and TMBr-3 are being transcribed early in development (Fig. 4), we were not able to determine whether both promoters are active in the same cell. Thus, there are two possibilities for regulation of the neuron-specific splicing event that uses exon 9c. One possibility is that the exon 1a and 1b promoters are used in different neurons and that the splicing of exon 9c is independent of the promoter. The other possibility is that both promoters are active in the same neuron and the splicing of exon 9c is influenced by events associated with transcription at the 5′ end of the pre-mRNA (e.g., giving rise to TMBr-1 in hippocampal neurons but suppressing the use of exon 9c in a pre-mRNA created by the exon 1a promoter in neurons of the

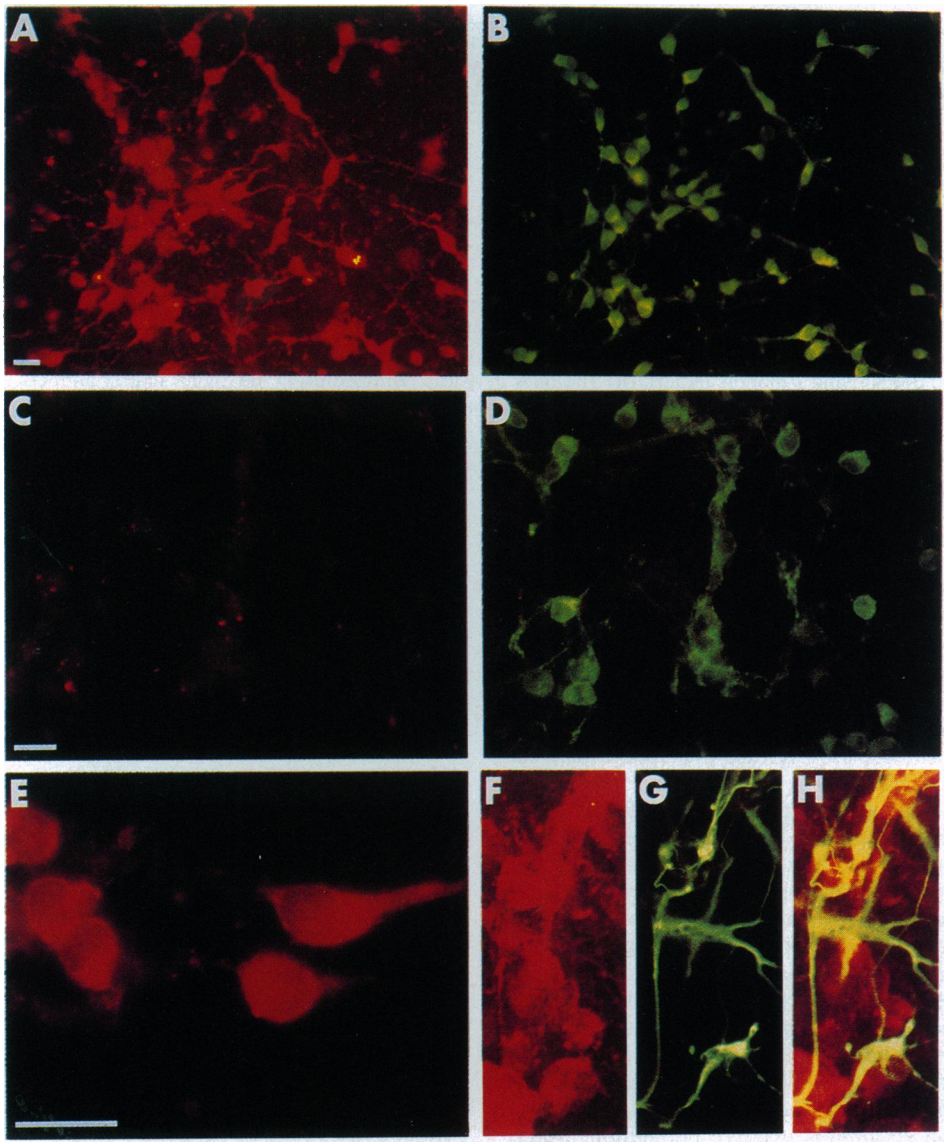

FIG. 5. Neuron-specific expression of exon 9c. Primary cultures from mesencephalon (*A–D*) or cortex (*E–H*) were made from E16 embryos and stained after 9 days of culture. Staining with anti-rTM9c was detected using rhodamine-conjugated antibody; all other antibodies were visualized using fluorescein-conjugated antibody, as indicated. (Bars = 20 μm.) (*A* and *B*) Exon 9c is expressed in all neurons. (*A*) Detection of anti-rTM9c immunoreactivity. (*B*) Same field stained using a mixture of anti-tau and anti-neurofilament antibodies. (*C–E*) Anti-rTM9c immunoreactivity can be blocked by preabsorbtion with TMBr-1. (*C*) The anti-rTM9c antibody was preabsorbed with recombinant TMBr-1 before staining the culture. (*D*) The same field stained with anti-tau antibody. (*E*) Higher magnification of neurons stained with anti-rTM9c. (*F–H*) Anti-rTM9c stains neurons and not glia. Cells were stained with anti-rTM9c (*F*) and with GFAP (*G*). (*H*) Field in *F* and *G* shown in a double exposure.

cerebellum). In this regard it is interesting to note that the 3-kb mRNA containing exon 9c does not contain sequences from exon 1a and appears to encode exclusively TMBr-3 (24). Similarly, the 1.8-kb mRNA containing exon 9c and exon 1a appears to encode TMBr-1 (24).

Expression of Exon 9c Is Specific for Neurons. In primary cultures of neurons and glia, we found that the use of exon 9c was restricted to neurons (Figs. 5 and 6). It is worth noting that we were unable to detect the expression of TMBr-1 in the primary cultures used in these studies. Since these cells were derived from embryonic brain, we did not expect to detect TMBr-1 because TMBr-1 was only expressed >2 weeks after birth. Furthermore, although we could not detect TMBr-1 in neuronal cultures, the absence of detectable TMBr-3 in glial cultures and the expression pattern of these isoforms in adult brain strongly suggests that both of these isoforms are specific to neurons. This specificity is in agreement with recent studies in PC12 cells demonstrating that expression of mRNA for TMBr-1 and TMBr-3 was concomitant with neuronal differentiation (24). Since the expression of these isoforms in PC12 cells was found to be temporally associated with neurite extension, it was hypothesized that these isoforms will be localized to dendritic spines and synapses where they would function in the establishment and maintenance of these structures (24). While we cannot rule out this possibility for TMBr-1, TMBr-3 was detected in the cell body as well as in the neuritic processes extending from it (Fig. 5). This staining pattern was consistently observed in cultures derived from midbrain and cortex and was examined at early and later time points in culture. The presence of immunoreactivity throughout the cell leads to the suggestion that TMBr-3 plays a more general role in neuronal cell structure.

Functions of TMBr-1 and TMBr-3. At present the functions of TMBr-1 and TMBr-3 are not known, but their expression pattern suggests a specialized role in the nervous system. The developmental study of neuron-specific TM expression presented here indicates that TMBr-3 and TMBr-1 are expressed relatively late during embryonic and neonatal development,

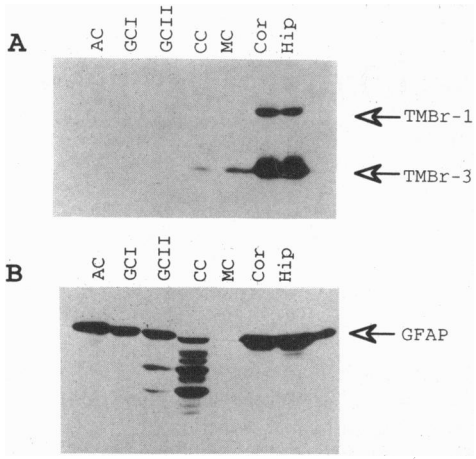

FIG. 6. Western blot analysis of TMBr-1 and TMBr-3 in glial and neuronal cultures. Lysates from astrocyte cultures (lanes AC), serum-free glia culture (lanes GCI), glia culture in the presence of fetal calf serum (lanes GCII), neuronal cultures from cortex (lanes CC) and mesencephalon (lanes MC), and cortex (lanes Cor) and hippocampus (lanes Hip) from adult rat brain were analyzed on a Western blot using anti-rTM9c (*A*) and anti-GFAP (*B*).

respectively, and remain at a constant level in the adult. Furthermore, when the expression of TMBr-1 in different areas of the brain is compared with other high molecular weight TMs that do not contain exon 9c, areas expressing high levels of TMBr-1 also express high levels of other TMs (Fig. 2). Both the developmental time course and the distribution indicate a replacement of nonbrain TM forms reflecting special positional or developmental needs of the neuronal cytoskeleton. The increase in expression of the brain-specific isoforms of TMs is coincident with processes of differentiation and maturation of the neuronal population. TMs are known to be associated with filamentous actin in both muscle and nonmuscle cell types. In neurons, actin filaments are thought to play a role in motile processes (2). Perhaps TMBr-1 and TMBr-3 play a role in process outgrowth, the formation of dendritic spines, and/or synapse formation, as TMs have been shown to be present in all of these structures (2). It is interesting to note that TMBr-1 has the strongest expression in areas of the brain thought to have the greatest plasticity after birth (5). The ratio of filamentous actin to globular actin has been shown to correlate with changes in visual cortical plasticity (29). In normal rats the amount of filamentous actin has been shown to be regulated by the amount of TM present. Furthermore, the potentially weaker actin binding properties of TMBr-1 and TMBr-3 (4) might allow for dynamic cytoskeletal changes and thus allow for continued plasticity in the adult brain. Finally, recent findings that the expression of TM is changed in some neurodegenerative diseases, such as Parkinson and Alzheimer disease (30) and in astrocyte transformation (31), furthermore, suggests the importance of different TM isoforms in the brain.

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