Tropomyosin 3 Increases Striated Muscle Isoform Diversity†

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ABSTRACT: Tropomyosin (TM), a component of the thin filament of the sarcomere, is encoded by a four-member multigene family: α-TM, *β*-TM, TPM 3, and TPM 4. The α-TM, *β*-TM, and TPM 3 genes each utilize an alternative splicing mechanism to encode a striated muscle isoform. Although the α-TM and *β*-TM striated muscle isoforms are well characterized, relatively little is known about the TPM 3 isoform. We cloned and sequenced the murine TPM 3 cDNA and found that it exhibits a 93% nucleotide homology and 99% amino acid homology to the human sequence. Results show that, unlike humans, TPM 3 is not expressed in any developmental stage of murine hearts. TPM 3 message is expressed in slow-twitch skeletal muscle but is not found in representative fast-twitch musculature. The soleus, a representative slow-twitch muscle, expresses transcript levels of 65% *β*-TM, 15% α-TM, and 20% TPM 3, but the TPM 3 protein accounts for approximately 31% of the total striated tropomyosin in slow-twitch muscle. In fast-twitch muscle, α-TM comprises 71% of the total striated muscle TM protein, and *β*-TM comprises 29%. The results demonstrate that a translational mechanism regulates the production of the TM proteins, with *β*-TM message not being efficiently translated. The unique distribution pattern of TPM 3 adds to the diversity of the tropomyosin family and strongly suggests functional significance for the different striated muscle TM isoforms.

Striated muscle tropomyosin (TM),[1] an integral thin filament component of the sarcomere, forms α-helical coiled-coil dimers which bind to actin and the troponin complex. Tropomyosin regulates Ca^{2+}-mediated actin−myosin cross-bridging through steric hindrance, allosteric, and cooperative mechanisms (*1*−*3*). Together with the troponin complex, TM inhibits actin−myosin interaction; however, with an increase in the cytosolic Ca^{2+} concentration, Ca^{2+} binds to troponin C, allowing actin−myosin cross-bridge activation.

Tropomyosins are encoded by a four-member multigene family: α-TM, *β*-TM, TPM 3, and TPM 4 (*4*). The four genes generate a multitude of tissue and developmental specific isoforms through alternative exon splicing, the use of different promoters, and differential 3′ end processing. Striated muscle specific isoforms are produced from the α-TM, *β*-TM, and TPM 3 genes (*4*, *5*). The α-TM and *β*-TM striated muscle isoforms are well characterized, but relatively little is known about the TPM 3 striated muscle isoform.

Although the striated muscle TM isoforms are highly homologous (α-TM and *β*-TM are 86% identical at the amino acid level), their unique expression patterns suggest that each isoform may have functional differences. For example, α-TM is predominantly expressed in fast-twitch skeletal and cardiac muscle, whereas slow-twitch skeletal muscle has an increased amount of *β*-TM (*4*). Recently, α-TM and *β*-TM have been shown to confer different contractile and relaxation parameters to muscle function (*6*). When *β*-TM is overexpressed in transgenic mouse hearts, various physiological assays show that the maximum rate of relaxation was reduced and the time to half-relaxation was prolonged in the absence of changes to other contractile proteins. Cardiac myofilaments from these transgenic mice exhibit an increased sensitivity to Ca^{2+}, and cardiomyocytes decrease their maximum rates of both relaxation and contraction (*7*, *8*). These studies demonstrate that, despite the high homology between α-TM and *β*-TM, each isoform contributes to produce unique sarcomeric contraction and relaxation properties.

Although the expression and function of α-TM and *β*-TM are well characterized, relatively little is known about the TPM 3 striated muscle isoform. The TPM 3 gene, approximately 42 kb in length, is located on human chromosome 1 (*9*). Through the use of two distinct promoters, a striated muscle specific mRNA (1.3 kb in length) and a nonmuscle isoform mRNA (2.5 kb in length) are produced (*10*). Also, recent data suggest that this gene generates additional neural specific isoforms (*11*).

The TPM 3 striated muscle isoform is often referred to as α-TM slow because of the homology with the chicken slow-twitch muscle isoform but has also been called skαTM.2 (*4*, *12*, *13*). The gene which encodes the TPM 3 striated muscle isoform is named TPM 3 or *γ*-TM but previously has been referred to as hTM30nm and hTMnm (*14*). In humans, TPM 3 is found in both adult heart and skeletal muscle, whereas in some smaller mammals (i.e., rat and

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[1] Abbreviations: TM, tropomyosin; TPM 3, tropomyosin 3; TPM 4, tropomyosin 4; EDL, extensor digitorum longus, UTR, untranslated region; RT-PCR, reverse transcriptase−polymerase chain reaction; EM, embryonic muscle; NM, newborn muscle; AM, adult muscle; T&E, tibialis and EDL.

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rabbit) it is solely expressed in skeletal muscle (*5*, *15*). The relative levels of TPM 3 RNA and protein in different muscle types and its developmental profile are unknown.

In the present study, we cloned and sequenced the mouse muscle cDNA isoform of TPM 3 and determined that the amino acid sequence is 99% homologous to the corresponding human sequence. The TPM 3 striated muscle isoform exhibits a 91% amino acid homology with the murine striated muscle α-TM coding region and an 86% homology with murine β-TM. The TPM 3 striated muscle isoform displays both tissue and developmentally regulated expression; it is solely expressed in skeletal muscle (not in cardiac tissue) and is found primarily in slow-twitch muscles. Analysis of quantified message and protein levels shows that translational regulatory mechanisms play a major role in controlling TM expression. As such, tropomyosin isoform diversity adds to the complexity of contractile protein isoforms which contribute to imparting functional differences during physiological performance.

MATERIALS AND METHODS

RT-PCR and Cloning. RNA was extracted using either RNAzol B (Tel-Test, Inc., Friendswood, TX) or Tri Reagent (Molecular Research Center, Cincinnati, OH). PCR primers were designed using the human sequence as a template (*10*). The forward primer, beginning at the 5′ coding region second methionine, is 5′-ATGGAGGCCATCAAGAAA-3′. The reverse primer corresponds to the nucleotide sequence between the poly(A) signal sequence and the cleavage site. The sequence of the reverse primer is 5′-TTTCCAG-CAGCTTAACAT-3′. The reverse transcriptase reaction was performed followed by 30 PCR cycles using the Expand High Fidelity PCR System (Roche Diagnostics Corp., Indianapolis, IN). Products were run on 0.8% agarose gels.

For cloning, a fresh PCR reaction was performed, mixed with the pCR-TOPO vector (Invitrogen, Carlsbad, CA), and ligated for 5 min. The ligated product was transformed into XL-1 Blue cells and grown overnight on ampicillin plates. Purified insert clone DNA was checked by endonuclease restriction digestion and confirmed by nucleotide sequencing.

A mouse diaphragm muscle Uni-ZAP XR cDNA library (Stratagene, La Jolla, CA) was plated and screened with the 1.1 kb TPM 3 probe. Positive clones were isolated, purified, and sequenced.

Northern Blot Analysis. RNA, extracted with either RNAzol B or Tri Reagent, was electrophoresed in formaldehyde gels. The gels were transferred to nylon membranes overnight and cross-linked using a UV Stratalinker (Stratagene, La Jolla, CA). Filters were prehybridized for 1 h, followed by hybridization in ExpressHyb (Clonetech Laboratories, Inc., Palo Alto, CA) with denatured radiolabeled probe (1 000 000 cpm/mL). The radiolabeled, PCR-generated probes corresponded to the nucleotide sequences of the TPM 3 3′UTR or GAPDH. After hybridization, membranes were washed in 2× SSC and 0.05% SDS for 20 min at 50 °C and then in 0.1× SSC and 0.1% SDS at 60 °C for 30 min. Blots were exposed to Kodak X-ray film.

Quantification of TM RNA Levels. To quantify different TM isoform transcript levels, predetermined amounts of total RNA (1, 2, and 4 μg) were slot blotted on nylon membranes. These membranes were probed with the radiolabeled 3′UTR sequence from α-TM, β-TM, TPM 3, and GAPDH. To determine specific activities among the radiolabeled probes, equivalent amounts of probe DNA (all ∼200 bp in length) were quantified via spectrophotometry and agarose gel analysis. Following incorporation of radiolabeled nucleotides, specific activities were calculated and found to be very similar. Equivalent numbers of counts were hybridized to the Northern slot blots. After hybridization, membranes were washed in 2× SSC and 0.05% SDS for 20 min at 50 °C and then in 0.1× SSC and 0.1% SDS at 60 °C for 0.5 h. Blots were exposed to a phosphorimager screen overnight, and the bands were quantified on Imagequant PhosphorImager V 4.2 (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis. Total protein homogenates were prepared from adult soleus, tibialis and EDL, limb skeletal muscle or cardiac tissue. A total of 40 μg of protein was electrophoresed in each lane of 3.4 M urea and 8% SDS−PAGE gels at 100 V. The separated proteins were transferred overnight to nitrocellulose membranes at 15 V.

The primary incubation for the Western analysis was done with the CH1 monoclonal striated muscle tropomyosin specific antibody (Sigma, St. Louis, MO) (*16*). The specificity of the antibody with each striated muscle TM isoform was tested by quantifying soleus muscle protein and reacting different amounts of the protein with the CH1 antibody. We then determined the amount of each TM isoform and found that there were no significant differences in CH1 antibody reactivity for the relative amounts of each isoform. Following incubation with the CH1 antibody, the secondary antibody, anti-[mouse IgG (H+L)]-peroxidase (Roche Diagnostic Corp., Indianapolis, IN), was incubated with the blot. Super Signal Substrate (Pierce, Rockford, IL) was used for chemiluminescent detection. Band intensity was measured on the Imagequant PhosphorImager V 4.2.

RESULTS

Cloning and Sequencing. Using the nucleotide sequence of the human TPM 3 gene, we designed primers to RT-PCR amplify the mouse striated muscle isoform of TPM 3. Since the start codon of TPM 3 is ambiguous (there is a double methionine in the translation initiation region), we designed the primer to start at the second methionine so the resulting product would be 284 amino acids in length, the invariant size of all striated muscle TM proteins. The 3′ primer was positioned to correspond to the 3′ untranslated nucleotide sequence at the end of the last skeletal exon. RT-PCR was used to produce the cDNA from mouse heart and skeletal muscle RNA. The expected 1.1 kb band was obtained from mouse skeletal muscle RNA but was not in cardiac tissue (Figure 1). This more sensitive technique confirms previous Northern blot analysis showing that the striated muscle TPM 3 isoform is not expressed in mouse heart (*5*).

The 1.1 kb fragment obtained from the RT-PCR procedure was cloned and sequenced (Figure 2). To confirm this sequence, we screened a mouse diaphragm skeletal muscle cDNA library using the PCR-generated cDNA fragment as a radiolabeled probe. Clones which hybridized to the probe were isolated, cloned, and sequenced. With this process, we confirmed the murine TPM 3 sequence, in addition to obtaining the 5′UTR sequence (Figure 2).

A sequence comparison between the human and murine TPM 3 sequences was conducted. They are highly homolo-

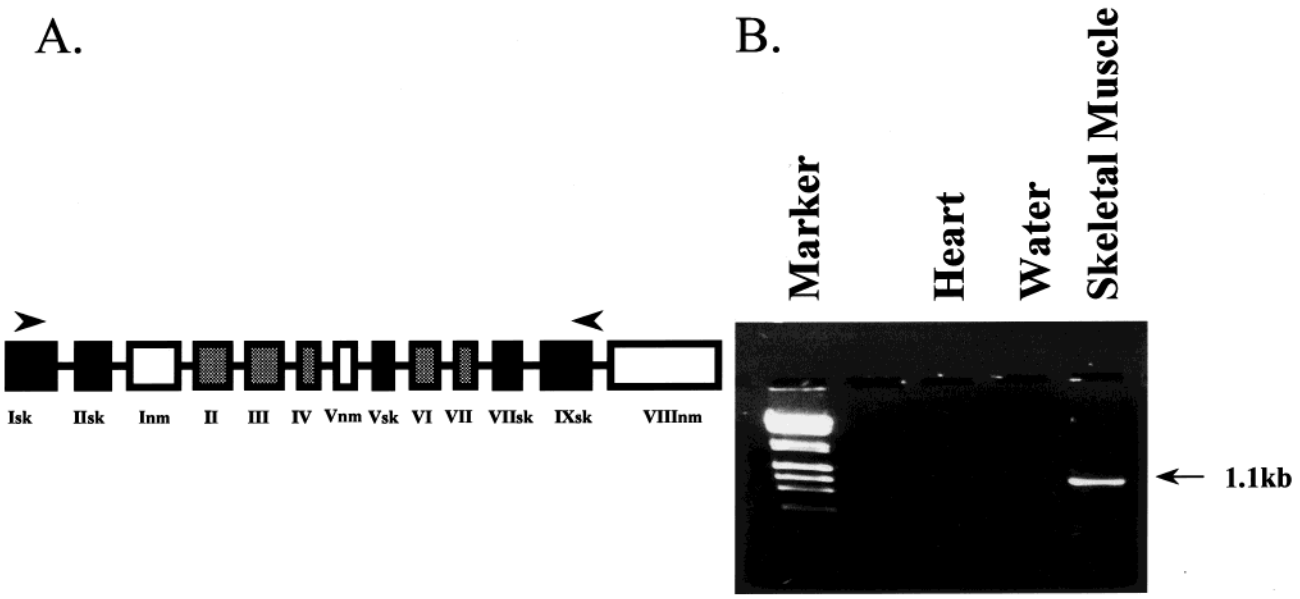

FIGURE 1: (A) Schematic picture of the tropomyosin 3 gene. Striated specific exons are solid, nonmuscle specific exons are in open boxes, and common exons are striped (*10*). Arrowheads indicate the position of the RT-PCR primers. The nomenclature for the exons is the same as used by Clayton et al. (*10*). (B) RT-PCR products electrophoresed on a 0.8% agarose gel. Lanes: 1, marker − ϕX174 digested with *Hae*III; 2, blank; 3, heart sample; 4, water control; 5, skeletal muscle sample. The expected 1.1 kb cDNA is seen only in the skeletal muscle sample.

gous, exhibiting a 93% nucleotide identity in the coding region and a 99% amino acid homology (Figure 2). Only three amino acids are different, and these changes are conservative: A18→V, A45→T, and E272→D. In addition to the high conservation of nucleotide and amino acid sequences exhibited between human and murine TPM 3, there is significant homology among TPM 3 and the other striated muscle isoforms. The murine, striated muscle α-TM and TPM 3 exhibit a 91% amino acid homology and the murine, striated muscle β-TM and TPM 3 are 86% homologous. An examination of the 3′ untranslated regions revealed that the murine and human sequences are approximately 75% homologous. Although the significance of this high degree of homology in the 3′UTR untranslated region is unknown, it may play a role in the regulation of tissue-specific expression or in influencing mRNA stability.

TPM 3 Developmental Expression Profile. Previous investigations show that TPM 3 is expressed in the human myocardium (*15*). Although our results show that the striated muscle isoform of TPM 3 is not expressed in adult mouse heart, it may be expressed during early cardiac development. Previous results in our laboratory found that, during murine fetal cardiac development, β-TM is expressed at significant levels (20% of TM mRNA in murine embryos is β-TM) (*17*). In adult mice, β-TM expression is reduced and the α-TM isoform accounts for 98% of the tropomyosin in the heart (*17*). To ascertain whether TPM 3 expression in the heart may be developmentally regulated, we addressed whether TPM 3 message could be detected during cardiogenesis.

Northern blot analysis was conducted to determine the developmental expression profile of TPM 3 in striated musculature. Total RNA was prepared from limb skeletal muscle and cardiac tissue obtained from embryonic day 17.5, newborn (days 1−3), and adult (over 2 months old) mice. A random primed, radiolabeled 200 bp fragment specific to the 3′UTR of TPM 3 was used as the probe. This probe does not cross-react with α- or β-TM sequences (data not shown). As seen in Figure 3, a 1.3 kb band corresponding to the TPM 3 message is found in all three stages in skeletal muscle tissue. However, TPM 3 is not expressed in any of the three developmental stages of the heart. These results were confirmed by RT-PCR analysis using the primers designed for the original isolation of the clone. The band corresponding to TPM 3 is amplified only in skeletal muscle samples of all three developmental stages but not in the heart or the liver samples (Figure 3). Unlike the expression profile of the α- and β-TM striated muscle isoforms, TPM 3 is restricted in its expression to the skeletal musculature and is not found in the cardiac tissue of the mouse.

Quantification of TPM 3 Expression in Skeletal Musculature. Northern blot analysis indicates that TPM 3 is expressed at greater levels in the adult than in the newborn or embryonic muscle. To quantify the relative amounts of α-TM, β-TM, and TPM 3 message which are expressed in embryonic, newborn, and adult leg muscle, we performed Northern slot-blot analyses. We also included RNA from adult tibialis/EDL and soleus as representative fast-twitch and slow-twitch muscles, respectively. The 3′UTR sequences were radiolabeled and used as specific probes for α-TM, β-TM, and TPM 3. The RNA blots were hybridized with these probes, quantified, and normalized to GAPDH levels (Figure 4).

In embryonic muscle, β-TM mRNA accounts for the largest percentage of the total TM message at 60%. α-TM mRNA levels are 37%, and TPM 3 is only expressed at 3% of the total TM levels. In the newborn mouse, the amounts do not change significantly (α-TM 35%, β-TM 62%, and TPM 3%). In the adult mouse, however, α-TM is the predominant isoform at 55% of the total tropomyosin, whereas β-TM accounts for only 43% and TPM 3 at 1%. Surprisingly, the adult TPM 3 percentage decreases from the newborn and embryonic mice to the adult mouse. On closer examination, we see that the total TM levels increase during

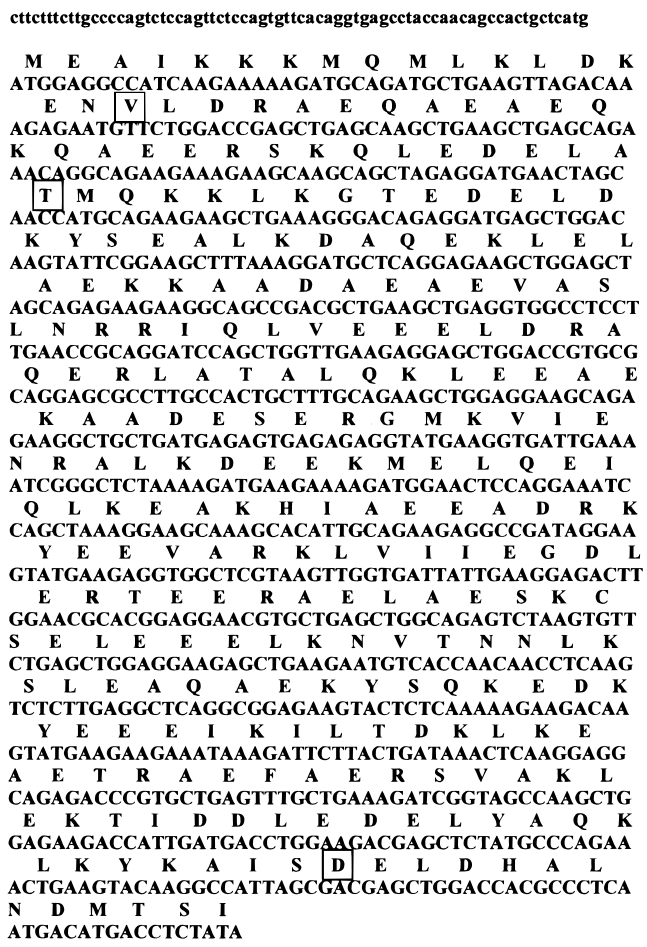

FIGURE 2: Nucleotide and amino acid (shown above the corresponding codons) sequence of the murine TPM 3 striated muscle isoform. The coding (capital letters) and 3′UTR/5′UTR (lower case letters) sequence was obtained from RT-PCR and confirmed with clones from a mouse skeletal muscle library. Amino acid differences from the human sequence are boxed (A18V, A45T, and E272D).

development from embryonic to the adult by 7.6-fold, as does the absolute value of TPM 3 (an increase of 4.5-fold; data not shown). However, direct comparison between embryonic and adult TM levels is complicated due to changes in expression of GAPDH levels in embryonic versus adult skeletal muscle. Also, the specific muscles selected from the adult can dramatically influence the amount of TPM 3 which is detected. For example, by examination of TM expression in the soleus and tibialis/EDL, it is apparent that muscle type dramatically affects the quantified TM isoform levels. The soleus, a representative slow-twitch muscle, expresses 65% β-TM, 15% α-TM, and 20% TPM 3, whereas tibialis/EDL express 35% β-TM, 63% α-TM, and no TPM 3. The adult limb muscle samples used in this current analysis involved muscles taken from five mice and are representative of musculature in the upper thigh region of the hind limbs.

Protein Expression Profile. Due to differences in RNA stability and translational efficiency, RNA levels do not necessarily correspond to protein levels. This is well documented for TM where studies show that translational control plays a major role in tightly regulating protein production over varying message levels (*6*, *18*, *19*). To address this area, we conducted quantitative Western blot analyses. Total protein was isolated from slow- and fast-twitch muscles (soleus and tibialis/EDL, respectively) and run on 3.4 M urea and 8% discontinuous SDS−PAGE gels. The addition of urea to the gel allows separation of the α-TM, β-TM, and TPM 3 proteins for distinct quantification of these three isoforms following immunoreactivity with the same CH1 striated muscle TM specific antibody. Preliminary studies show that there is no preferential reactivity of the CH1 TM antibody with the different TM isoforms (see Materials and Methods).

Results show that soleus muscle expresses all three isoforms but at different levels. The level of TPM 3 protein is 31% of the total striated muscle tropomyosin, whereas α-TM accounts for 47% and β-TM 22% (Figure 5). As expected, from the RNA analysis, tibialis and EDL did not express TPM 3 protein; α-TM is approximately 71% and β-TM 29% of the total striated muscle tropomyosin protein in these representative fast-twitch muscles. Wild-type and β-TM transgenic hearts were used to show the relative positions of α-TM and β-TM, respectively. TPM 3 was bacterially expressed to determine its position relative to α-TM and β-TM. TPM 3 is only expressed in slow-twitch muscle but at a much higher concentration than expected on the basis of mRNA levels which enhances the TM isoform diversity present in striated muscle fibers.

DISCUSSION

The unique expression patterns of the striated muscle isoforms of tropomyosin imply that these isoforms may have distinct functions. The α-TM and β-TM isoforms have been well characterized, but relatively little was known about TPM 3. This isoform has become more important since the discovery of a point mutation (M9R) in TPM 3 which is associated with a disease called nemaline myopathy (*20*). This disease is characterized by the accumulation of rodlike structures in muscle tissue and associated muscle weakness (*21*).

The murine striated muscle TPM 3 cDNA sequence is highly homologous to the human sequence (99% amino acid homology and 93% nucleotide identity). Considering the high homology of tropomyosin isoforms across species, it is not surprising that there are only three amino acid changes (A18→V, A45→T, and E272→D) between the human and murine isoforms. These amino acid changes are conservative and would not be expected to result in functional differences. In the mouse, the TPM 3 striated muscle message is found exclusively in the skeletal muscle but not in the cardiac tissue at any developmental stage. However, this isoform is expressed in the adult human skeletal and cardiac musculature (*15*). Previous studies have shown differences between the mouse and human expression profile for other contractile proteins. For example, in the mouse ventricle, α-MHC is the predominant isoform, whereas in humans, β-MHC is a principal adult ventricular isoform. Also, β-TM represents only 2% of the total cardiac TM message in mice, but in humans, it accounts for 20% of the TM in the heart. Interestingly, the relative levels of TPM 3 message are low throughout development; however, they account for a large percentage of TM in the slow-twitch muscles. TPM 3 protein accounts for approximately one-third of the total tropomyosin

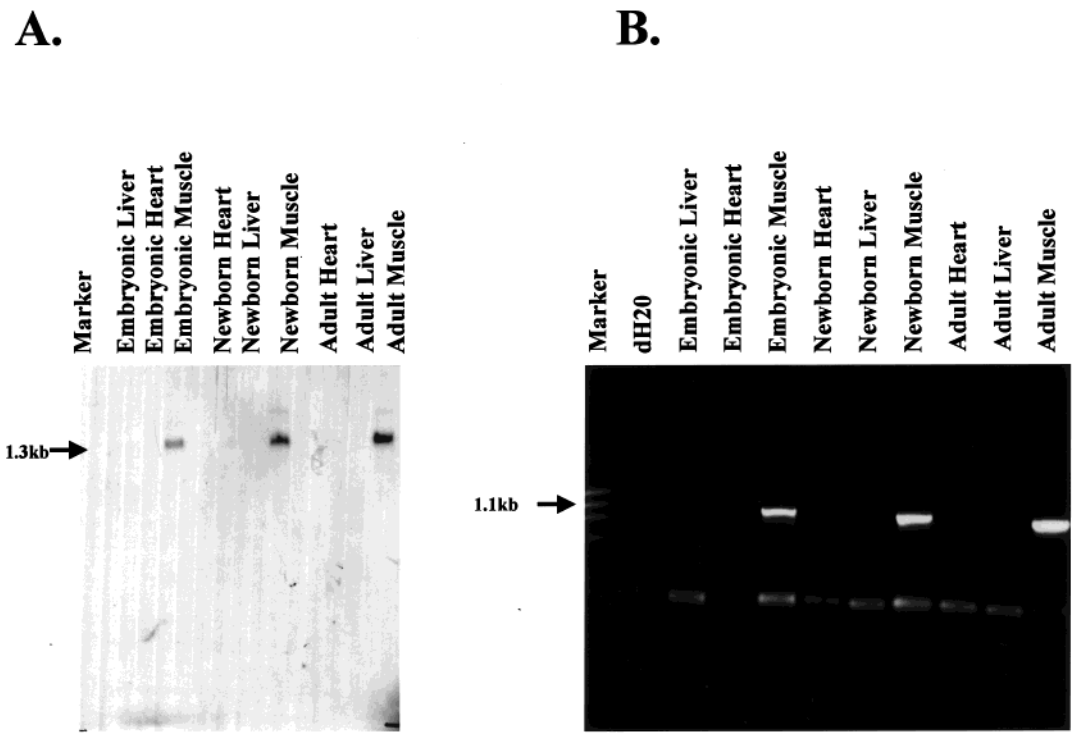

FIGURE 3: TPM 3 expression profile. (A) Northern blot analysis of embryonic (embryonic day 17.5), newborn (1−3 days), and adult mouse (>2 months) tissues with a radiolabeled 3′UTR probe from TPM 3. Liver was used as a negative control for all stages. The expected 1.3 kb band is seen only in the skeletal muscle samples from all three developmental stages. (B) RT-PCR analysis of TPM 3 expression. RT-PCR of the TPM 3 cDNA was done using RNA extracted from embryonic, newborn, and adult tissues. The expected 1.1 kb band is seen only in the skeletal muscle samples. Note that the size of the RT-PCR product is smaller than the Northern band because the 5′UTR is not amplified with the chosen primers.

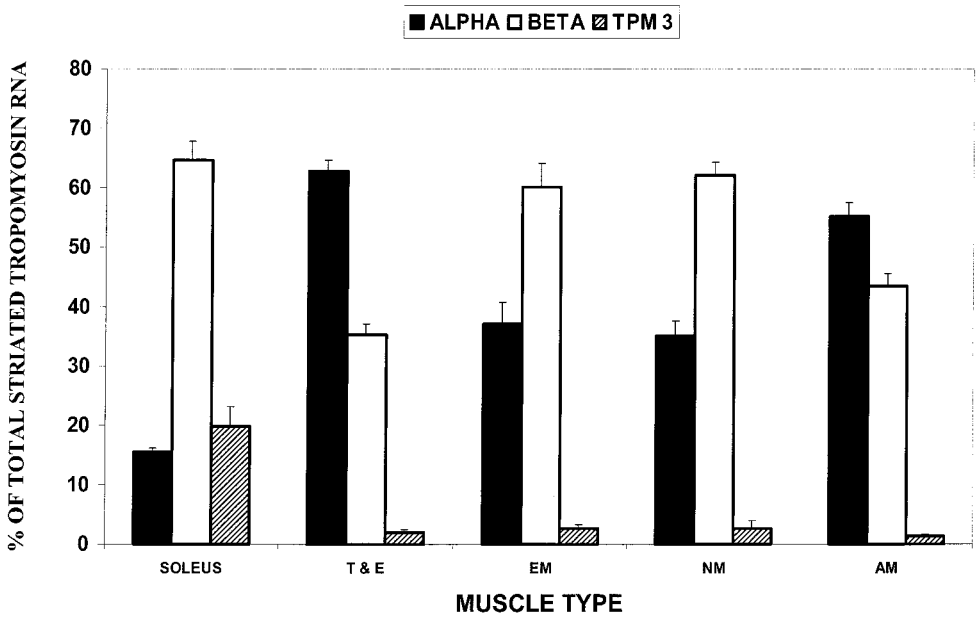

FIGURE 4: Quantification of α-TM, *β*-TM, and TPM 3 RNA expression. Slot blot analysis was used to quantify the relative transcript levels of each isoform. The radiolabeled probes were specific to the 3′UTRs to prevent cross-hybridization. Results were normalized to GAPDH levels. A total of nine slot blots for each tissue were analyzed. α-TM is in black, *β*-TM is open, and TPM 3 is hatched. EM = embryonic muscle, NM = newborn muscle, AM = adult muscle, and T&E = tibialis and extensor digitorum longus.

protein in slow-twitch muscles. We did not find TPM 3 expression in murine fast-twitch musculature; however, other studies have shown that psoas (a fast-twitch muscle) expresses low levels of TPM 3 RNA (*5*). Since most muscles are a mixture of various fiber types, this difference in results may reside in the variance in fiber composition for distinct fast-twitch muscles.

The message levels in the soleus and tibialis/EDL samples do not directly correlate to the protein levels. In soleus, TPM 3 RNA accounts for 20%, α-TM for 15%, and *β*-TM for 65% of the total striated muscle TM message. However, quantified protein analysis shows that TPM 3 is 31%, α-TM is 47%, and *β*-TM is 23% of the total striated protein. In fast-twitch muscles, TPM 3 mRNA levels are negligible,

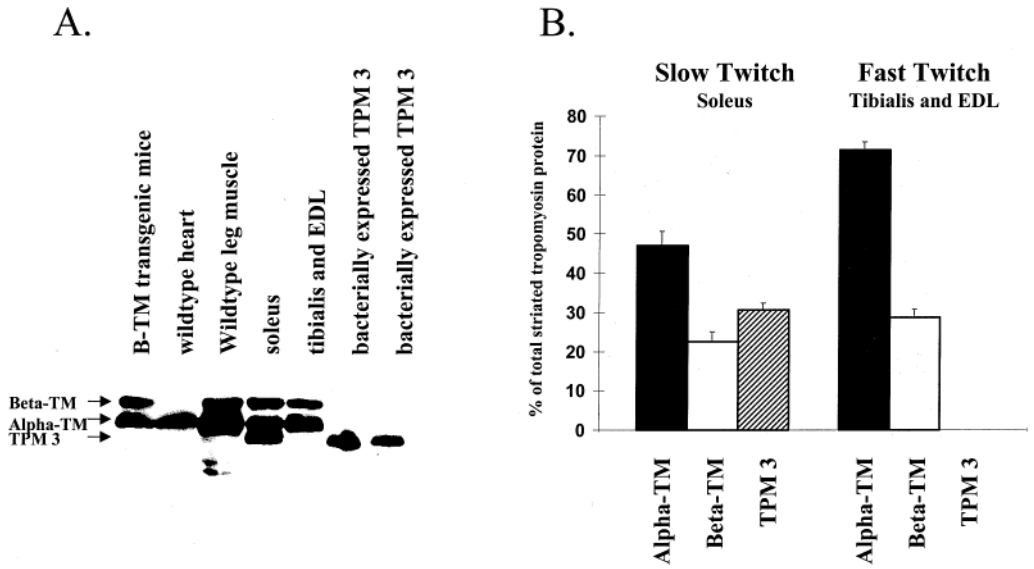

FIGURE 5: TPM 3 protein expression. (A) Western blot analysis. A total of 40 μg of protein was electrophoresed on 8% 3.4 M urea SDS−PAGE gels. The gels were transferred to nitrocellulose and probed with the CH1 antibody, which recognizes striated muscle tropomyosin specifically. β-TM overexpressing hearts were used to show the relative positions of α-TM and β-TM. Wild-type hearts show the position of α-TM, and bacterially expressed TPM 3 was used to show its position relative to α-TM and β-TM. Wild-type leg muscle was a mixture of mostly upper leg muscles. (B) Quantification of Western blot analysis. α-TM is in black, β-TM is open, and TPM 3 is hatched.

α-TM mRNA is 63%, and β-TM is 35% of the total striated muscle TM. Protein results show that there is 71% α-TM and 29% β-TM. From these results, it appears that β-TM is not translated as efficiently as α-TM and TPM 3. This conclusion is supported by previous work done in our laboratory on β-TM transgenic mice that exhibit a 150-fold excess of β-TM message but only a 34-fold increase in protein levels. The total levels of TM in these hearts are constant. These results demonstrate that a translational mechanism regulates the production of the striated muscle TM proteins and that β-TM message may not be translated as efficiently as the α-TM and TPM 3 transcripts.

The unique expression patterns of TPM 3 suggest that it may have distinct functions from those of α-TM and β-TM. The mouse TPM 3 amino acid sequence is 91% homologous to α-TM but only 86% homologous to β-TM, suggesting that TPM 3 may impart functional properties more similar to α-TM than β-TM. There are 26 amino acid positions at which the TPM 3 molecule differs from α-TM. Of particular interest is a charge change of amino acid lysine at position 29 in α-TM which corresponds to a glutamine in TPM 3, giving the TPM 3 a more negative charge than α-TM. (β-TM differs in two charges from α-TM, which make it more negative.) The charge change between α-TM and TPM 3 is not in the troponin T binding regions. Therefore, this charge difference may alter the interaction of TPM 3 with actin rather than the troponin complex. Also, cooperativity among adjacent TM molecules may be affected by different tropomyosin isoform compositions. Since TPM 3 is predominantly expressed in slow-twitch fibers as is the β-TM isoform, TPM 3's physiological contribution to thin filament regulation may indicate functional properties which are similar to β-TM. However, the β-TM troponin T binding region has a more negative charge than the α-TM and TPM 3 troponin T binding regions. Therefore, perhaps TPM 3's contribution to the thin filament will be more like α-TM than β-TM. Furthermore, the TPM 3 troponin T binding region is more polar than the α-TM binding region. These differences in polarity may also impart unique phyiological characteristics to the TPM 3 isoform. Current experiments employing transgenic mice are in progress to directly address these questions of TPM 3's contribution to sarcomeric function.

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