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Three Novel Brain Tropomyosin Isoforms Are Expressed from the Rat α-Tropomyosin Gene through the Use of Alternative Promoters and Alternative RNA Processing

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cDNA clones encoding three novel tropomyosins, termed TMBr-1, TMBr-2, and TMBr-3, were isolated and characterized from a rat brain cDNA library. All are derived from a single gene, which was previously found to express striated muscle α-tropomyosin and a number of other tropomyosin isoforms via an alternative splicing mechanism (N. Ruiz-Opazo and B. Nadal-Ginard, J. Biol. Chem. 262:4755–4765, 1987; D. F. Wieczorek, C. W. J. Smith, and B. Nadal-Ginard, Mol. Cell. Biol. 8:679–694, 1988). The derived amino acid sequences revealed that TMBr-1 contains 281 amino acids, TMBr-2 contains 251 amino acids, and TMBr-3 contains 245 amino acids. All three proteins contain a region that is identical to amino acids 81 through 258 of skeletal muscle α-tropomyosin. TMBr-1 is identical to striated muscle α-tropomyosin from amino acids 1 through 258 but contains a novel COOH-terminal region from amino acids 259 through 281. TMBr-2 and TMBr-3 both contain identical NH_2-terminal sequences from amino acids 1 through 44 which were found to be expressed from a novel promoter. TMBr-3 contains the same COOH-terminal region as TMBr-1, whereas TMBr-2 contains a second novel COOH-terminal region. The genomic organization of the exons encoding TMBr-1, TMBr-2, and TMBr-3 were determined. These studies revealed a previously uncharacterized promoter located in the internal region of the α-TM gene as well as two novel COOH-terminal coding exons. The α-TM gene is a complex transcription unit containing 15 exons including two alternative promoters, two internal mutually exclusive exon cassettes, and four alternatively spliced 3′ exons that encode four different COOH-terminal coding regions. A total of nine distinct mRNAs are known to be expressed from the α-TM gene in a cell type-specific manner in tissues such as striated muscle, smooth muscle, kidney, liver, brain, and fibroblasts. The mRNAs encoding TMBr-1, TMBr-2, and TMBr-3 were found to be expressed only in brain tissue, with TMBr-3 being expressed at much greater levels than TMBr-1 and TMBr-2. The individual structural characteristics of each brain α-tropomyosin isoform and their possible functions are discussed.

Tropomyosins are a diverse group of proteins with distinct isoforms present in striated muscle, smooth muscle, and nonmuscle cells (2, 9, 23, 36, 40, 55, 56). They are elongated structural proteins that have a simple dimeric structure consisting almost entirely of an alpha-helical coiled-coil (reviewed in reference 58). The coiled-coil structure is based on a 7-amino-acid pattern that is repeated along the full length of the molecule (49, 56). The tropomyosins bind to actin in a lateral manner, with each molecule encompassing six or seven actin monomers (12, 16). A pattern of 19.66 residues is repeated 14 times in muscle tropomyosins and is thought to be involved in actin binding (50). At present it is known that the striated muscle tropomyosins mediate the calcium ion-dependent regulatory effect of troponin on actomyosin ATPase activity (reviewed in reference 34). The role of tropomyosin in smooth muscle and nonmuscle cells is not well defined.

Tropomyosins can generally be grouped into low-M_r and high-M_r forms. The high-M_r tropomyosins are 284 amino acids in length and have an apparent M_r on sodium dodecyl sulfate (SDS)-polyacrylamide gels of between 33,000 and 45,000. They are typified by the striated muscle tropomyosins (43), which bind strongly to actin filaments with a stoichiometry of one molecule per seven actin monomers (16, 50), exhibit a strong tendency for head-to-tail polymerization (29), and show a 39.5-nm repeat in Mg^{2+} paracrystals (7). The striated muscle tropomyosins can be separated into two bands on SDS-polyacrylamide gels that are named alpha (faster mobility) and beta (slower mobility) (13). Other 284-amino-acid tropomyosins include those found in smooth muscle (24, 36, 56, 64) and fibroblasts (46, 47), including TM-1 (41, 65), TM-2 (61), and TM-3 (37).

The low-M_r tropomyosins are 247 or 248 amino acids in length and have an apparent M_r on SDS-polyacrylamide gels of 28,000 to 34,000. They are typified by an equine platelet β isoform (35) which is homologous to fibroblast TM-4 of rats (66) and $TM30_{pl}$ (42) of humans. Equine platelet tropomyosin shows a repeat of 33.3 nm in Mg^{2+} paracrystals (11) and binds to actin filaments with a stoichiometry of one molecule per six actin monomers (12). Relative to striated muscle tropomyosin, it requires a higher concentration of magnesium ions for actin binding and has a weak propensity for head-to-tail polymerization (10). Other low-M_r isoforms with a defined primary structure include human $TM30_{nm}$ (9), chicken fibroblast TM-3b (2), and *Drosophila melanogaster* cTM (22). Tropomyosins with physical properties characteristic of 247-amino-acid isoforms have also been purified from fibroblasts (28), erythrocytes (20), thyroid (32), brain (4, 6, 14, 18, 31), and intestinal epithelium (6). The low-M_r isoforms from erythrocytes and intestinal epithelium are distinctive in that their actin-binding capabilities are similar to that of the muscle tropomyosins (6, 20).

The tropomyosins of chickens (2, 24, 36, 39, 56), *D. melanogaster* (22, 30), humans (9, 40–42), quails (21, 53), and rats (23, 54, 64, 66) are all encoded by multigene families. Further diversity results from the presence of

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alternative transcriptional promoters (2, 9, 22) and alternative splicing of the primary gene transcripts (2, 9, 21–23, 30, 36, 40, 41, 54). The tropomyosins of rats are expressed from at least three genes, including α-TM (54, 64), β-TM (23), and the gene encoding fibroblast TM-4 (66). In humans, a fourth gene termed TM_{nm} has been characterized in addition to the three genes equivalent to those found in rats (9). Prior to this study, the rat α-TM gene was shown to possess three sets of mutually exclusive alternate exons (54, 64) encoding amino acids 39 to 80, amino acids 189 to 213, and amino acids 258 to 284. Alternative splicing of the α-TM gene transcript results in mRNAs that encode a distinct tropomyosin in striated muscle and smooth muscle (54, 55). In addition, RNA protection studies have indicated the presence of at least three alternate mRNAs in nonmuscle cells (64). We have recently found that this gene also encodes four isoforms in rat fibroblasts termed TM-2, TM-3, TM-5a, and TM-5b (L. O. Goodwin, J. P. Lees-Miller, and D. M. Helfman, manuscript in preparation).

In this study, we characterize three tropomyosin mRNAs that are products of the α-TM gene and show that they are detected only in brain. They encode isoforms that are 245, 251, and 281 amino acids in length and that differ from each other and striated muscle α-tropomyosin only at their NH_2-terminal and COOH-terminal regions. Their expression involves transcription initiation from alternate promoters and the use of two previously uncharacterized splice choices located at the 3′ end of the gene. We have also determined the location of the exons encoding the novel 5′ and 3′ ends of the brain tropomyosin mRNAs on the α-TM gene map.

MATERIALS AND METHODS

Genomic and cDNA clones. The cDNA clones were isolated from a rat brain cortex cDNA library constructed in a pcD vector (51). The library was provided by Hiroto Okayama. It was screened with a ^{32}P-labeled cDNA insert that was prepared by nick translation that contained the coding sequence for amino acids 96 to 284 and the 3′-untranslated region of rat striated muscle α-tropomyosin. Hybridization conditions were as described previously for screening a rat fibroblast cDNA library (23). Three 20-min washes were carried out in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C. Eighteen positive clones were isolated; 3 of these cDNA clones, pOk4, pOk10 and pOk15, are described in this paper.

The genomic clones used to map α-TM gene structure, λ-TM2 and λ-TM4, have been previously described (54). They were provided by Christopher Smith and Bernardo Nadal-Ginard. All clones were mapped with restriction digests and Southern blotting (60).

DNA sequencing. Restriction fragments of cDNA and genomic clones were subcloned into bacteriophage plasmids M13mp18 and M13mp19 (67), with JM101 as a host bacterial strain. All sequencing reactions were carried out by the dideoxy chain termination method (57), as modified in the Sequenase (United States Biochemical Corp.) kit protocol. All novel coding sequences and areas containing compressions were sequenced on both strands.

Subcloning brain tropomyosin cDNAs into the pGEM-4 plasmid. We obtained a full-length striated muscle α-tropomyosin cDNA inserted into the *Eco*RI site of pGEM-4 (Promega Biotec) from Bernardo Nadal-Ginard. It contains 63 bases of 5′-untranslated sequence and an *Nco*I restriction site at the AUG initiation codon. We replaced sequences downstream of the translation initiation site with an *Nco*I-*Xba*I fragment of pOk4 (see Fig. 2) to make pG4-Ok4 and a *Sty*I-*Sty*I fragment of pOk15 to make pG4-Ok15. These constructs contained the complete coding sequences for TMBr-1 and TMBr-2, respectively. We then used a common *Sac*I restriction site at the codon for amino acid 184 of TMBr-1 and amino acid 148 of TMBr-2 to replace the 5′ end of pG4-Ok4 with that of pG4-Ok15 to make pG4-Ok154, which encoded TMBr-3.

RNase protection. ^{32}P-labeled antisense transcripts were synthesized with T7 RNA polymerase by using cDNA inserts cloned into pGEM-4 as templates. RNA transcription reactions were carried out with an in vitro transcription kit from Promega. DNA templates were digested with DNase I (Promega). RNA protection experiments were carried out as previously described (68). The antisense transcripts (50,000 cpm) were hybridized with total tissue RNA, which had been isolated by the guanidinium-cesium chloride centrifugation method (38), in 30 μl of hybridization solution containing 80% formamide, 0.4 M NaCl, and 10 mM 1,4-piperazinediethanesulfonic acid (pH 6.4) at 45°C for 6 h. Transcripts that did not hybridize were digested with RNases A (Worthington Diagnostics) and T_1 (Pharmacia, Inc.) at 20°C for 1 h. Protein was digested with proteinase K (Bethesda Research Laboratories, Inc.). Protected transcripts were separated on a 5% polyacrylamide gel containing 8 M urea and detected by autoradiography.

RESULTS

Isolation and characterization of cDNA clones encoding brain α-tropomyosins. We determined the sizes of mRNAs expressed from the rat α-TM gene in rat brain, uterus, kidney, and skeletal muscle by Northern blot analysis (Fig. 1). The blot was probed with a ^{32}P-labeled cDNA that contained sequences common to all known rat α-TM gene mRNAs. The 1.3-kilobase (kb) message detected in skeletal muscle and the 1.8-kb message seen in uterus corresponded, respectively, to the striated muscle and smooth muscle α-tropomyosins described previously (54, 55). Because of alternative splicing, the 1.8-kb bands detected with kidney and brain RNAs represented a heterogeneous population of α-tropomyosin mRNAs, including those encoding TM-2, TM-3, TM-5a and TM-5b (see Fig. 8). Interestingly, rat brain RNA was found to contain a 3.0-kb mRNA, in addition to the 1.8-kb message, that hybridized to α-tropomyosin sequences. To analyze the structure of the 3.0-kb message, we isolated corresponding cDNA clones from a cDNA library made from rat brain cortex mRNA. At least three classes of clones were identified based on restriction enzyme analysis and differential hybridization under various conditions of stringency. cDNA clones for each of these three classes are shown in diagrammatic form in Fig. 2. Their DNA and derived amino acid sequences are shown in Fig. 3 and 4, respectively.

The first class of cDNA clones isolated is represented by pOk10, which contains an insert 2.5 kb in length. Sequence analysis of pOk10 (Fig. 3) indicated that it encodes a sequence identical to skeletal muscle α-tropomyosin from amino acids 82 to 257 (54, 55) but encodes a novel COOH terminus that extends 24 residues past amino acid 257, as compared with the 27 residues found in other tropomyosins. The 3′-untranslated region of pOk10 extends through two potential polyadenylation signals and then continues on to include the COOH-terminal coding sequence for amino acids 258 to 284 and 3′-untranslated region of the smooth muscle and fibroblast α-tropomyosins (Fig. 3).

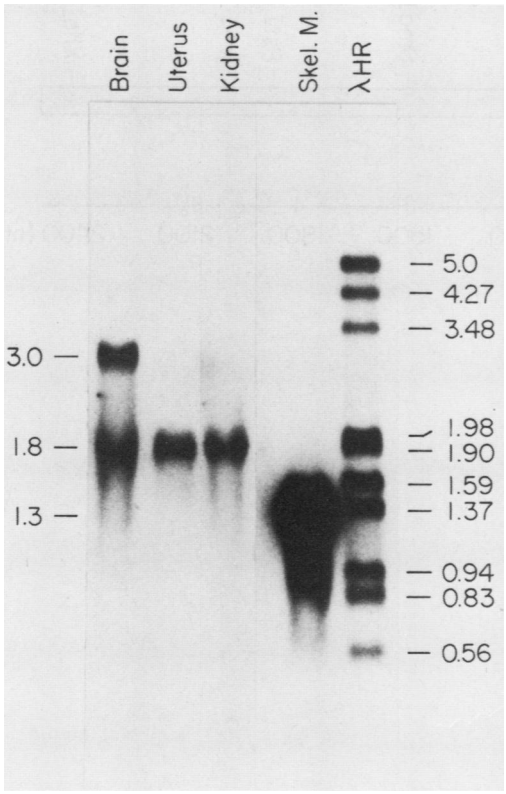

FIG. 1. Detection of RNA coding for α-tropomyosins in adult rat tissues. Total RNA was prepared from adult brain, uterus, kidney, and skeletal muscle (Skel. M.). The total RNA in brain (15 μg), uterus (3 μg), kidney (15 μg), and skeletal muscle (3 μg) was separated on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated ^{32}P-labeled probe prepared from cDNA insert pG4-Ok154, which encodes TMBr-3 (see Fig. 4). The filter was washed in 0.15× SSC containing 0.1% SDS at 60°C and autoradiographed for 2 days with Cronex Lighting-Plus X-ray intensifying screens (Dupont, NEN Research Products). λ DNA digested with *Hin*dIII and *Eco*RI (λHR) and end labeled with ^{32}P was used for approximate sizing of mRNAs. The restriction fragment sizes are indicated in kilobases on the right. The approximate sizes of α-tropomyosin mRNAs are indicated on the left in kilobases.

The second class of clones is represented by pOk4, which is 1.1 kb long and encodes a full-length (281-amino-acid) tropomyosin molecule, herein referred to as TMBr-1 (Fig. 2, 3, and 4). Clone pOk4 contains 40 nucleotides (nt) of 5′-untranslated sequence which is 90% identical to the same region of the striated muscle α-tropomyosin mRNA (54), followed by a region identical to the coding sequence of the striated muscle mRNA from amino acids 1 to 257. The COOH-terminal coding region is identical to that in clone pOk10, although pOk4 contains a relatively short (168-nt) 3′-untranslated sequence due to the use of a polyadenylation signal (attaaa) different from that used in pOk10.

The third class of cDNA clone identified is represented by pOk15, which is 1.1 kb in length and encodes a full-length tropomyosin (251 amino acids), herein referred to as TMBr-2 (Fig. 2, 3, and 4). Clone pOk15 contains 136 nt of 5′-untranslated sequence and 115 nt of 3′-untranslated sequence which terminates in a poly(A) tract beginning 20 base pairs downstream of a polyadenylation signal (aataaa). TMBr-2 possesses a 44-residue amino-terminal sequence unlike that of muscle α-tropomyosins but similar to that of fibroblast TM-4 (see Fig. 9). From amino acid residues 45 to 221, it is identical to the muscle α-tropomyosins over the region encoding amino acids 81 to 257 (54, 55) but contains a novel 30-amino-acid COOH terminus different from those of all known tropomyosin isoforms.

RNase protection analysis of mRNAs transcribed from pOk4 and pOk15 (see Fig. 5) indicated that a third α-tropomyosin mRNA containing the 5′ end of pOk15 and the 3′ end of pOk4 was abundant in brain. We therefore constructed a plasmid, pG4-Ok154, encoding this message (see Materials and Methods). We refer to the protein encoded by pG4-Ok154 as TMBr-3. It is 245 amino acids in length and is identical to TMBr-2 from amino acids 1 to 222 but contains the COOH-terminal 24 amino acids of TMBr-1 (Fig. 4).

The cDNA clones pOk15, pOk4, and pG4-Ok154 were transcribed and translated in vitro to determine the apparent M_rs of the brain α-tropomyosins on SDS-polyacrylamide gels (data not shown). They are 36,000 for TMBr-1, 31,000 for TMBr-2, and 31,000 for TMBr-3. The M_rs of the brain α-tropomyosins, as calculated from amino acid compositions, are 32,477 for TMBr-1, 28,678 for TMBr-2, and 28,315 for TMBr-3.

Tissue distribution of the α-TM gene-encoded mRNAs. RNase protection analysis was performed to estimate the relative abundance of the various α-tropomyosin messages in brain, kidney, liver, uterus, and skeletal muscle. Since the α-TM gene is a complex transcription unit, the mRNAs that can contribute to the protection pattern include those for TMBr-1, TMBr-2, TMBr-3, smooth muscle α-tropomyosin, striated muscle α-tropomyosin (54, 64), and fibroblast TM-2, TM-3, TM-5a, and TM-5b (Goodwin et al., in preparation).

The antisense probe for TMBr-1 was 1,030 nt in length and was complementary to 843 nt of coding sequence, 85 nt of 3′-untranslated sequence, 63 nt of 5′-untranslated sequence, and 39 nt of vector sequence. Full-length protection of the TMBr-1 sequence (991 nt) was detected only in brain, where it represented less than 5% of the α-tropomyosin message (Fig. 5a). Two protected bands of greater abundance were also found in brain. These included a 685-nt fragment that resulted from hybridization to TMBr-3 mRNA and a 533-nt fragment that was also present in the uterine RNA protection assay. The latter fragment corresponded to hybridization of the coding sequence for amino acids 81 to 258 of smooth muscle α-tropomyosin. Other α-TM gene mRNAs, including TMBr-2 and fibroblast TM-5a (see Fig. 8), could also give the same length protection. Skeletal muscle RNA yielded a protected product of 836 nt that corresponded to the 5′-untranslated sequence and the coding sequence for amino acids 1 to 258 of the striated muscle α-tropomyosin transcript (Fig. 5a). Fibroblast TM-2 was expected to yield a protected fragment of a similar length. The major band in kidney was 324 nt in length. It represented hybridization of the coding sequence for amino acids 45 to 152 of fibroblast TM-5b (Goodwin et al., in preparation). A second protected fragment of 627 nt in kidney resulted from the fibroblast TM-3 message, including 63 nt of the 5′-untranslated sequence and the coding sequence for amino acids 1 to 188. The presence of TM-5b and TM-3 also resulted in a protected fragment of 137 nt in kidney corresponding to the coding sequence for amino acids 178 to 222 and 214 to 258, respectively.

The antisense probe for TMBr-2 was 891 nt in length and was complementary to 753 nt of coding sequence, 36 nt of 3′-untranslated sequence, and 100 nt of vector sequence. Full-length protection of the TMBr-2 sequence (791 nt, 2 of which are homologous to the vector sequence) was detected

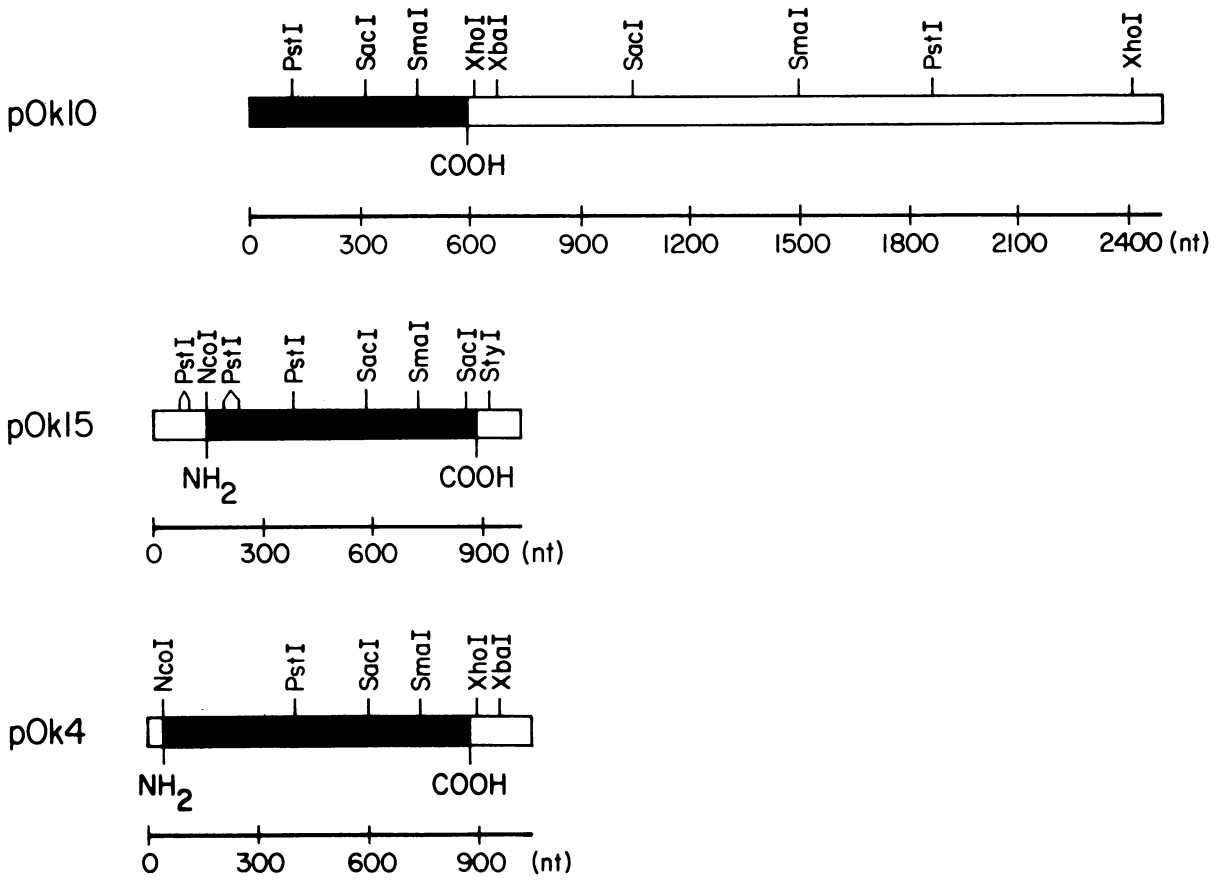

FIG. 2. Schematic representation of cDNA clones pOk10, pOk4, and pOk15. The coding sequences are represented by black bars. Restriction enzyme sites are indicated above the clones.

only in brain (Fig. 5b) and was less than 5% of the RNA protection in brain. The major protected band was 667 nt in length and corresponded to the coding region for amino acids 1 to 222 of TMBr-3 and fibroblast TM-5a. A second major band of 533 nt was also found in skeletal muscle and uterus. It corresponded to the hybridization of the coding sequence for amino acids 81 to 258 of striated muscle α-tropomyosin, smooth muscle α-tropomyosin, and fibroblast TM-2. Note the presence of a 125-nt doublet in skeletal and cardiac tissues. This band resulted from the hybridization of 89 nt of the TMBr-2 coding sequence (amino acids 222 to 251) and 36 nt of the 3′-untranslated sequence identical to the 3′-untranslated sequence of striated muscle α-tropomyosin. In kidney and liver, the major protected product was 458 nt in length and corresponded to the coding sequence for amino acids 1 to 152 of fibroblast TM-5b. In adenovirus-transformed rat embryo fibroblast cells, the major protected fragment was 327 nt in length and represented the coding sequence for amino acids 81 to 188 of fibroblast TM-3.

The antisense probe for TMBr-3 was 922 nt in length and was complementary to 735 nt of coding sequence, 85 nt of 3′-untranslated sequence, and 100 nt of vector sequence. Full-length protection of the TMBr-3 sequence (822 nt, 2 of which are homologous to the vector sequence) was detected only in brain, where it represented approximately 50 to 60% of the total α-TM gene mRNA protection (Fig. 5c). A minor protected band of 688 nt in brain corresponded to the message for TMBr-1. An abundant band of 667 nt resulted from the hybridization of TMBr-2 and fibroblast TM-5a mRNAs over the coding region from amino acids 1 to 222. Protection of the coding sequence from amino acids 45 to 222 (533 nt) was found in uterus, skeletal muscle, and kidney, where it corresponded to protection of transcripts for striated muscle α-tropomyosin, smooth muscle α-tropomyosin, and fibroblast TM-2. Protection of the coding sequence for amino acids 1 to 152 (458 nt) of fibroblast TM-5b was observed in liver and kidney.

Mapping of the α-TM gene. The α-TM gene has been

FIG. 3. nt sequences of cDNA clones encoding rat brain α-tropomyosins. The sequences listed are from cDNA clones selected from a rat brain cortex library constructed in the pcD vector (51). They were selected with a clone encoding rat skeletal muscle α-tropomyosin, subcloned into M13, and sequenced by a modified dideoxy chain termination method (57). The amino-terminal coding regions of pOk4 and pOk15 are aligned on the basis of sequence identities and gene structure. Such an alignment leads to a 126-nt gap in the sequence of pOk15 corresponding to the absence of exon 2b, indicated by a blank space. Clone pOk10 begins in the codon corresponding to amino acid 81 of long or 45 of short α-tropomyosins. Identities to pOk15 are indicated by dashes. Putative polyadenylation signals attaaa and aataaa are underlined. The coding sequence for the COOH terminus of smooth muscle α-tropomyosin (54) is presented in lowercase boldface type within the 3′-untranslated region of pOk10.

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pOk15                       cttagccgcaggggcgccggcgttgcaaagcgggcctgggcgggacgaaccggacc -80

pOk15 tggctccgtaactgcaggagcccagcagtagcagctgcagagcgcggaggcagagcacagcctcagccgccgcctcgcc -1
pOk4                                          ctctggctcctctgcctaagggcccctcgccaccgccacc -1

pOk15 ATG GCG GGT AGC AGC TCG CTG GAG GCG GTG CGG AGA AAG ATC CGG AGC CTG CAG GAA CAG 60
pOk4                        ATG GAC GCC ATC AAG AAG AAG ATG CAG ATG CTG AAG CTC GAC 42

pOk15 GCA GAT GCC GCG GAG GAG CGT GCG GGC AGC CTG CAG CGA GAG CTG GAC CAG GAG CGG AAG 120
pOk4  AAA GAG AAC GCC TTG GAT CGA GCA GAG CAG GCG GAG GCT GAC AAG AAG GCT GCG GAA GAC 102

pOk15 CTG CGG GAA ACC                                                              132
pOk4  CGG AGC AAG CAG CTG GAA GAT GAG CTG GTG TCA CTG CAA AAG AAA CTC AAG GGC ACT GAA 162

pOk15
pOk4  GAT GAA CTG GAC AAA TAC TCC GAG GCT CTC AAA GAT GCC CAG GAG AAA CTG GAG CTG GCG 222

pOk10                           - --- --- --- --- --- --- --- --- --- --- --- --- --- 40
pOk15                       GCT GAA GCT GAC GTA GCA TCT CTG AAC AGA CGC ATC CAG CTG 174
pOk4  GAG AAA AAG GCC ACA GAT --- --- --- --- --- --- --- --- --- --- --- --- --- --- 282

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 100
pOk15 GTT GAG GAG GAG TTG GAT CGC GCT CAG GAG CGT CTG GCC ACA GCT CTA CAG AAG CTG GAG 234
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 342

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 160
pOk15 GAG GCT GAG AAG GCT GCA GAT GAG AGT GAG AGA GGC ATG AAA GTC ATT GAA AGC CGA GCC 294
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 402

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 220
pOk15 CAA AAA GAT GAA GAA AAG ATG GAG ATT CAG GAG ATC CAG CTG AAA GAG GCC AAG CAC ATT 354
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 462

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 280
pOk15 GCT GAA GAT GCT GAC CGA AAG TAT GAA GAG GTG GCC CGT AAG CTG GTC ATC ATC GAG AGC 414
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 522

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 340
pOk15 GAT CTG GAG CGT GCG GAG GAG AGG GCT GAG CTC TCG GAA GGC AAA TGT GCC GAG CTT GAA 474
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 582

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 400
pOk15 GAA GAG TTG AAA ACG GTG ACG AAC AAC TTG AAG TCA CTG GAG GCT CAG GCT GAG AAG TAC 534
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 642

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 460
pOk15 TCT CAG AAA GAA GAC AAG TAT GAA GAG GAG ATC AAG GTT CTC TCT GAC AAG CTG AAG GAG 594
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 702

pOk10 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 520
pOk15 GCT GAG ACC CGG GCT GAG TTT GCA GAG AGA TCA GTA ACC AAA TTG GAG AAA AGC ATT GAT 654
pOk4  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 762

pOk10 --- --- --- -AT CAA CTC TAC CAT CAA CTC GAG CAA AAC CGC CGT CTA ACT AAT GAA CTA 580
pOk15 GAC TTA GAA GAT AAG TTT CTT TGC TTC TCT CCT CCC AAG ACT CCT TCA TCA AGC CGG ATG 714
pOk4  --- --- --- -AT CAA CTC TAC CAT CAA CTC GAG CAA AAC CGC CGT CTA ACT AAT GAA CTA 822

pOk10 AAG CTG GCC CTG AAT GAG GAT TAA aaccctgggccaagttctaggaacggagtccatgttcagaaaatcta 651
pOk15 TCC CAC CTC TCT GAG CTC TGC ATC TGT CTG CTC TCC AGC TGA cccaggtttctttctagtgccca 779
pOk4  AAG CTG GCC CTG AAT GAG GAT TAA aaccctgggccaagttctaggaacggagtccatgttcagaaaatcta 895

pOk10 tgactctcggggttaaaaaatgataacttctagagaaatgggaaggaaatgcattggctggaaggccagccttgcctat 730
pOk15 cccaccctagggccaggcacagaccgtgctttctattgtacagaggtgatcctcccagtgtaaaataaacactgtacgc 858
pOk4  tgactctcggggttaaaaaatgataacttctagagaaatgggaaggaaatgcattggctggaaggccagccttgcctat 974

pOk10 gttttcctctgtatatcttaattaaatcttcaataaccaagaagtttttatgatacattatgtatatgggaaaatattt 809
pOk15 tagtcctgtt(poly A)
pOk4  gttttcctctgtatatcttaattaaatcttcaataacc(poly A)

pOk10 ataccaatcaattcagctcttcctgcatcagcaggccctctttgaagtgatgataatgaggactcccccaccaccacca 888
      tccccccaaaaaaaggaaaaaattttctatttcctctaaacagctgtgagtcctgctttaaatatctcttctacatttg 967
      tattcctaacatcctcctaacatgtatagtgtttgaaatgtgatccaacgtgtacctatgcttgggcaaaatagctttt 1046
      gaaaacaggagctcgtaccacaagtccctggttgaaaggtatgctttgttaagtttaacggtgttggaatctgattatc 1125
      gtgtcatgctgttaagcgggatactataaaaagactaagagaatgtccctaatgaagtgtgcatgagatgtgttcatat 1204
      aaccatttgcgagccgtagaaataaatctatttaaaaagtggtcagaaccttccttacgtcatcttcgttttctgtgag 1283
      gtcgtgttctgacccacctacccgatctttaactgcctgtaactcagaatcctgtgctggtattaacgtcttcttcggt 1362
      ctgtgtaacaatgctgtacctgtatttggtttttgaccctcccttgttccccgtagcacgcagtgggtgagccttcacc 1441
      aaacacaccctgcacgttacgtcagggtgggttgcaggtgtaggtgcacccggggccttgtgtagatgtgagtgacttc 1520
      caaagcatgtgtagtgccactcatctgtgtgtgactggtgtttgcttactaaatgtacaaaccactaccgagtgtgaag 1599
      ctcagccattcatgactcaatttttctgatcccatcacagagaaagtggcacatgccaaagaagaaaaccttagtatgca 1678
      ccagatgctggaccagactttactggagctaaacaacatgtgaaaccctccttagctgcggccacattctctcgtttcc 1757
      ccctgttgttttattttgtctttaaacacatgcttaccatgaaaccccttcaatgcttttttttttttatatatatatat 1836
      acctttaccactgtcactgcaacatctgcagagagccagctagggcaggggtggggaaagacacacagaaagcaggtg 1915
      tacttgttatttaaatgtgccatttcccaggttgacattgccacacttcacagagtttaggcatgcagtttgcttagcc 1994
      agtgtaggaagcctcacgaaaacaaaaggattaacagcctaagtgccaatggtggggtcatcaggaagactgatgttgg 2073
      agacaatcaggtgcagattggtgctaatttaaacaaaagaaccccctggtgtcttttgttcagtctaatacaatttagac 2152
      ttctgtccaacactaatttatttttgtcttgagtagcactgcgagaggatatgggttccatgtgcccgtgttcctcaca 2231
      gccaagggtttgaaagccacgctggtcttgtaagattcccattccttcccagctgacatgcttgtggtcccttccaact 2310
      cagtggggagagggctcagtgctgttcgccccccccccccccaattctccatgtcagggtaagagatctaggcactaca 2389
      tatgtaattggtaaagaaaaggcattttctcgagagttataactatgtaaacattgtataatgatatgaaataaaatgc 2468
      acattgtaggacattttcta(poly A)
```

```
       1
TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Met Ala Gly Ser Ser Ser Leu Glu Ala Val Arg Arg Lys Ile Arg Ser Leu Gln Glu Gln
TMBr-1                         Met Asp --- Ile Lys Lys --- Met Gln Met --- Lys Leu Asp
                                1

TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Ala Asp Ala Ala Glu Glu Arg Ala Gly Ser Leu Gln Arg Glu Leu Asp Gln Glu Arg Lys
TMBr-1 Lys Glu Asn --- Leu Asp --- --- Glu Gln Ala Glu Ala Asp Lys Lys Ala Ala Glu Asp

                   44
TMBr-3 --- --- --- ---
TMBr-2 Leu Arg Glu Thr
TMBr-1 Arg Ser Lys Gln Leu Glu Asp Glu Leu Val Ser Leu Gln Lys Lys Leu Lys Gly Thr Glu
                        39

TMBr-3
TMBr-2
TMBr-1 Asp Glu Leu Asp Lys Tyr Ser Glu Ala Leu Lys Asp Ala Gln Glu Lys Leu Glu Leu Ala

                               45
TMBr-3                         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2                         Ala Glu Ala Asp Val Ala Ser Leu Asn Arg Arg Ile Gln Leu
TMBr-1 Glu Lys Lys Ala Thr Asp --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                               81

TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Val Glu Glu Glu Leu Asp Arg Ala Gln Glu Arg Leu Ala Thr Ala Leu Gln Lys Leu Glu
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

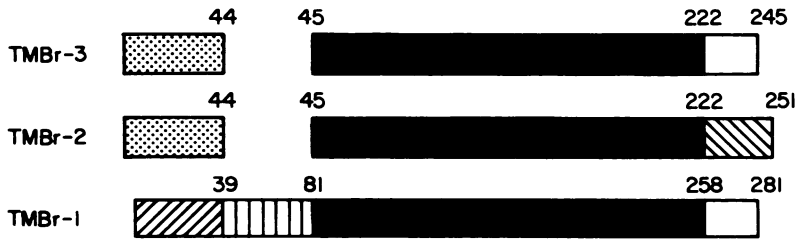
                                           89
TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Glu Ala Glu Lys Ala Ala Asp Glu Ser Glu Arg Gly Met Lys Val Ile Glu Ser Arg Ala
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                           125

TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Gln Lys Asp Glu Glu Lys Met Glu Ile Gln Glu Ile Gln Leu Lys Glu Ala Lys His Ile
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                           129
TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Ala Glu Asp Ala Asp Arg Lys Tyr Glu Glu Val Ala Arg Lys Leu Val Ile Ile Glu Ser
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                           165
                                                       152
TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Asp Leu Glu Arg Ala Glu Glu Arg Ala Glu Leu Ser Glu Gly Lys Cys Ala Glu Leu Glu
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                       188
                                                                                   179
TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Glu Glu Leu Lys Thr Val Thr Asn Asn Leu Lys Ser Leu Glu Ala Gln Ala Glu Lys Tyr
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                   214

TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Ser Gln Lys Glu Asp Lys Tyr Glu Glu Glu Ile Lys Val Leu Ser Asp Lys Leu Lys Glu
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

       199
TMBr-3 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TMBr-2 Ala Glu Thr Arg Ala Glu Phe Ala Glu Arg Ser Val Thr Lys Leu Glu Lys Ser Ile Asp
TMBr-1 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       235
                   222
TMBr-3 --- --- --- --- Gln Leu Tyr His Gln Leu Glu Gln Asn Arg Arg Leu Thr Asn Glu Leu
TMBr-2 Asp Leu Glu Asp Lys Phe Leu Cys Phe Ser Pro Pro Lys Thr Pro Ser Ser Ser Arg Met
TMBr-1 --- --- --- --- Gln Leu Tyr His Gln Leu Glu Gln Asn Arg Arg Leu Thr Asn Glu Leu
                   258
                                  245                  251
TMBr-3 Lys Leu Ala Leu Asn Glu Asp
TMBr-2 Ser His Leu Ser Glu Leu Cys Ile Cys Leu Leu Ser Ser
TMBr-1 Lys Leu Ala Leu Asn Glu Asp
                                  281
```



mapped and found to express striated muscle α-tropomyosin and a number of other tropomyosin isoforms via an alternative splicing mechanism (54, 64). From our analysis of brain tropomyosin cDNA clones, we noted the presence of three new sequences, encoding one NH_2- and two COOH-terminal regions, that had not been mapped in the existing gene structure. We subsequently determined the position of the exons encoding these sequences in the α-TM gene by comparison of the brain cDNA sequences and genomic subclones (Fig. 6).

Sequences corresponding to the 5'-untranslated region and amino-terminal coding region (amino acids 1 to 44) of TMBr-2 (cDNA clone pOk15) are contained in exon 1b. This exon is located between exon 2b and exon 3 (Fig. 6). The sequence of this exon, including its upstream promoter elements, is shown in Fig. 7. The region from −1 to −350 is 72% G+C. Binding sites for the transcription factor Sp1 (ccgccc or gggcgg) are located at −95, −185, and −225 relative to the translation initiation codon (15). Two putative CCAAT elements are present within the G+C-rich region at −250 and −290. The CCAAT element is also known to bind proteins that regulate transcription (8). A third putative CCAAT box is present at −540, but no TATA element is identifiable downstream of any of the CCAAT sequences. From sequencing of cDNAs, S1 protection assays, and RNase protection assays, we have detected what appear to be multiple transcription initiation sites between positions −140 and −200 (data not shown). Strong primer extension stops have prevented unequivocal confirmation of these sites by primer extension.

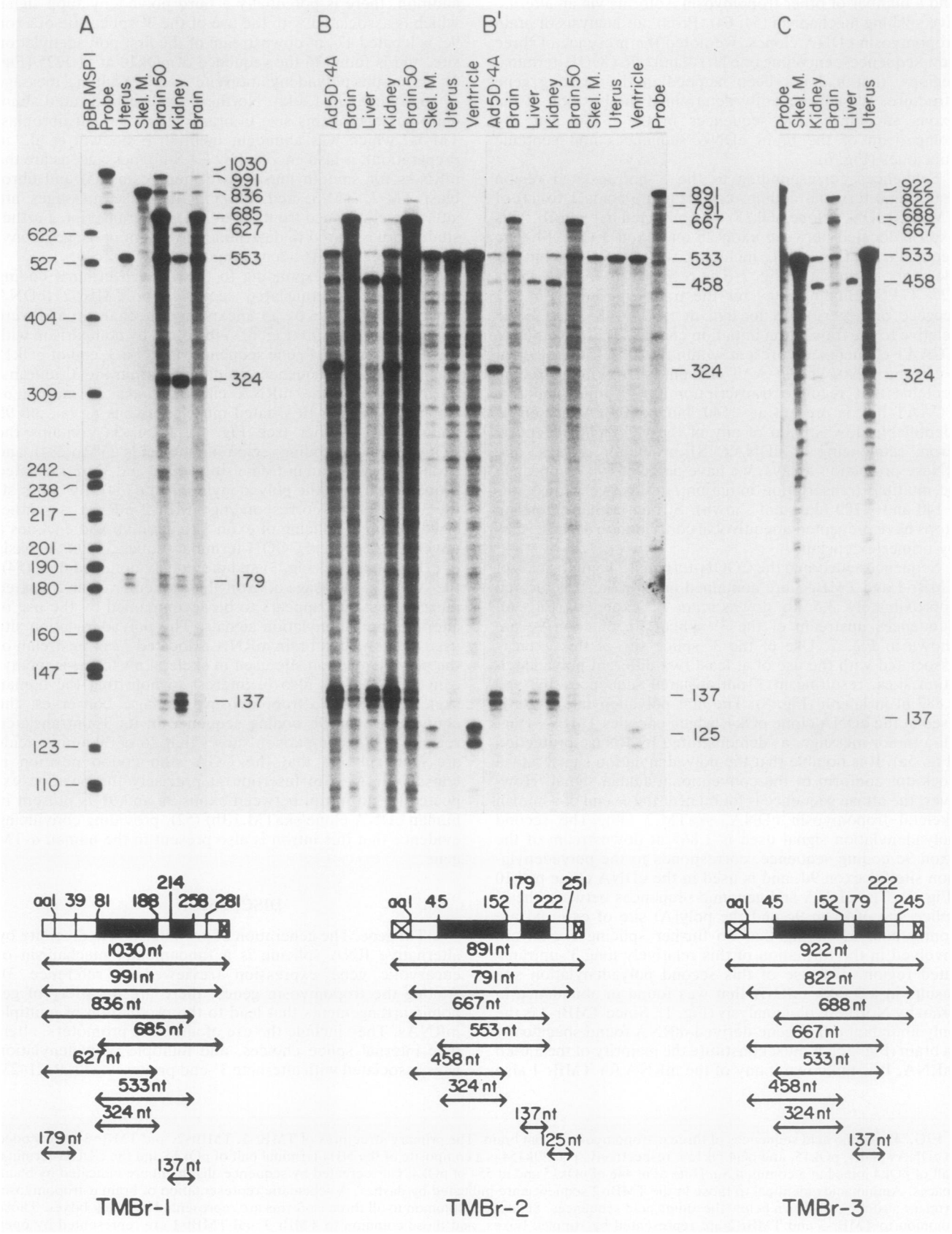
Sequences encoding the COOH-terminal 24 amino acids of TMBr-1 and TMBr-3 are contained in exon 9c. It is located approximately 3.5 kb downstream of exon 9b (Fig. 6). Sequences upstream of the 3' splice site of exon 9c are shown in Fig. 7. Use of the 3' splice site of this exon is associated with the use of at least two different polyadenylation sites, resulting in 3'-untranslated sequences 168 and 1,887 nt in length (Fig. 3). The first polyadenylation site is used in the cDNA clone pOk4, which encodes TMBr-1 (Fig. 3), a minor message, as demonstrated by RNase protection (Fig. 5a). It is notable that the polyadenylation signal attaaa does not conform to the conventional aataaa signal. However, the attaaa sequence is found near the 3' end of a human skeletal tropomyosin cDNA, skaTM.2 (40). The second polyadenylation signal used is 1,865 nt downstream of the exon 9c coding sequence, corresponds to the polyadenylation site for exon 9d, and is used in the cDNA clone pOk10 (Fig. 3). The cDNA and genomic sequences between the 3' splice site of exon 9c and the poly(A) site of exon 9d are contiguous, indicating that no further splicing events are involved in the formation of this relatively long 3'-untranslated region. The use of this second polyadenylation site results in a 3.0-kb mRNA that was found in abundance in brain by Northern blot analysis (Fig. 1). Since TMBr-3 is the only abundant α-TM gene-derived mRNA found specifically in brain (Fig. 5c), it must constitute the majority of the 3.0-kb mRNA. It is unknown if any of the mRNA for TMBr-1 also contains this relatively long 3'-untranslated sequence. In addition, there is potentially a third polyadenylation signal which is associated with the use of the 3' splice site of exon 9c, is located 474 nt downstream of the first polyadenylation site, and is found in the sequence of pOk10 at nt 1225 (Fig. 3). Use of this polyadenylation site would result in a message of approximately 1.8 kb. Northern analysis indicated abundant message of this size in brain. However, rat fibroblast TM-5a, which was abundant in brain (Goodwin et al., in preparation), is also encoded by a 1.8-kb message, as are the mRNAs for smooth muscle α-tropomyosin (55) and fibroblast TM-2, TM-3, and TM-5b. All these messages are alternatively spliced from the α-TM gene transcript. Further studies are required to determine the extent of usage, if any, of this potential polyadenylation site.

Sequences corresponding to the COOH-terminal coding region and 3'-untranslated sequence of TMBr-2 (cDNA clone pOk15) arose by an unexpected mechanism and were contained in exon 9b (Fig. 6). We noted, by comparison with the published α-TM gene sequence, that the 3' end of pOk15 was identical to sequences contained within the 3'-untranslated region of the mRNA encoding striated muscle α-tropomyosin (55). In striated muscles, exons 8, 9a, and 9b are spliced together (see Fig. 8). Exon 9a contains the COOH-terminal coding region (amino acids 258 to 284), and exon 9b provides an in-frame stop codon and 3'-untranslated sequence through the polyadenylation site (54). By contrast, the 3' end of the corresponding TMBr-2 mRNA is synthesized by direct splicing of exon 8 to exon 9b and encodes a novel 30-amino-acid COOH-terminal sequence. On the basis of cDNA analysis (Fig. 3) and previously obtained data (54), the switch in the usage of exon 9b between brain and striated muscle messages appears to be accompanied by the use of alternate polyadenylation signals. The polyadenylation site used in exon 9b in brain mRNA is located 77 nt upstream of the polyadenylation site used in skeletal muscle α-tropomyosin mRNA. It is also of interest to note that the human skeletal muscle α-tropomyosin message conserves the equivalent exon 9b coding sequence in its 3'-untranslated region (40). A comparison shows that 26 of 30 amino acids are identical and that the UGA stop codon position is conserved. A 48-nt insertion at precisely the position expected for an intron between exons 9a and 9b is present in human cDNA clone skaTM.1(fb) (52), providing convincing evidence that this intron is also present in the human α-TM gene.

DISCUSSION

α-TM gene. The generation of protein isoform diversity by alternative RNA splicing is a fundamental mechanism of eucaryotic gene expression (reviewed in reference 3). Among the tropomyosin genes, there are a variety of genomic arrangements that lead to the production of multiple mRNAs. They include the use of alternate promoters, alternate internal splice choices, and multiple polyadenylation sites associated with alternate 3'-end processing (2, 9, 21–23,

FIG. 4. Amino acid sequences of three α-tropomyosins from brain. The primary structures of TMBr-1, TMBr-2, and TMBr-3 are encoded in cDNAs pOk4, pOk15, and pG4-Ok154, respectively. pG4-Ok154 is a composite of the NH_2-terminal half of pOk15 and the COOH-terminal half of pOk4 joined at a common *Sac*I site at nt 446 of pOk15 and nt 554 of pOk4. Gaps created by sequence alignment are indicated by blank spaces. Amino acids identical to those in the TMBr-2 sequence are indicated by dashes. A schematic representation of brain α-tropomyosin primary structure is shown below the amino acid sequences. Sequences common to all three isoforms are represented by black boxes. Those common to TMBr-3 and TMBr-2 are represented by stippled boxes, and those common to TMBr-3 and TMBr-1 are represented by open boxes. Unique sequences are represented by hatched boxes.


A
pBR MSP I
Probe
Uterus
Skel. M.
Brain 50
Kidney
Brain
1030
991
836
685
627
553
324
179
137
622
527
404
309
242
238
217
201
190
180
160
147
123
110
B
B'
Ad5D·4A
Brain
Liver
Kidney
Brain 50
Skel. M.
Uterus
Ventricle
Probe
891
791
667
533
458
324
137
125
C
922
822
688
667
533
458
324
137
214
aa1 39 81 188 258 281
1030 nt
991 nt
836 nt
685 nt
627 nt
533 nt
324 nt
179 nt
137 nt
TMBr-1
179 251
aa1 45 152 222
891 nt
791 nt
667 nt
553 nt
458 nt
324 nt
137 nt
125 nt
TMBr-2
222
aa1 45 152 179 245
922 nt
822 nt
688 nt
667 nt
533 nt
458 nt
324 nt
137 nt
TMBr-3

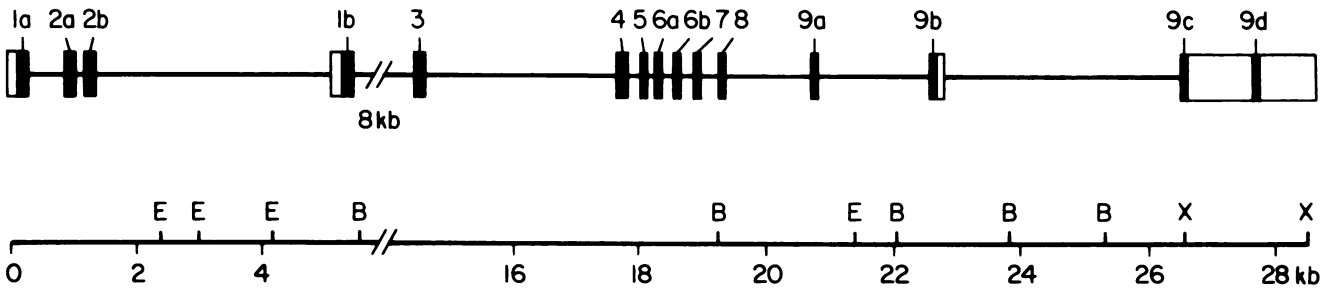


FIG. 6. Exon-intron organization of the α-TM gene. The gene is depicted in the 5'-to-3' direction and is 28 kb in length. The 15 exons of the α-TM gene are represented as black boxes and are numbered in a manner which reflects alternative splice choices. A partial structure of the rat α-TM gene has been previously presented (54, 64). We have added exons 1b and 9c to the previous maps. The amino acids encoded in each exon for high-M_r tropomyosins (281 to 284 amino acids) are as follows: 1a, 1 to 38; 2a and 2b, 39 to 80; 3, 81 to 125; 4, 125 to 164; 5, 165 to 188; 6a and 6b, 188 to 213; 7, 214 to 234; 8, 235 to 258; 9a and 9d, 258 to 284; and 9c, 258 to 281. Those for low-M_r tropomyosins (245 to 250 amino acids) are as follows: 1b, 1 to 44; 3, 45 to 89; 4, 89 to 128; 5, 129 to 152; 6a and 6b, 152 to 178; 7, 179 to 198; 8, 199 to 222; 9a and 9d, 222 to 248; 9b, 222 to 251; and 9c, 222 to 245. The positions of all *Bam*HI (B) and *Eco*RI (E) restriction sites are indicated above the distance markers. Two *Xho*I (X) sites are also indicated.

30, 36, 40–42, 54). The most complex of the vertebrate tropomyosin genes described to date is the rat α-TM gene, as presented in this study (Fig. 6 and 8). A gene possessing an even greater number of alternative splice choices has been found in *D. melanogaster* (22). The complexity of the rat α-TM gene results from alternate promoters (exons 1a and 1b), two sets of alternate internal splice choices (exons 2a and 2b and exons 6a and 6b), and five polyadenylation signals associated with four unique COOH-terminal coding sequences (exons 9a, 9b, 9c, and 9d). At least five different mRNAs are generated by alternative splicing of transcripts initiated from the exon 1a promoter, including messages for TMBr-1, TM-2, TM-3, and smooth and striated muscle α-tropomyosins, while four mRNAs initiate at the exon 1b promoter, including messages for TMBr-2, TMBr-3, TM-5a, and TM-5b (Fig. 8). There are several reports of multiple transcripts initiating from an upstream (exon 1a) tropomyosin gene promoter (23, 30, 36, 41, 53, 54). However, this appears to be the first incidence of multiple transcripts initiating from an internal (exon 1b) tropomyosin gene promoter. The α-TM gene has a total of 15 exons and can theoretically generate mRNAs for at least 24 proteins. We are now aware of nine mRNAs that are derived from the α-TM gene (Fig. 8). These mRNAs can be assigned to four size classes, including the 3.0-kb (TMBr-3), 1.8-kb (TM-2, TM-3, TM-5a, TM-5b, and smooth muscle α-tropomyosin), and 1.3-kb (striated muscle α-tropomyosin) classes and a predicted 1.2-kb (TMBr-1 and TMBr-2) class. It is clear from this study and a previous study (64) that each tissue examined expresses a limited subset of the nine known α-tropomyosin isoforms (Fig. 1 and 5). This finding implies that multiple, tissue-specific factors are required to regulate the expression of α-tropomyosin mRNAs. Some of the *cis*-acting elements involved in the regulation of tropomyosin gene alternative splicing have been elucidated (24a, 25, 59), but the *trans*-acting factors have not been characterized.

The tropomyosin gene family found in animals appears to have arisen through duplication of an ancestral gene. The primordial gene was suggested to have possessed a complex alternate splicing pattern (64). It is now clear that several tropomyosin genes contain two alternate promoters, including the human TMnm gene, the chicken β-TM gene, the rat α-TM gene, and the *D. melanogastor* TmII gene (2, 9, 22). In each gene the alternate promoters are associated with distinct NH_2-terminal coding sequences. In the rat α-TM gene, we have termed the coding sequences and their associated 5'-untranslated region exons 1a and 1b. A comparison of both the DNA and derived amino acid sequences of these exons between *D. melanogaster* and vertebrates (data not shown) reveals that exon 1a is highly conserved within the vertebrates and moderately conserved between *D. melanogaster* and the vertebrates. By contrast, exon 1b is moderately conserved within the vertebrates but highly divergent between *D. melanogaster* and the vertebrates. Exon 1a sequences are expressed within tropomyosins present in the muscle contractile apparatus, while exon 1b sequences are not. The greater divergence of exon 1b coding sequences than of exon 1a coding sequences implies that functional constraints on the evolution of the amino-terminal region of nonmuscle tropomyosins are not as severe as those placed on the evolution of their muscle counterparts.

The present study revealed two previously unidentified COOH-terminal coding exons in the rat α-TM gene, termed exons 9b and 9c. Exon 9b is found in the mRNA for TMBr-2, while exon 9c is found in the mRNAs for TMBr-1 and TMBr-3 (Fig. 8). Thus, the rat α-TM gene contains four COOH-terminal coding exons. The *D. melanogaster* TmII gene has also been reported to contain four COOH-terminal

FIG. 5. Expression of α-tropomyosin mRNAs in different rat cells and tissues. ^{32}P-labeled antisense RNAs were synthesized for TMBr-1 (A), TMBr-2 (B and B'), and TMBr-3 (C). They were incubated with 25 μg of total cellular RNA from the cells and tissues indicated. Ad5D.4A cells are rat embryo fibroblasts that have been transformed with a plasmid encoding all adenovirus type 5 E1 proteins (46). One-half of the hybridized and RNase-treated samples were electrophoresed on a 5% polyacrylamide gel. Muscle samples were diluted 1 to 25 in A, 1 to 50 in B and B', and 1 to 10 in C. Gels were exposed on X-ray film for 16 to 24 h, with the exception of B, which was exposed for 60 h so that full-length protection by the TMBr-2 mRNA would be more readily visible. Schematic representations of the antisense probes are presented beneath each autoradiograph. The predicted lengths of hybridizing fragments are indicated in nt to the right of each autoradiograph. The regions of the antisense probe that these fragments hybridized to are indicated by arrows below the probe representations. Markers were a restriction digest of pBR322 DNA with *Msp*I and are indicated in nt to the left of panel A (lane pBR MSPI). Skel. M., Skeletal muscle; Brain 50, samples that contained 50 μg of RNA.

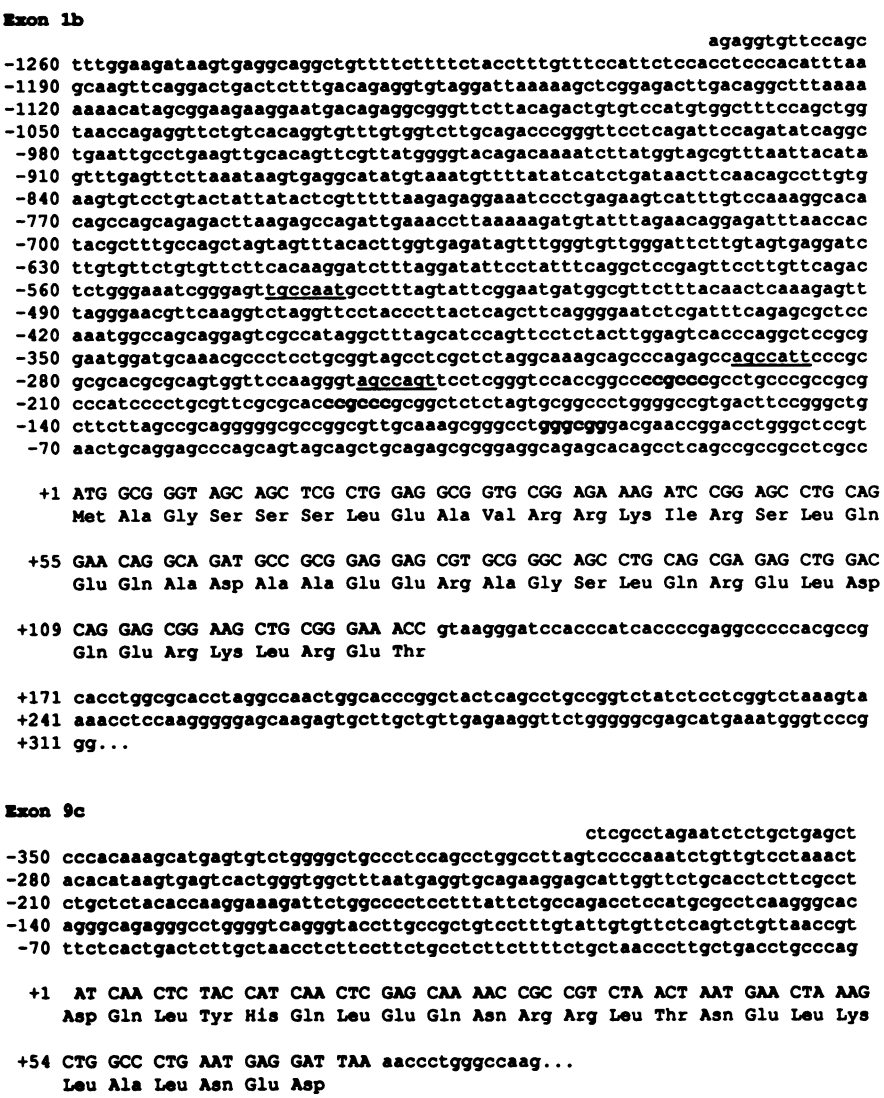


FIG. 7. Nucleotide sequence of α-TM gene exons 1b and 9c. In the exon 1b sequence, residue +1 denotes the A of the ATG presumed to encode the initiator methionine of the low-M_r tropomyosins. nt preceding this are indicated by negative numbers. The positions of three putative Sp1-binding sites (ccgccc or gggcgg) are indicated by boldface type at −95, −185, and −225. The positions of three putative CCAAT boxes are underlined at −250, −290, and −540. The nucleotide sequence of the region downstream of exon 9c is not presented but can be found in the 3′-untranslated sequence of cDNA pOk10 from nt 601 to 1642 (Fig. 3).

coding exons (22, 30). The evolution of these exons, however, appears to have followed distinctly different pathways. In the *D. melanogaster* TmII gene, exon 9a is associated with nonmuscle transcripts, while exons 9b, 9c, and 9d are expressed in striated muscles. This situation is reversed in the vertebrate α-TM gene. All vertebrate tropomyosin genes that have been characterized have been reported to encode no more than two different COOH-terminal domains. We have therefore searched for exon 9b- and 9c-like regions in the published sequences of other tropomyosin genes. The human TMnm gene contains an exon 9b-like sequence which provides a stop codon and 3′-untranslated sequence when spliced to the exon 9a equivalent present in this gene (9). However, when spliced directly to exon 8, this sequence does not encode a COOH-terminal sequence similar to that encoded by exon 9b of the rat α-TM gene. We have found an exon 9c-like sequence between exons 9a and 9d of the chicken β-TM gene (36). This sequence is flanked by a 3′ splice site consensus sequence (tag), possesses an appropriate translation stop codon, and encodes a sequence more similar to that encoded by rat exon 9c than to that encoded by rat exon 9a or 9d. It therefore appears that exon 9c may have a broad distribution within the vertebrates.

Brain α-tropomyosins. In this study we have elucidated the structure of three new tropomyosins, termed TMBr-1, TMBr-2, and TMBr-3, which are expressed in brain. The mRNAs for TMBr-1 and TMBr-2 are expressed at relatively low levels as compared with that for TMBr-3 (Fig. 5). In the present study, whole brain tissue (brain stem through cerebral cortex) was used for the RNA analysis (Fig. 1 and 5). Since brain is a heterogeneous tissue comprised of multiple cell types, it remains to be determined if these isoforms are cell type or brain region specific. In addition, the functional significance of these isoforms remains to be determined. Tropomyosins are known to be associated with filamentous actin in both muscle (52) and nonmuscle (33, 47) cell types.

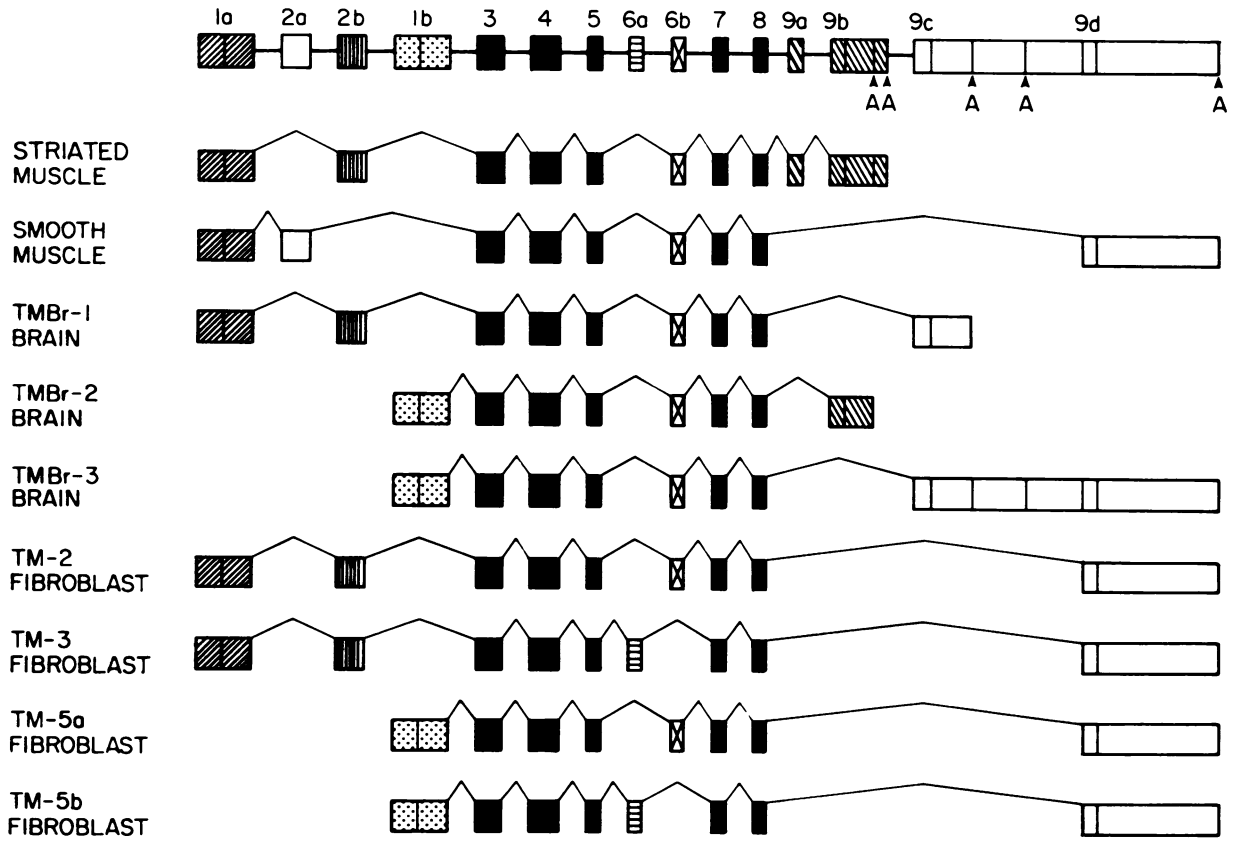


FIG. 8. Schematic representation of the organization of the α-TM gene and its associated mRNA products. Exons are represented by boxes, and introns are represented by horizontal lines. Polyadenylation signals are marked with an A. The exons are numbered from 1a through 9d to facilitate simple comparison with other tropomyosin genes. The 3'-untranslated sequence of TMBr-3 includes all of exon 9d. Cloning and characterization of TM-2, TM-3, TM-5a, and TM5b will be presented elsewhere (Goodwin et al., in preparation).

Actin-based filaments are affiliated with a variety of neural structures, such as growth cones (19), dendritic spines (45, 48), plasma membranes (26, 62), axoplasm (17), presynaptic termini (27), and postsynaptic densities (48). They are thought to play a role in motile processes such as axoplasmic transport, growth cone movements, synaptic rearrangement, and vesicle movement.

TMBr-1, TMBr-2, and TMBr-3 all share a common 177-amino-acid internal sequence with striated muscle α-tropomyosin. The structural properties of the latter 284-amino-acid isoform have been well characterized. They include two repeating patterns, one of 7 residues that is repeated 40.5-fold and is responsible for α-helical coiled-coil structure and one of 19.66 residues which is repeated 14-fold and is thought to be important for actin binding (49, 50, 58). TMBr-1 possesses a 27-amino-acid COOH-terminal sequence which differs considerably from that found in other tropomyosins (Fig. 9). Sequence conservation is, however, maintained at the first and fourth (a and d) amino acids of the 7-residue pattern, which are most critical for coiled-coil structure. The 19.66-residue pattern is not conserved in the COOH-terminal region of TMBr-1, because of the absence of appropriately located nonpolar residues on the external face of the coiled coil. TMBr-1 may therefore be found to have weaker actin-binding properties than the muscle tropomyosins.

TMBr-2 possesses a 44-residue NH_2-terminal sequence which is similar to that of TM-4 but differs greatly from the 39-residue NH_2-terminal sequence of striated muscle tropomyosin. Despite these differences, the features of the 7-amino-acid pattern required for α-helical coiled-coil structure are present (Fig. 9). The actin-binding pattern is not maintained, however. TMBr-2 also contains a unique COOH-terminal sequence which is 30 amino acids in length. This sequence does not possess the features required for helical structure and contains three prolines which are rarely found in tropomyosins (Fig. 9). Some *D. melanogaster* tropomyosins also have prolines near their ends (22, 30). The sequences encoding the COOH-terminal end of TMBr-2 (exon 9b) are also present in the 3'-untranslated region of the rat striated muscle α-tropomyosin mRNA (55). We therefore examined the 3'-untranslated region of human clone skaTM.1, which encodes a striated muscle α-tropomyosin (40), and found an appropriately located region capable of encoding a 30-amino-acid sequence highly similar to the COOH terminus of TMBr-2 (Fig. 9).

TMBr-3 has an NH_2-terminal sequence identical to that of TMBr-2 and a COOH-terminal sequence identical to that of TMBr-1. It is therefore expected to possess predominantly coiled-coil structure along its entire length and to exhibit weak actin-binding properties. Relatively weak actin binding of low-M_r brain tropomyosin mixtures as compared with that of muscle tropomyosins has been well documented (4, 6, 14, 18, 31). Other functions that may be affected by the ends of brain tropomyosin isoforms include their ability to polymerize in a head-to-tail manner and their interaction with other actin filament-associated proteins (1, 5, 29, 44, 56, 63).

In summary, this and previous studies have demonstrated

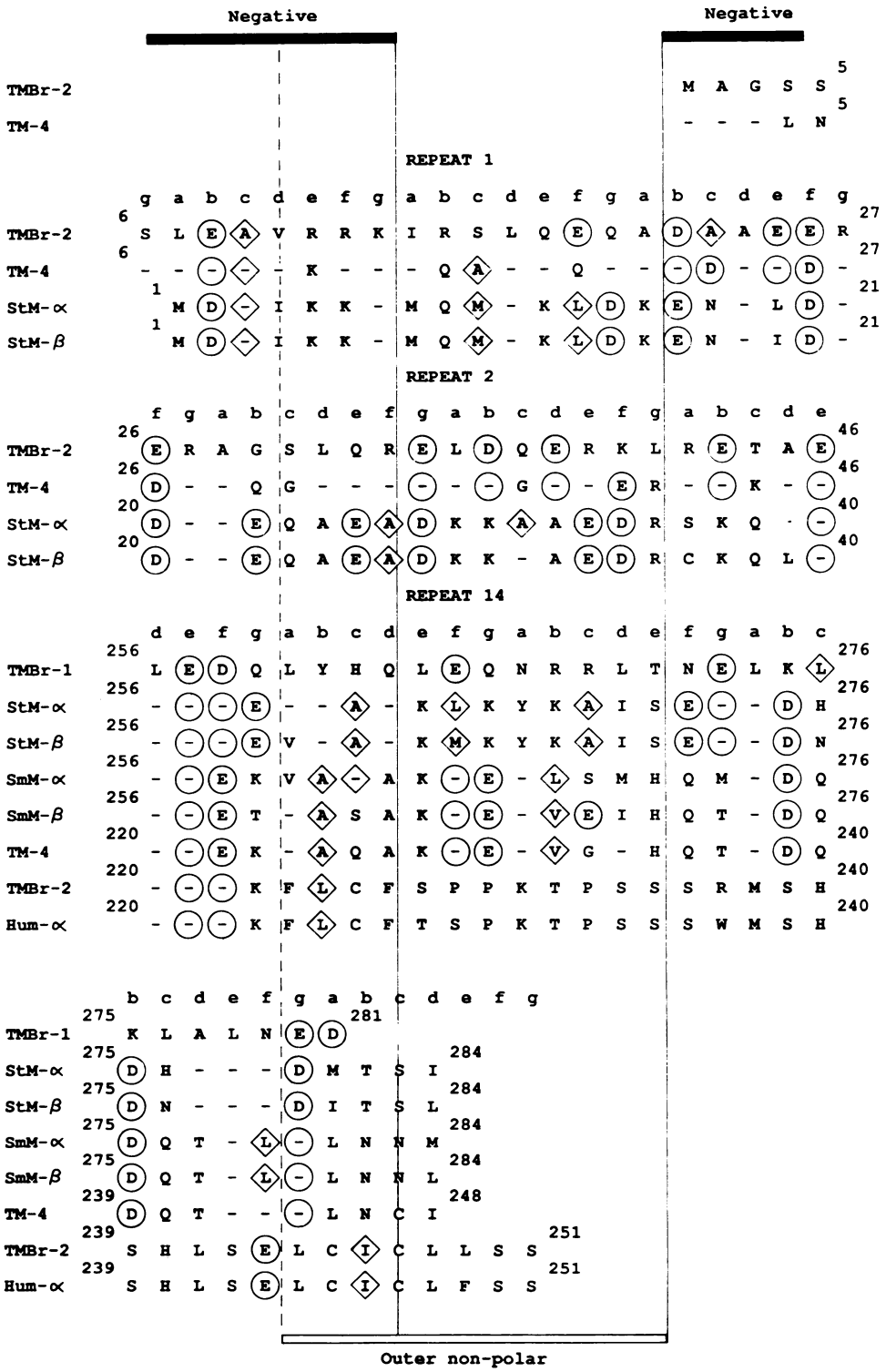


FIG. 9. Structural comparison of the NH_2-terminal and COOH-terminal sequences of rat tropomyosins. Abbreviations: StM, striated muscle; SmM, smooth muscle; Hum, human. The heptapeptide repeat is indicated by a, b, c, d, e, f, and g, where a and d are found in the nonpolar core of the tropomyosin dimer; b, c, and f are on the external face; and e and g occupy a position between outer and inner residues (49, 58). A 14-fold repeat of a 19.66-amino-acid pattern has been postulated to be responsible for actin filament binding by striated muscle α-tropomyosin (50). The typical actin-binding repeat consists of a central region that contains some nonpolar residues in the b, c, or f positions (◇) that is flanked by regions possessing negatively charged residues (○). Here we have compared actin-binding repeats 1, 2, and 14 of striated muscle α-tropomyosin with the equivalent regions of other rat tropomyosins. Identities to the first sequence presented in each repeat are indicated by a dash. The COOH-terminal amino acid sequence of Hum-α was obtained by translating the 3′-untranslated sequence of cDNA clone skaTM.1 (40). The tropomyosin sequences for rat striated muscle α (54) and β (65), smooth muscle α (54) and β (23), and TM-4 (66) were obtained from the literature.

that at least 12 distinct tropomyosin isoforms are expressed from three separate genes in rats. The α-TM gene encodes nine isoforms (54, 64; Fig. 8), the β-TM gene encodes two isoforms (23, 65), and the TM-4 gene encodes at least one isoform (66). The cell type-specific expression of these gene products raises some intriguing questions with regard to the mechanism of their regulation at both the transcriptional and the posttranscriptional levels. In addition, the functional significance of tropomyosin diversity remains to be determined. The expression of a diverse group of tropomyosin isoforms in a highly tissue-specific manner via alternative promoters and alternative RNA processing strongly suggests that each isoform is required to carry out specific functions in the actin-based filaments of various muscle and nonmuscle cells.

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