Splicing of Two Internal and Four Carboxyl-terminal Alternative Exons in Nonmuscle Tropomyosin 5 Pre-mRNA Is Independently Regulated during Development*

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Four nonmuscle tropomyosin isoforms have been reported to be produced from the rat Tm5 gene by alternative splicing (Beisel, K. W., and Kennedy, J. E. (1994) Gene (Amst.) 145, 251–256). In order to detect additional isoforms that might be expressed from that gene, we used reverse transcriptase-polymerase chain reaction assays and evaluated the presence of all product combinations of two alternative internal exons (6a and 6b) and four carboxy-terminal exons (9a, 9b, 9c, and 9d) in developing and adult rat brain. We identified five different combinations for exon 9 (9a + 9b, 9a + 9c, 9a + 9d, 9c, and 9d), and the exon combinations 9a + 9c and 9a + 9d were previously unreported. Each of these combinations existed with both exon 6a and exon 6b. Thus, the rat brain generates at least 10 different isoforms from the Tm5 gene. Northern blot hybridization with alternative exon-specific probes revealed that these isoforms were also expressed in a number of different adult rat tissues, although some exons are preferentially expressed in particular tissues. Studies of regulation of the 10 different Tm5 isoform mRNAs during rat brain development indicated that no two isoforms are coordinately accumulated. Furthermore, there is a developmental switch in the use of exon 6a to exon 6b from embryonic to adult isoforms. Tm5 protein isoforms show a differential localization in the adult cerebellum.

Tropomyosins are rod-like proteins that are associated with actin filament in muscle and nonmuscle cells. Multiple isoforms of tropomyosins exist in both muscle and nonmuscle cells (1). In muscle cells, tropomyosins play a pivotal role in regulating the interaction between the actin and myosin filaments. The role of tropomyosins in nonmuscle cells is beginning to be more defined. They are thought to differentially affect the stability of actin filaments (2) and have been shown to be implicated in various cellular functions including the regulation of cell transformation (3–5), cytokinesis (6), motility (7, 8), and morphogenesis (9–13).

Alternative splicing accounts for the majority of tropomyosin isoform diversity. In mammals, four tropomyosin genes have been identified, α-Tm(14), β-Tm(15), Tm4(16), and Tm5(17). Except for the Tm4 gene, it is now clear that the other three rat tropomyosin genes contain two alternate promoters (exons 1a and 1b), two alternative internal exons (6a and 6b), and four alternative COOH-terminal exons (9a, 9b, 9c, and 9d). Therefore, the tropomyosin genes can each theoretically generate mRNAs for at least 16 isoforms. However, only 20 isoforms have been characterized from all four rat tropomyosin genes (14–24). This suggests that some exon combinations either do not occur or are unique to cell types that have not yet been studied.

In the case of the rat Tm5 gene, one muscle isoform and four nonmuscle isoforms have been reported to date. The muscle product of this gene is known as α-Tm, since it is preferentially expressed in slow-twitch skeletal muscle (25). The four nonmuscle isoforms have been termed Tm5 NM1 to NM4 (23), but Tm5 NM1 was previously referred as the fibroblastic TM30 nm isoform (22) or Tm5 isoform (26). These four nonmuscle isoforms have been identified in the rat cochlea and varied in their usage of alternative exons 6a and 6b, 9a + 9b, 9d and 9c (Fig. 3).

In order to detect additional isoforms that might be expressed from the Tm5 gene, we used systematic RT-PCR1 assays to evaluate the presence of all product combinations of two alternative internal exons (6a and 6b) and four carboxy-terminal exons (9a, 9b, 9c, and 9d) of the Tm5 gene in the developing and adult rat brain. We have identified novel splice combinations, and at least 10 nonmuscle isoforms are produced from that gene in the rat brain. These isoforms are independently regulated during rat brain development. Furthermore, a switch occurs in the use of exon 6a to exon 6b from embryonic to the adult isoforms. In the case of two isoforms, Tm5 NM1 and NM2, isoform switching coincides with a shift in sorting to different neuronal compartments.

EXPERIMENTAL PROCEDURES

Tissue Sampling and RNA Isolation

Embryonic and adult rat tissues were collected from Sprague-Dawley rats, immediately frozen in liquid nitrogen, and stored at −70 °C.

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1 The abbreviations used are: RT-PCR, reverse transcriptase-polymerase chain reaction; Tm, tropomyosin; a.a., amino acid; bp, base pairs; UTR, untranslated region; kb, kilobase pair(s).
Nonmuscle Tropomyosin 5 Isoforms

Frozen tissues were homogenized in phenol/guanidine isothiocyanate, and RNA was extracted as described (27).

**Southern Blot Probes**

Two oligonucleotide probes were synthesized from the rat Tm5 cDNA sequence (published in Ref. 23) as follows: the H192 probe (position 552–880), specific for exons 7 and 8 (a.a. 179–221 from nonmuscle Tm5), and the H169 probe (position 683–761), specific for exon 9a (a.a. 222–248 from nonmuscle Tm5). 100 ng of oligonucleotides were 3’-end labeled using a Gigaprimere labeling kit (Bresatec, Australia) by incubation with 50 μCi of [α-35S]dCTP and the large Klenow fragment of *Escherichia coli* DNA polymerase at 37 °C for 15 min. 1 μg of antisense nucleotides specific to the 3’-end of each DNA probe was used to prime the reactions.

**Northern Blot Probes**

**Exons 7 + 8**—A human Tm5 cDNA probe, pHM-Tm5-SA, which is a gene-specific and well conserved cross-species, has been characterized previously (25). This DNA fragment encodes a.a. 213–284 plus 48 bp of the 3’-UTR from Tm5, or the corresponding a.a. 177–248 from nonmuscle Tm5.

9d 3’-UTR—A 1,500-bp probe corresponding to the entire 9d 3’-UTR, pMMTm5-3’ UT, and generated from a mouse Tm5 nonmuscle cDNA by PCR amplification has been characterized previously (28). Two other probes, 3’UT244, corresponding to the 5’ end of the 9d 3’-UTR (bp 760 to 1190), and 3’UT110, corresponding to the central region of the 9d 3’-UTR (bp 1200 to 1800), were generated by PCR amplification using the following sets of primers: 5’-GAGATGTACCTCGTCCGATC 3’/5’-GTATCCATTAGGTAAGTGGTC 3’ and 5’-ATGCGAGAGAGAACAAGCAGGAATG 3’/5’-GAGATTTGCCGACGACTGAC 3’. Amplification was carried out using rat fibroblast cDNA.

9c 3’-UTR—Two oligonucleotide probes, H135 (position 774–909) and H136 (position 910–1056) corresponding to the entire 9c 3’-UTR, were synthesized from the rat Tm5 cDNA sequence and used simultaneously for hybridization.

**Exon 9a—**To obtain a probe that could recognize specifically exon 9a, the human Tm5 cDNA previously described (25), pHM-Tm5-SA, was digested by *SnaI*. This DNA fragment encodes a.a. 259–284 plus 48 bp of the 3’-UTR from Tm5 or the corresponding a.a. 223–248 from nonmuscle Tm5.

Exon 6a and Exon 6b—Oligonucleotide probes specific for exon 6a (H182) and exon 6b (H184) were synthesized from the rat Tm5 cDNA sequence (23). These probes are identical in sequence to the four last a.a. encoded by exon 5 (a.a. 149–152) plus the entire exon 6a or exon 6b (a.a. 153–178) plus the four first a.a. encoded by exon 7 (a.a. 179–182) from nonmuscle Tm5 or the corresponding a.a. 185–218 from Tm5.

**RT-PCR, Southern Blot, and Sequence Analysis of RT-PCR Products**

Rat Tm5-specific oligonucleotide probes were chosen from the rat Tm5 cDNA (23) as follows: exon 1b, GCAAGATCCAGGTTCTGCAG (position 53–72); exon 6a, CAGAGGACAGGATGTAGTAG (position 476–495); exon 6b, GTGCTGAGGAGTTGGC (position 477–496); exon 9b, CACCTGAGAGAAGGGTGT (position 767–786); exon 9c, CAGGCTTAGAGTGCTCCTAGG (position 843–863). First strand cDNA was synthesized from 1 μg of total RNA in a final volume of 50 μl containing 20 units of RNase H reverse transcriptase (Superscript II, Life Technologies, Inc.), 750 ng of random hexamers (Promega), 0.4 mm each dNTPs, 5 units of RNasin, and 100 mM dithiothreitol. Reaction was performed for 75 min at 37 °C. PCR reactions were then conducted for 35 cycles in a total volume of 20 μl using 6 μl aliquots of a 5-fold dilution of the cDNA and Tm5-specific primers, 80 ng each, in the presence of 1 unit of Taq polymerase (Boehringer Mannheim), and 0.2 mm each dNTPs. RT-PCR products were resolved in agarose gels and visualized by staining with ethidium bromide. For Southern blot analysis, RT-PCR products were transferred onto Hybond N membranes (Amersham Pharmacia Biotech) according to Ref. 29. Oligonucleotide probes specific for rat Tm5 exons 7 and 8 (H92) or exon 9b (H189) were labeled using specific 3’ antisense primers and hybridized to DNA blots at 106 cpm/ml in a solution of 4× SSC, 5× Denhardt’s solution, 50 mM NaH2PO4 (pH 7.0), and 10% dextran sulfate at 60 °C for 16 h. The blots were then washed once with 1× SSC, 0.1% SDS at 60 °C for 30 min and twice with 0.5× SSC, 0.1% SDS at 60 °C for 30 min each. Filters were exposed to Kodak Biomax film for a few hours. For sequencing, amplified Tm5 cDNA fragments were excised from agarose gel, isolated by glass-wax extraction, and purified by ethanol precipitation. DNA sequence was determined with a DNA sequencer (373A Applied Biosystems) using dye fluorescent labels. Sequence data were determined in the sense and antisense orientations.

**Antibodies and Immunohistochemistry**

The CG3 antibody is a monoclonal antibody whose epitope has been mapped to a.a. 29–44 encoded by exon 1b of the Tm5 gene (30) and has been kindly provided by Jim J.-C. Lin. The mouse monoclonal antibody was directed against a peptide DKLKCT corresponding to a.a. 222–227 of exon 9d and raised in rabbit, as described previously (12). The peptide ESYLRQLESNLSENLKLTL corresponding to a.a. 222–241 of exon 9c was used to generate rabbit antisera and referred to as WD 5/9c. The secondary antibodies were used goat anti-mouse and anti-rabbit IgG-alkaline phosphatase conjugated (Jackson ImmunoResearch). Adult rat cerebellum was fixed and prepared for sectioning as described previously (11, 12). Primary antibodies were used at a dilution of 1:100. Secondary antibodies were used at 1:250.

**RESULTS**

Samples of total RNA were electrophoresed on 1% agarose gels containing 6% formaldehyde and blotted to Hybond N nylon membranes (Amersham Pharmacia Biotech) as described (29). Probes were labeled using random or specific 3’ antisense primers and hybridized to RNA blots under identical conditions as for Southern blots, but hybridization and washes were conducted at 65 °C. For probes specific for exon 6a (H182), exon 6b (H184), and exon 9a (pHM-Tm5-SaeI), hybridization was performed at 60 °C and washing at 55 °C. Filters were exposed for 24–48 h to phosphor screens which were scanned by a Molecular Dynamics Laser PhosphorImager (Molecular Dynamics). To correct for loading and transfer errors, Northern blots were hybridized to an 18 S specific ribosomal RNA oligonucleotide. mRNA levels were quantified using the Molecular Dynamics Laser PhosphorImager.

**Splicing Patterns of Tm5 mRNA**—To evaluate the extent of Tm5 isoform diversity, total RNA was isolated from developing and adult rat brain and analyzed by RT-PCR. Specific oligonucleotide primers were used to amplify the products resulting from each possible splicing combination occurring between the two internal exons 6a and 6b and the four carboxyl-terminal exons 9a, 9b, 9c, and 9d (Fig. 1). The specificity of the RT-PCR products was then confirmed by Southern blot hybridization to an oligonucleotide probe identical to the region coding for constitutive exons 7 and 8 (Fig. 1A). For both primer pairs 6a/9d (Fig. 1A, panel 1, upper) and 6b/9d (Fig. 1A, panel 1, lower), two bands were detected. The size of the smaller (and major) band was consistent with the expected size of PCR products containing, respectively, the exon combinations 6a + 9d, 6b + 9d, based on the published patterns of alternative splicing for Tm5 NM1 and NM2 isoforms (23). For both primer pairs, the two bands differing by 80 base pairs. This raised the possibility that an additional exon may be included in the larger band. The presence of exon 9a in the larger product was demonstrated by hybridization to an oligonucleotide probe specific for this exon (Fig. 1B, panel 1). The exon combination 9a + 9d was a previously unreported splice event, and we found it existed with both exon 6a and 6b.

For both primer pairs 6a/9c and 6b/9c, two bands differing by 80 base pairs were also detected (Fig. 1A, panel 2). The smaller band was in agreement with the exon combinations 6b + 9c, corresponding to NM4 isoform, and 6a + 9c, respectively, which was a novel splice variant. Again, exon 9a was shown to be present in the larger band, resulting in two additional splice variants containing the exon combination 9a + 9c with both exons 6a and 6b (Fig. 1B, panel 2).

Finally, primer pairs 6a/9b and 6b/9b detected only a single band of the predicted size for products containing the exon combinations 6b + 9a + 9b, corresponding to NM3 isoform, and 6a + 9a + 9b, respectively, which was previously unreported (Fig. 1A, panel 3).
To determine the exact nucleotide sequence identity of the novel splice variants containing exon 9a, amplified cDNA fragments were extracted from the gel and sequenced. The nucleotide sequences for the fragments amplified with primer pairs 6b/9d and 6b/9c from adult rat brain and the amino acid sequences predicted by the new mRNA splice junctions are given in Fig. 2A. The novel RNA splicing patterns occur between the 5'-splice donor site of exon 9a and the 3'-acceptor sites of exon 9d or exon 9c. When they are spliced to exon 8, exons 9d and 9c are each capable of encoding a unique 27 carboxyl-terminal amino acid residue protein fragment. The splice event exon 9a to 9d introduces a termination codon in the second to fourth nucleotides of exon 9d, leaving the entire 9d sequence as a 3'-UTR. The exon 9a to 9d splice variant cDNA was previously cloned from a mouse macrophage library, and its 3'-UTR was partially sequenced (31). The splice event 9a to 9c creates a novel reading frame with a termination codon in the first seven codons of exon 9c, resulting in an amino acid change in the first six residues normally encoded by exon 9c, and leaving the remaining 9c sequence as a 3'-UTR. This novel reading frame is conserved in the rat and human genes. These two splicing events exist with both exon 6a and 6b.

Moreover, in some of the RT-PCR assays performed on adult rat brain samples, the larger band amplified with primer pair 6a/9c did not hybridize to the exon 9a probe, suggesting that this band contained distinct Tm5 products of similar size which did not possess exon 9a (Fig. 1, A and B, panels 2, adult lane). This band was sequenced and corresponds to exon 6a spliced to exon 6b. The nucleotide sequence at the junction between exon 6a and exon 6b for the fragment amplified with primer pairs 6a/9c from adult rat brain is given in Fig. 2B. This splicing pattern introduces a premature termination codon in the first codon of exon 6b, resulting in a truncated Tm protein of 178 amino acid residues. We also identified a product containing both exons 6a and 6b when amplification was performed with primer pair 6a/9d (data not shown).

The amino-terminal region of the Tm5 gene contains two alternative promoters, associated with exons 1a and 1b. To determine from which promoter the novel variants were produced, RT-PCR assays were performed on developing and adult
rat brain samples with primer pairs 1b/9d, 1b/9c, and 1b/9b. Positive hybridization to oligonucleotide probes specific for exons 6a and 6b, A, the PCR-amplified 467-bp cDNA fragment designated "6b-9a-9d" and the PCR-amplified 465-bp cDNA fragment designated "6b-9a-9c" (Fig. 1, A and B) were purified and sequenced as described under "Experimental Procedures." The nucleotides and deduced amino acid sequences are shown for exons 9d and 9c according to their splicing pattern to either exon 8 (lower line) or exon 9a (upper line). The boundaries between exons are derived from the human genomic sequence (17). The amino acids are indicated above the second nucleotide of the codon. The termination signals are marked with ***. The novel 9a-9d splice junction results in the introduction of a termination codon in the first four nucleotides encoded by exon 9d, leaving the entire 9d sequence as a 3'-UTR. The novel 9a-9c splice junction results in the introduction of a termination codon in the first seven codons of exon 9c, resulting in an amino acid change in the first six residues encoded by exon 9c, and leaving the remaining 9c sequence as a 3'-UTR. B, the PCR-amplified 467-bp cDNA fragment designated "6a-9a-9c" from adult rat brain was purified and sequenced as described under "Experimental Procedures." The novel 6a-6b splice junction results in the introduction of a termination codon (***). These data are available through GenBank™ accession numbers AF053359, AF053360, and AF053361.

**Nonmuscle Tropomyosin 5 Isoforms**

**Tissue Specificity of Tm5 mRNA Splice Variants**—To determine the pattern of expression of these variants in various adult rat tissues, total RNA was isolated from adult rat soleus (slow muscle), skeletal muscle from the leg (slow and fast muscle), testes, kidney, liver, and brain. These samples were analyzed by Northern blot hybridization using four Tm5 probes: a probe specific for exons 7 and 8, full-length 9d 3'-UTR, full-length 9e 3'-UTR, and exon 9a (Fig. 4). Hybridization with the first probe, which is in a constitutive region, confirmed that the muscle-specific 1.3-kb transcript, α-Tm, is highly expressed in soleus and skeletal muscle and is the major isoform of the Tm5 gene. This probe also detected a 2.5-kb transcript expressed at very similar levels in both muscle and nonmuscle tissues and a 1.0-kb mRNA in testes. Two minor mRNAs were also detected in brain with sizes estimated at 2.9 and 1.5 kb. Hybridization with 9d 3'-UTR cDNA recognized the 2.5-kb mRNA whose pattern of expression is characteristic of a nonmuscle tropomyosin, as expected (28). According to our RT-PCR results, the 2.5-kb mRNA could account for four isoforms, corresponding to NM1 (exon combination 6a + 9d), NM2 (6b + 9d), NM5 (6a + 9a + 9d), and NM6 (6b + 9a + 9d), which were not independently resolved by this Northern blot analysis. The 9d 3'-UTR probe also detected the 1.0-kb mRNA in testes. In skeletal muscle, two novel transcripts of approximately 1.0 and 1.7 kb were detected. The 1.7-kb mRNA was not clearly detected in the soleus muscle by the full-length 9d 3'-UTR probe which gave a strong 1.0-kb signal. These transcripts will be explained below.

The 9e 3'-UTR probe, within the limit of detection in this experiment, hybridized only to the brain 1.5-kb mRNA, suggesting that exon 9e is preferentially expressed in brain. The 1.5-kb transcript could represent NM7 (6a + 9c), NM4 (6b + 9c), NM8 (6a + 9a + 9c), and NM9 (6b + 9a + 9c). Finally, the exon 9a probe detected the muscle 1.3-kb transcript but virtually nothing in other tissues, indicating that the α-Tm isoform is the major product expressing exon 9a. Moreover, the 2.9-kb transcript detected in brain with the exon 7 and 8 probe did not show any positive hybridization to the known 3'-UTRs, suggesting that the 2.9-kb mRNA must contain a novel exon 9 of the Tm5 gene.
In addition to the nonmuscle 2.5-kb transcript, the full-length 9d 3'-UTR probe recognized the 1.0-kb mRNA in testes and two novel transcripts of 1.7 and 1.0 kb in muscle. Two probes containing, respectively, the 5' end (position 760–1190) and the central region (position 1190–1800) of the 9d 3'-UTR were produced by PCR amplification and hybridized to soleus, skeletal muscle, and testes RNAs (Fig. 5). The 5' probe recognized the testes-specific 1.0-kb mRNA, suggesting that this transcript may derive from use of an alternative polyadenylation signal located in the 5' end of the 9d 3'-UTR. A putative consensus site, ATTA, is located in this region. The central region probe recognized the 1.7-kb mRNA in soleus and skeletal muscle, whereas the full-length probe yielded a strong 1.0-kb signal. These transcripts did not hybridize to a 9d coding region probe either. We hypothesize that these two transcripts may be generated through splicing of exon 8 to an alternative splice acceptor site located in the 3' end of the 9d 3'-UTR.

We then compared the tissue distribution of transcripts containing exon 6a to those containing exon 6b (Fig. 4). It appeared that exon 6a is universally used with an expression pattern similar to that of the nonmuscle exon 9d and that NM1 (6a + 9d) isoform is the commonly used isoform. In contrast, the use of exon 6b is more restricted since it is primarily expressed in muscle and in brain.

**Temporal Relationship between the Expression of Alternative Exons**—We have noted differences in the regulation of Tm5 variants during rat brain development with the RT-PCR assays (Fig. 1). We therefore chose to evaluate the temporal regulation of the mRNA splice variants and to compare expression of alternative exons. Total RNA isolated from developing to adult rat brain was size-fractionated and hybridized initially with the exons 7 + 8, 9d 3'-UTR, 9c 3'-UTR, and exon 9a probes (Fig. 6). Direct quantification of 32P on the hybridization membrane by PhosphorImager analysis indicated that mRNAs with the 9d 3'-UTR undergo a 5-fold decrease in accumulation from embryo to the adult. In contrast, the level of transcripts containing 9c 3'-UTR is constant. Hybridization with the exon 9a probe indicated that exon 9a is expressed in about equal amounts in all splicing combinations producing the 2.5-kb (9a + 9d), 1.5-kb (9a + 9c), and 1.3-kb (9a + 9b) mRNAs and that accumulation of transcripts containing exon 9a slightly increases from embryo to the adult. Thus, transcript accumulation of Tm5 mRNAs is subject to temporal regulation and is different with the use of each alternative carboxyl-terminal exon.

We then compared expression of transcripts differing in the use of internal exons 6a and 6b during rat brain development. Fig. 7A shows that NM1, NM5, NM7, and NM8 mRNAs containing exon 6a are down-regulated from embryo to the adult. Reciprocally, the NM2, NM6, NM4, and NM9 mRNAs containing exon 6b are up-regulated. A switch in the preferential use of exon 6a to 6b from the embryonic stages to the adult was also seen with the PCR assays (Fig. 1). This developmental switch from exon 6a to 6b is independent of the terminal exon. To determine if this regulatory pattern was a general phenomenon of Tm5 transcripts, expression of these mRNAs during skeletal muscle development was examined. Fig. 7B shows that the change from the expression of the nonmuscle exon 9d in embryo limbs (2.5-kb transcript) to the muscle-specific exon 9b (1.3-kb transcript) in the adult is associated with the same developmental switch in the use of internal exon from 6a to 6b.

**Fig. 3.** Diagram of the Tm5 mRNA splice variants expressed in the rat brain. Exons are represented by boxes and introns by lines. Polyadenylation signals are marked with an A and termination signals with a T. The 3' untranslated regions (3'-UTR) are indicated in white. The Tm5 gene structure shown is a combination of data obtained from human genomic sequence (17) and rat cDNA (23). The precise order and location of exon 9c is unknown. The α-Tm, isoform expressed in skeletal muscle is also shown. Nomenclature of mRNA transcripts is based on that introduced in Ref. 23.
as the one observed during brain development.

Tm5 Protein Isoforms Are Localized to Specific Structures within the Adult Rat Cerebellum—Antibodies that identify amino acids encoded by specific exons were used to investigate whether protein isoforms from the Tm5 gene were expressed in adult cerebellum. This brain region was chosen because of its well studied histology and clearly defined polarity orientation of its main neuronal cell types. The CG3 monoclonal antibody recognizes the amino acids encoded by exon 1b and therefore potentially identifies all the nonmuscle isoforms derived from the gene. Fig. 8A shows that CG3 staining is broadly distributed within the cerebellum. Most prominent staining is present in the Purkinje neuron cell bodies as well as the molecular layer which is densely packed with axons. Within the granule cell layer, staining is seen within the synaptic rich glomeruli but not in the granule cells themselves. In contrast, WS5/9d a polyclonal serum shows a very different expression pattern. This antiserum was raised against a unique sequence within exon 9d and therefore potentially identifies the isoforms NM1 and NM2, although in the adult brain NM2 is primarily expressed (see Fig. 7A). This antiserum has been previously well characterized (11, 12) and gave a localization pattern that was a specific subset of the pattern seen with CG3. Only the Purkinje cell soma and dendrites were identified by this antiserum (Fig. 8C), which is the most prominent cell type identified by CG3. Another antiserum, WD5/9c, was raised against exon 9c of the Tm5 gene and therefore potentially identifies the isoforms NM7 and NM4. In contrast to WS5/9d, the distribution of these isoforms is broader and very similar to CG3 (Fig. 8B). In primary cortical neuron/gliar mixtures grown in culture this antibody was neuron-specific as was exon 9c expression from the α-Tm5 gene (12). In summary, it appears that multiple protein isoforms from the Tm5 gene are expressed in the adult cerebellum. They are associated with specific neuronal structures suggesting potentially different functions of these isoforms in the maintenance of morphology.

DISCUSSION

Our results indicate that Tm5 isofrm diversity is much greater than previously thought and can be generated by alternative splicing of coding and noncoding regions. These results also raise the question of whether the other tropomyosin genes also have the capability to generate such a large number of isoforms.

We have identified novel exon combinations for alternative splicing of terminal exon 9. The 9a + 9c mRNAs encode potential new variants carrying six additional residues in their carboxyl-terminal part. In contrast, the 9a + 9d mRNAs produce the same 9a-encoded COOH-terminal amino acid residue proteins as the 9a + 9b mRNAs, and the differences between these variants lie in their 3'-UTR regions. Regions of the 3'-UTRs have been conserved between mammals (26, 23), indicating that these regions may be functionally important. Alternative splicing occurring in the 9b 3'-UTR of the α-Tm5 gene was previously reported (32). In this case, skeletal muscle and fibroblast produce α-Tm5 tropomyosin isoforms which are identical except for the last COOH amino acid but are generated from two mRNAs differing by 48 bp in their 9b 3'-UTR sequence. Similarly, the skeletal muscle β-Tm isoform can also be produced from an unusual mRNA with an elongated 3'-UTR caused by the retention of a 1-kb intron (20). Moreover, the 3'-UTR regions can undergo alternative polyadenylation. The Tm5 testes-specific transcript may be derived from use of an alternate polyadenylation signal, as the 9b 3'-UTR and 9c 3'-UTR regions of the α-Tm5 gene contain at least two different polyadenylation sites each (24). These results therefore suggest that a functional difference between tropomyosin isoforms may also exist at the mRNA level. The 3'-UTR regions may endow the mRNAs with isoform-specific functions, such as specific intracellular localization or translation efficiency of mRNAs.

We have also identified in skeletal muscle two mRNAs with deletions of the 5' end of the 9d 3'-UTR, and we hypothesized that these two transcripts may be generated through splicing of exon 8 to an alternative splice acceptor site located in the 3' end of the 9d 3'-UTR. Indeed, a mouse embryonic muscle Tm5 cDNA in which the last nine codons of exon 8, the coding region of exon 9d, and the first 400 bp of the 9d 3'-UTR were missing was reported in the Expressed Sequence Tag (EST) data base of GenBankTM (accession number W82438). This cDNA potentially encodes a 217-amino acid nonmuscle Tm5 (or a 253 amino acid muscle Tm5) as a termination codon is created after the first five codons of the 9d 3'-UTR sequence. The respective amino acid sequences are conserved only between rodents, but the termination codon is conserved from rat to human. Thus, it is likely that all splicing possibilities occur in the Tm5 gene and give rise to additional isoforms.

Although the two alternative exons are not usually spliced to each other, we identified an additional splice variant with exons 6a and 6b spliced together. Several sequences involved in the choice of exons 6a and 6b of the β-Tm gene have been identified (33, 34), and the mechanism required for exon 6a inclusion appears to be independent of the one responsible for exon 6b exclusion. There is no intrinsic barrier to splicing between exons 6a and 6b in the Tm5 gene (35), and some previous reports also suggest that the exons 6a and 6b of the

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2 R. P. Weinberger, unpublished observations.
\(\beta\)-Tm gene could splice together in vitro (33) and in vivo (36). This splice variant potentially produces a truncated tropomyosin of 178-amino acid residues which may function as a "poison molecule" by incorporation into polymers and subsequently disrupting the head-to-tail overlaps.

The differential expression of \(Tm5\) isoform mRNAs in various adult rat tissues suggests that, although some exons are preferentially expressed in particular tissues, alternate exons are not used in a strictly tissue-specific manner. Rather, we hypothesize that these isoforms are expressed in any type of cell where they may be used to define specific intracellular compartments. Tropomyosin isoforms are indeed spatially segregated in a variety of cell types where they maintain specific microfilament domains (12, 37). The differential localization of NM1, 2 isoforms (exon 9d), and NM4, 7 (exon 9c) isoforms in rat cerebellum also supports functionally distinct roles. The existence of extensive \(Tm5\) isoform diversity may thus provide a means of creating multiple spatial compartments with distinct functions inside the same cell.

The study of expression of the different \(Tm5\) isoform mRNAs indicates that no two transcripts are coordinately regulated during rat brain development. The selection of alternative internal and terminal exons is thus independently controlled. We show that a developmental switch occurs in the preferential use of internal exon 6a to 6b from embryonic to adult isoforms. This is not restricted to the brain but also occurs during skeletal muscle development. A similar switch was described for
Alternative splicing may also regulate the final protein localization by selection of specific coding exons that may contain information for preferred protein-protein interaction. A shift in localization of two isoforms, Tm5 NM1 and NM2, to different neuronal compartments during development was revealed by staining with an antibody specific for amino acids encoded by exon 9d. These isoforms are located in the growing axon in developing neuron but in the cell body and the dendrites in mature neurons (12). Protein distribution did not correlate exactly with corresponding mRNA localization in the mature neuron (11). Given the switch in the use of exon 6a to exon 6b from the developing to the adult isoforms described in this paper, we expect that the NM1 isoform (6a + 9d) is targeted to the somatodendritic compartment of a mature neuron. Thus, the differential use of exons 6a and 6b may regulate the targeting of isoforms and therefore the development of specific cellular compartments.

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