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Original Article

Identification of a novel TPM4 isoform transcript and comparison to the expression of other tropomyosin isoforms in bovine cardiac and skeletal muscles

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Abstract: In mammals, there are four tropomyosin (TPM) genes (TPM1, TPM2, TPM3, and TPM4) each of which generate a multitude of alternatively spliced mRNAs. TPM isoform diversity in bovine unlike in humans are not well characterized. The purpose of this investigation is to perform an extensive analysis of the expression of both transcripts and corresponding protein of sarcomeric TPMs in bovine strated muscles. We have cloned and sequenced the transcripts of the sarcomeric isoform of the TPM4 gene designated as TPM4α as well as a new splice variant TPM4ε from bovine striated muscles. Additionally, we have determined the expression of various sarcomeric TPM isoforms and TPM4ε in bovine heart and skeletal muscles. Relative expression as well as absolute copy number determination by qRT-PCR suggests that TPM1α expression is significantly higher in bovine cardiac muscle, whereas TPM2α is higher in skeletal muscle. The relative expression of TPM3α in bovine heart and skeletal muscle is very similar. The relative expression of TPM4α and TPM4ε is higher in bovine heart and skeletal muscle, respectively. We have evaluated the protein expression levels of various TPM isoforms by 2D western blot analyses in commercially available protein extracts of heart and skeletal muscles with the CH1 monoclonal antibody against TPM. Protein from each CH1-positive spot was extracted for LC-MS/MS analyses, which show that bovine heart extract contains 91.66% TPM1 and 8.33% TPM2, whereas skeletal muscle extract contains 57% TPM1 and 42.87% TPM2. We have failed to detect the presence of unique peptide(s) for TPM3α, TPM4α, and TPM4ε.

Keywords: Novel splicing, qRT-PCR, absolute copy number, 2D western blot, mass spectrometry, LC-MS/MS

Introduction

Muscle contraction is dependent upon a cooperative interaction between thick and thin filament sarcomeric proteins. TPM, a component of thin filament, interacts with actin and the troponin complex to regulate contractile activity. During muscle contraction, an increase of calcium (Ca^{2+}) in the myofilament space promotes binding of Ca^{2+} to troponin C, which alters the conformational state of TPM and facilitates actomyosin interactions. There are four known TPM genes (*TPM1, TPM2, TPM3*, and *TPM4*) in vertebrates [1-7] except for zebrafish where six TPM genes have been reported [3, 8, 9]. By the use of different promoters, alternate polyadenylation sites, and/or alternate splicing, each of the TPM genes generate a multitude of isoforms. TPMs are often categorized into two groups, muscle and non-muscle isoforms. Striated muscle isoform(s) from each TPM gene have identical exon compositions encoding 284 amino acid residues. There are two types of vertebrate striated muscles-cardiac and skeletal. Skeletal muscles are composed of different types of fibers, which may be classified into type 1 (slow type) and type 2 (fast type). The latter again can be divided into three subtypes by histological techniques [10].

Different isoforms of myofibrillar proteins may variably regulate muscle contraction and may also define the quality of bovine-derived meats with respect to texture and tenderness. Molecular analyses indicate that myosin heavy chain (MyHC) isoforms are the major proteins responsible for the different fiber types [11]. In order to elucidate the role(s) of various TPM isoforms in

different skeletal muscle types in relation to meat quality [12], Oe et al. characterized the sarcomeric TPM isoforms of different muscles from Holstein cows of various ages. They reported the differential expression of TPM1, TPM2, and TPM3 proteins in various tissues, for example *masseter*, diaphragm, *psoas major, longissimus thoracis* and *semitendinosus*. Further, Oe et al. reported the distribution of various TPM isoforms in different types of single fibers isolated from bovine skeletal muscle [13].

The expression of sarcomeric isoforms TPM1α, TPM2α, and TPM3α in bovine skeletal muscle are known but there is no report of the expression of either TPM4α transcripts or TPM4α protein in bovine skeletal muscle. As far as we know, there is no evidence in the literature on the characterization and/or the expression of TPM4 isoforms in bovine hearts. As a matter of fact, very little work, if at all, has been done on the expression of TPM isoforms in bovine cardiac tissues. In this study, we have cloned and sequenced the transcripts of various sarcomeric isoforms of TPM1, TPM2, TPM3, and TPM4 known as TPM1α, TPM2α, TPM3α, and TPM4α, respectively, from bovine heart and skeletal muscle. Interestingly, we have characterized a new unusual splice variant of the *TPM4* gene, TPM4ε. However, we have failed to detect TPM1κ, the other known sarcomeric TPM1 isoform in vertebrates [2, 14]. In this study, we have also quantified various TPM transcripts in heart and skeletal muscle by qRT-PCR determining the absolute copy number as well as determining the relative expression in heart and skeletal muscle. To the best of our knowledge this is the first report of cloning, sequencing, and expression analyses of TPM4α and the new variant TPM4ε from bovine striated muscles. Finally, we have performed 2D Western blot analysis of proteins extracted from bovine heart and skeletal muscle with CH1 monoclonal antibody that is universally used for detection of all vertebrate sarcomeric TPM isoforms. Proteins from all CH1-positive spots were extracted for subsequent mass spectra analyses.

Materials and methods

Ethical statement

The present study was carried out with commercially available bovine tissue extracts and tissue-specific bovine RNAs. Hence, a specific Institutional Animal Care and Use Committee animal use protocol is not required at our institution. However, the protocol(s) was reviewed and approved by the Institutional Biosafety Committee IBC# 169 (D.K. Dube), IBC # 321 (J.W. Sanger), and IBC # 212 (B.J. Poiesz).

RT-PCR for amplification of TPM1α, TPM2α, TPM3α, TPM4α, and TPM4ε

Total RNAs from bovine whole heart (Cat # R1B 34122) and skeletal muscle (Cat # R1B 34171) were procured from BioChain (Newark, CA). We do not know the gender, breed or muscle type of the bovine source of the samples; the heart and skeletal muscle samples were not necessarily obtained from the same muscle type or animal. cDNAs were made from various RNAs using oligo dT using our published protocols [2, 14]. PCR-amplification of various gene-specific TPM isoforms were performed subsequently with gene-specific and/or isoform-specific primer-pairs as displayed in **Table 1**. The PCR amplified DNA were used for agarose gel electrophoresis and stained with ethidium bromide. Subsequently, MiniElute Gel extraction kit (Qiagen, Valencia, CA) was used for extraction of DNA from the excised ethidium-stained DNA band from the agarose gel for determining direct nucleotide sequences and also for cloning into T/A cloning vectors (Life Technologies) following our published protocols. The DNA from the positive clones were extracted by using Qiagen mini-prep kit (Valencia, CA). Each of the isolated DNA was sequenced from both sides (Cornell University Life Science Core Laboratories Center, Ithaca, NY).

Real-time quantitative RT-PCR (qRT-PCR)

In order to quantify transcript level in a given tissue one can determine both relative and absolute amounts. qRT-PCR was carried out using the LightCycler 480 Real-Time PCR System as described before [3, 15, 16]. Relative quantification of qRT-PCR data was performed using the DCT (sample Ct minus 18S rRNA CT) and DD CT (sample DCt minus comparator DCt) methods. Efficiencies (X) were determined using dilution series of isoform-specific plasmid clones with respective isoform-specific primers pairs. Efficiency of 18S rRNA was determined by serial dilution of cDNAs generated with specific primers. The reaction mixture contained 12.5 µl of the SYBR green supermix, 1 µl of

Table 1. Primer-pairs and probes used for amplification and detection of TPM1α, TPM1κ, TPM2α, TPM3α, TPM4α, and TPM4ε

Gene		Type of amplification		Nucleotide sequence
Isoform	Used for	Conventional RT-PCR	qRT-PCR	
TPM1α/κ (+)	Con RT-PCR forward	TPM1α/k		5'-CGCCGCTCACCGCGCGCTC-3'
TPM1α/κ (-)	Con.RT-PCR reverse	TPM1α/k	cDNA also	5'-GAAACTTATATGGAAGTCA-3'
TPM1κ (+)	Con.RT-PCR forward	TPM1κ		5'-CGAGGAGGACATAGCGGCCAA-3'
TPM1α Probe	Probe for TPM1α	-		5'-ATGAACTGGACAAATACTCTGAG-3'
TPM1κ Probe	Probe for TPM1κ	-		5'-GCCAAGGAGAAGCTGCTGCG-3'
TPM1α (+)	TPM1α forward		X	5'-TGGAAGATGAGCTGGTGTC-3'
TPM1α (-)	TPM1α reverse primer		X	5'-AGAGGCATGA AAGTCATTGA-3'
TPM2α (+)	qRT-PCR forward		X	5'-CTCAAGGAGGCAGAGACCCG-3'
TPM2α (-)	qRT-PCR reverse		X	5'-TCTTTGGTGCATTTCAGTTT-3'
TPM2 Ex 9 (-)	For making cDNA			5'-CTTGTACTTCATCTTCTGGGCATAG-3'
TPM3α (+)	qRT-PCR forward		X	5'-CTTGGAGCGCACAGAGGAAC-3'
TPM3α (-)	qRT-PCR reverse		X	5'-GATCCAGAACAGAGCAGAAAC-3'
TPM3 cDNA	For making cDNA			5'-AACAGAGCAGAAACGGTGA-3'
TPM4α/n (+)	Con RT-PCR forward	Full length TPM4α or TPM4ε & for Making cDNA also		5'-GCCCTGGCTGCTCACTTGA-3'
TPM4α/n (-)	Con RT-PCR reverse			5'-CAGACTTCAGTATTGCTA-3'
TPM4α (+)	qRT-PCR forward		X	5'-GACCTGGAAGATGAGCTA-3'
TPM4α (-)	qRT-PCR reverse		X	5'-AGAGGGTGGCACCCCAGCCA-3'
TPM4ε (+)	qRT-PCR forward		X	5'-CTGTACCTCTTCTGAACT-3'
TPM4v (-)	qRT-PCR reverse		X	5'-AGAGGGTGGCACCCCAGCCA-3'
18S rRNA (+)	qRT-PCR forward		X	5'-TGCTGCAGTTAAAAAGCTCGTA-3'
18S rRNA (-)	qRT-PCR reverse		X	5'-ACCAACAAAATAGAACCGCGGT-3'

both positive and negative 10 μM primer, 9.5 μl DEPC-treated H_2O, 1 μl of cDNA for the unknowns, or 1 μl of DNA from the dilution series of each bovine TPM TA clones for the standards, or 1 μl of H_2O for the primer control. To verify the specificity of the primer pair, PCR products were run on an agarose gel after real-time analysis. For qRT-PCR of TPM1α, TPM2α, TPM3α, and TPM4α, cDNA for each isoform was made with the corresponding gene and isoform-specific oligonucleotide designed from the exon 9 A/B of the respective TPM genes. The strategy of qRT-PCR was used for maintaining the specificity (or avoiding the cross amplification) of the highly conserved genes like TPMs. The nucleotide sequences for isoform-specific oligonucleotides used for making cDNA are given in **Table 1**.

qRT-PCR was carried out to determine absolute copy numbers using a standard curve of TPM1α, TPM2α, TPM3α, TPM4α, and TPM4ε RNAs from adult bovine heart and skeletal muscle. First, optical density was taken of the corresponding TPM TA clone plasmid using a spectrophotometer. The copy number per volume of cloned DNA in solution was determined using the equation, number of copies (ng of plasmid DNA × 6.02 × 10^{23})/(bp length of plasmid 1 × 10^9 × 650), which was simplified by Andrew Staroscik at the URI Genomics and Sequencing Center. A dilution series of each clone was done for 1 × 10^1-1 × 10^6 copies of template, which would be used to create a standard curve post amplification. iQ1 SYBR green supermix (Bio-Rad) and a Bio-Rad iCycler thermocycler were used and melt curve was determined using manufacturer's protocol.

2D Western blot analysis of commercially available bovine heart and skeletal muscle extracts

2D (Two-dimensional) analyses of the commercial bovine whole heart (Cat # BT-801) and skeletal muscle (Cat # BT-102) extracts were obtained from Zyagen (California) were performed for us by Kendrick Labs, Inc. (Madison, WI). Again, we do not know the gender, breed or muscle type of the sample source. Further, the protein samples are not matched with the previously described RNA samples. Kendrick Labs' procedures that we have published several times [3, 20] are as follows: The protein concentrations of the samples were determined using the BCA Assay [21, and Pierce Chemical Co., Rockford, IL]. Two-dimensional electropho-

resis was performed according to the carrier ampholine method of isoelectric focusing [21, 22] by Kendrick Labs, Inc. (Madison, WI) as described previously [3, 20]. The blots were incubated in primary antibody (CH1 monoclonal antibody) diluted in 2% NFDM TTBS overnight and rinsed 3-10 min in TTBS. Blot #1 was placed in secondary antibody (anti-mouse IgGHRP [GE Healthcare, Cat # NA 931V, Lot # 399402] diluted 1:2,000 in 2% NFDM TTBS) for 2 h, rinsed as above, treated with Enhanced ChemiLuminescence (ECL), and exposed to x-ray film.

The ECL reagent used during Western blotting was Pierce™ ECL Western Blotting Substrate (Cat. # 32106). Image from films were captured with an Agfa Arcus 1200 scanner using Agfa FotoLook software. The CB stained gels and CB stained PVDF membranes were captured with a GE ImageScanner III running SilverFast software (v. 6.6.0r5).

Spots identified were subjected to subsequent mass spectral analyses as follows.

LC-MS/MS

The peptides mixture was analyzed by reverse phase liquid chromatography (LC) and MS (LC-MS/MS) using a NanoAcuity UPLC (Micromass/Waters, Milford, MA) coupled to a Q-TOF Ultima API MS (Micromass/Waters, Milford, MA), according to published procedures [23-25]. The entire procedure used was previously described [3, 20].

Data processing and protein identification

The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software. The following parameters were used: background subtraction of polynomial order 5 adaptive with a threshold of 30%, two smoothings with a window of three channels in Savitzky-Golay mode and centroid calculation of top 80% of peaks based on a minimum peak width of 4 channels at half height. The resulting pkl files were submitted for database search and protein identification to the in-house Mascot server (www.matrixscience.com, Matrix Science, London, UK) for database search using the following parameters: databases from NCBI (Bovine), parent mass error of 0.5 Da with 1 ^{13}C, product ion error of 0.8 Da, enzyme used: trypsin, three missed cleavages, propionamide as cysteine fixed modification and Methionine oxidized as variable modification. To identify the false negative results, we used additional parameters such as different databases or organisms, a narrower error window for the parent mass error (1.2 and then 0.2 Da) and for the product ion error (0.6 Da), and up to two missed cleavage sites for trypsin. In addition, the pkl files were also searched against in-house PLGS database version 2.4 (www.waters.com) using searching parameters similar to the ones used for Mascot search. The Mascot and PLGS database search provided a list of proteins for each gel band. To eliminate false positive results, for the proteins identified by either one peptide or a mascot score lower than 25, we verified the MS/MS spectra that led to identification of a protein.

Statistical analysis and interpretation of Mascot results from Mascot database search using Mascot server. Database search operated independent on the investigator. The only parameters that can be modified in database search are the number and type of the fixed and variable modifications, error of the precursor and product ions, number of ^{13}C atoms and number of missed trypsin cleavages. In standard proteomics experiments, we use default parameters: parent mass error of 0.5 Da with 1 ^{13}C, product ion error of 0.8 Da, enzyme used: trypsin, three missed cleavages, propionamide as cysteine fixed modification and Methionine oxidized as variable modification. Therefore, most of the probability-based analysis is done automatically by the Mascot software.

Results

Cloning and sequencing of TPM4α and a new splice variant of the TPM4 gene (TPM4ε) from RNA of bovine heart and skeletal muscle

The nucleotide as well as deduced amino acid sequences of bovine TPM1α, TPM2α, and TPM3α are already published. However, the nucleotide sequence of bovine TPM4α is not in the literature. Hence, we decided to clone and sequence TPM4α cDNA from bovine striated muscles. We designed a number of primer-pairs for RT-PCR amplification using the predicted *Bos taurus* tropomyosin 4 sequences (Accession number: XM-005208510) with cDNAs made from the RNA of bovine heart and skele-

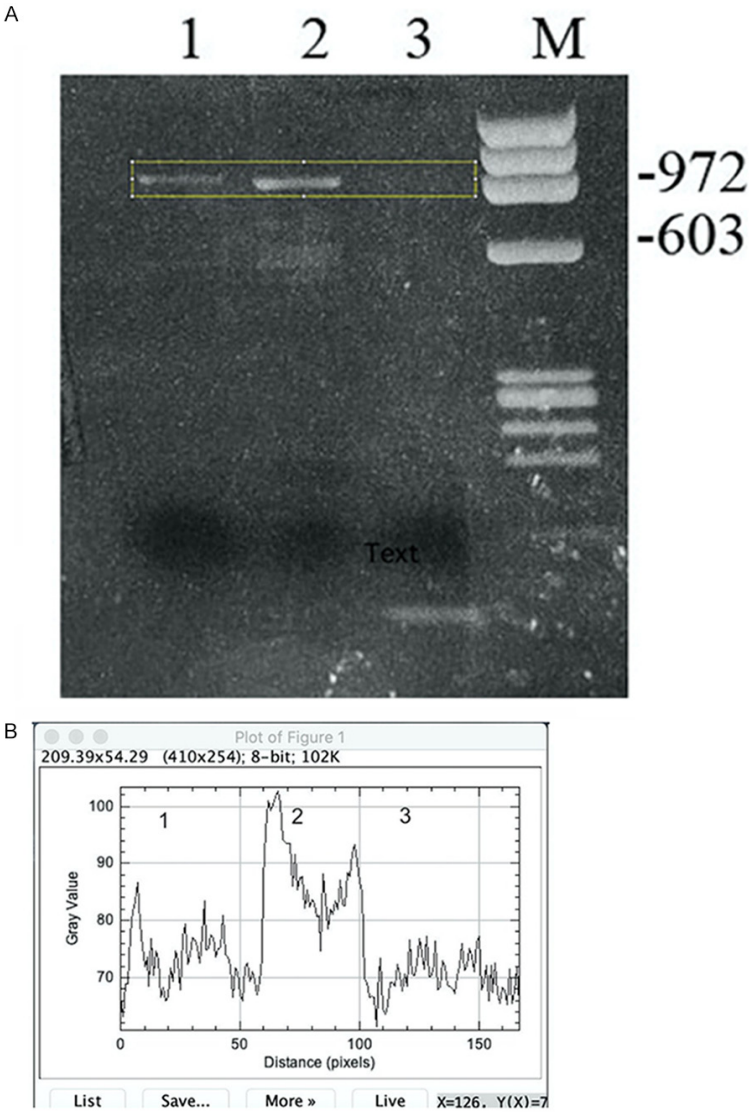


Figure 1. A. Amplification of tpm4α by conventional rt-pcr of cdna from bovine heart and skeletal muscle. CDNAs were made with RNA from bovine heart and skeletal muscle with oligodT as described under Materials and Methods. TPM4α/n (+) and/TPM4α/n (-) primer pair was used for PCR amplification. Sequences of primer-pair are shown in **Table 1**. Lane 1: heart; Lane 2: skeletal muscle, and Lane 3: negative control. ~972 high molecular weight bands were excised for extraction of amplified DNA as stated under Materials and Methods. Gel extracted DNAs were cloned in T/A cloning vector (Company) as described earlier (Dube et al., 2016). DNA were extracted for subsequent nucleotide sequence determination as described under Materials and Methods. B. Grayscale analysis of **Figure 1A** using Plot profile software for image analysis.

tal muscle. The results depicted in **Figure 1A** show the correct size band of the amplified DNA in both heart (lane 1) and skeletal muscle (lane 2). **Figure 1B** depicts the gray scale analysis for **Figure 1A**. As this is a qualitative conventional RT-PCR, we do not prefer to quantify the expression of TPM4α in bovine heart and skeletal muscle. However, we extracted the amplicons from the gel and determined the nucleotide sequences of the DNA from both lanes. Direct sequencing was not ideal, yet it gave enough information to search the data base for sequences similar to existing bovine TPM4 sequences. Once we confirmed the sequences, we cloned each PCR hyper-amplified DNA and picked 10 colonies from each amplified DNA clone, isolated DNA, and determined nucleotide sequences. From heart clones we found 4 out of 10 having full length TPM4α nucleotides as well as deduced amino acid sequences identical to the predicted sequences in the data base (XM-005208510) (**Figure 2A**). We found two identical clones having a larger open reading frame compared to the predicted TPM4α sequences although both 5'- and 3'-ends are identical with TPM4α sequences. There is an insertion of 48 nucleotides between exon 8 and exon 9a (**Figures 2B** and **3A**). This insertion however did not cause a premature termination; rather the open reading frame encoded 16 additional amino acid residues between exon 8 and exon 9a (**Figures 2B** and **3B**). After extensive data base searching we found out that the inserted 48 nucleotides are coming from the 3'-end of the intron in between exon 8 and exon 9a. The model of the new splicing is shown in **Figure 4A**. Dr. S.H. Rangwala, ReSeq Scientist, NCBI contractor, suggested that the insertion begins with an "AG" which can align at either end. The insertion in this variant may represent alternate splicing with the terminal exon that initiates at a different earlier position. Based on this argument, we

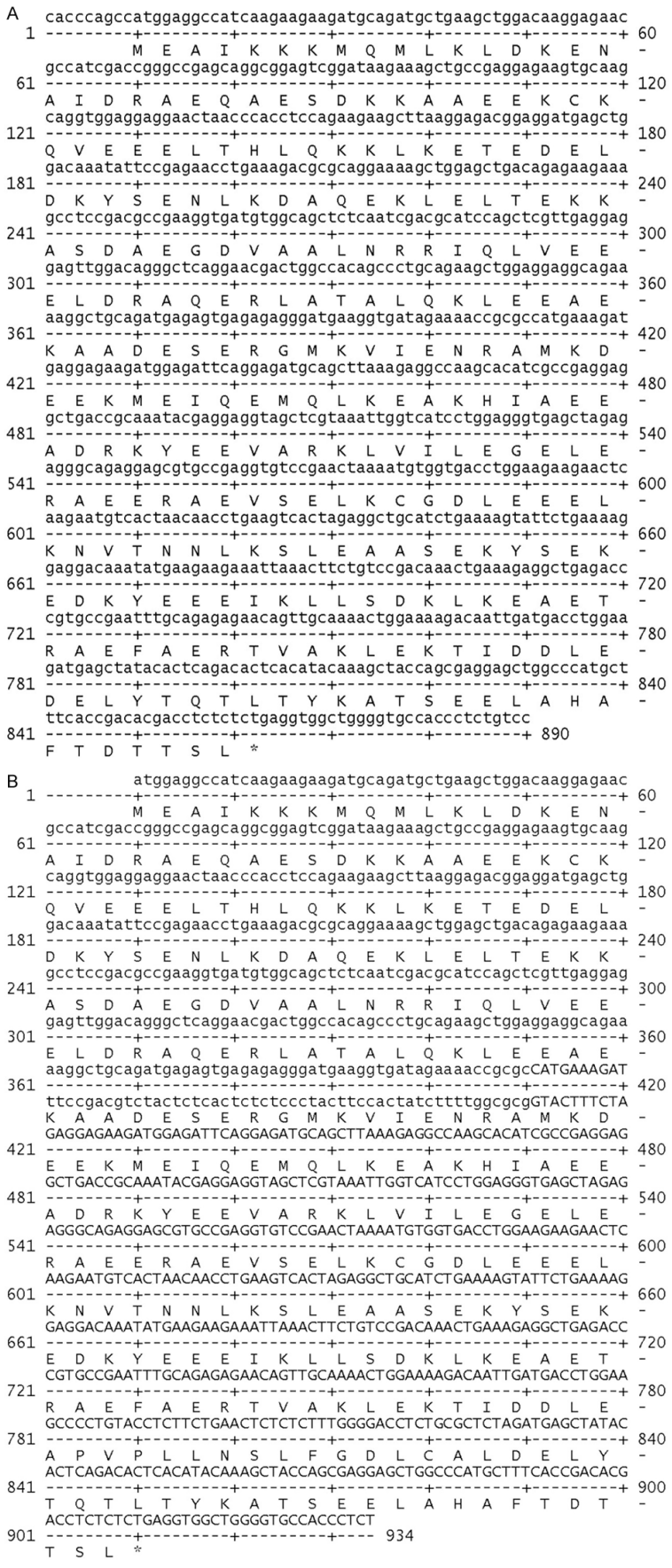


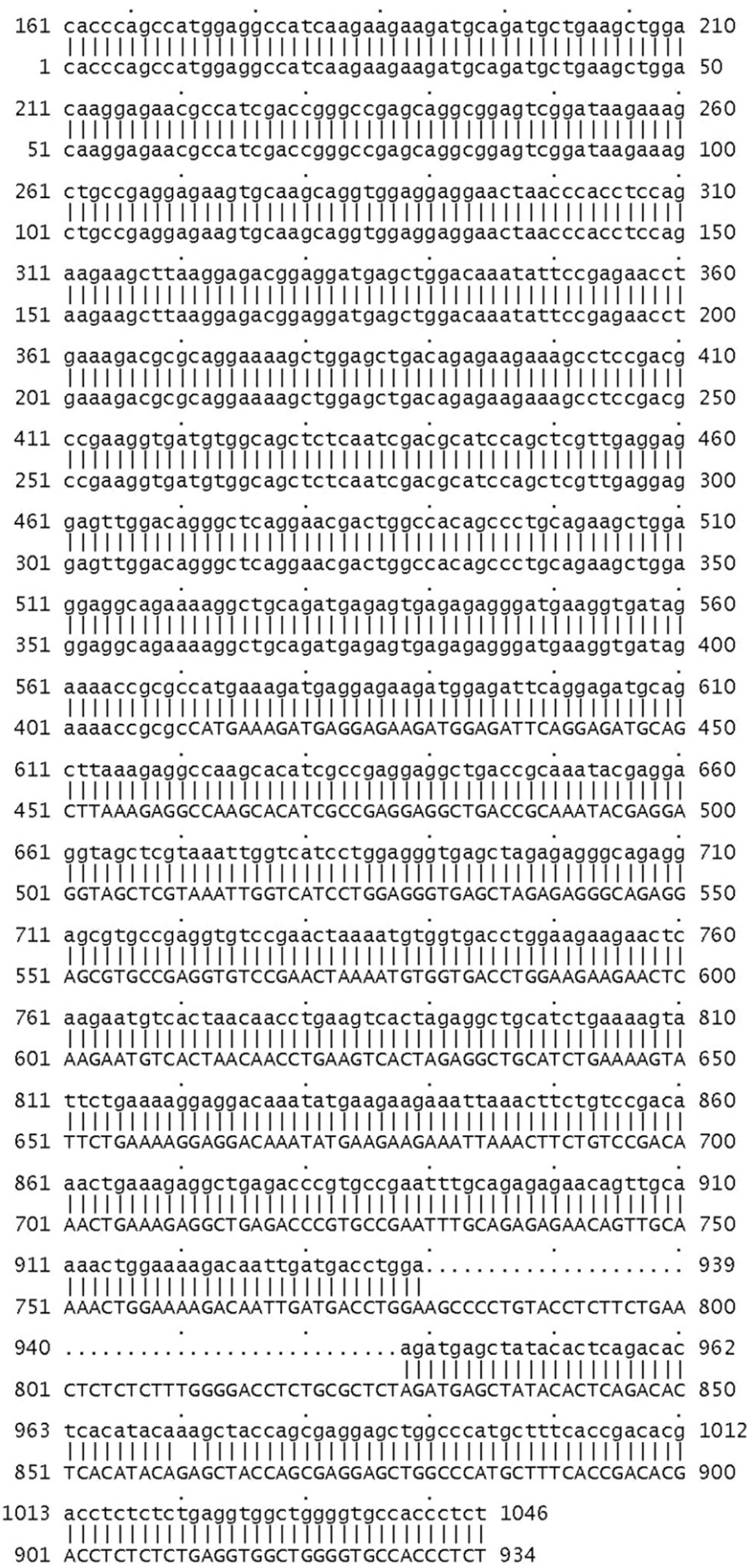
Figure 2. Nucleotide and deduced amino acid sequences of TPM4α (A) and TPM4ε (B).

have drawn a schematic diagram for the alternate splicing pattern of the *TPM4* gene of *Bos taurus* as shown in **Figure 4B**. One may ask the question whether this is unique for *Bos taurus* or is it universal for the mammalian TPM4 gene. We did extensive data base searches for the sequences of the intron in between exon 8 and exon 9a that may contain similar "AG" (as shown in bold in **Figure 4C**) sequences that may lead to similar alternate splicing of the TPM4 gene in other mammals. The information depicted in **Figure 4C** show that in most species actual splicing take place with the second AG (at the end) (**Figure 4C**, Top line). But in *Bos taurus* there is another AG (at the beginning) that may have caused the new splice variant of the *TPM4* gene.

Amplification and detection of TPM1α and TPM1κ cDNAs with RNA from bovine heart and skeletal muscle

Sarcomeric TPM1α cDNA from bovine striated muscle has long been cloned and sequenced (Accession # NM-001013-590.2). Although the predicted nucleotide sequences of TPM-1κ, the other sarcomeric isoform of the TPM1 gene, has been posted in GenBank (Accession number: XP0248530-22.1), no one has yet reported whether TPM1κ mRNA or protein is expressed in bovine tissues. Hence, following our published protocols for RT-PCR and nested RT-PCR [15, 26] we attempted to detect whether TPM1κ mRNA is

A



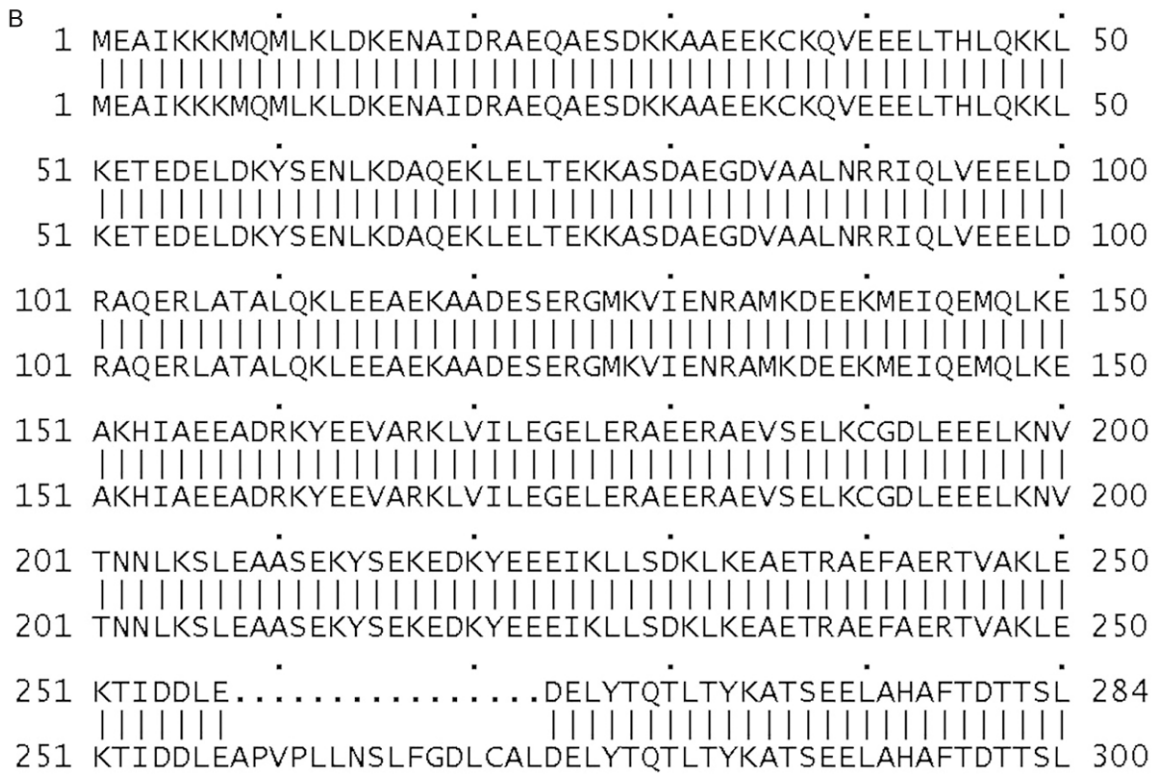


Figure 3. Alignment of nucleotide and peptide sequences of TPM4ε with predicted bovine TPM4 sequences. A. Alignment of TPM4ε nucleotide sequences with the nucleotide sequences of the predicted TPM4α sequences. B. Alignment of the peptide sequences of TPM4α (as in **Figure 2B**) which is identical with predicted sequences available from the data base with the deduced amino acid sequences of TPM4ε as in **Figure 2A**.

expressed in bovine striated muscles. The primer-pairs and probes were designed from the predicted TPM1κ cDNA sequence (Predicted XP024853022.1) and are shown in **Table 1** along with primer-pairs for TPM1κ. It is to be noted that these two isoforms, TPM1α and TPMκ, are only different in exon 2-the former contains exon 2B and the latter contains exon 2A. Although we successfully cloned and sequenced TPM1α, we failed to detect/amplify TPM1κ. We believe TPM1κ transcripts are either not expressed or are expressed in such a low quantity that we have failed to detect them by conventional and nested RT-PCR. Hence, we have excluded TPM1κ in subsequent expression analyses by qRT-PCR.

Relative expression of TPM4α and TPM4ε transcripts in bovine heart and skeletal muscle by qRT-PCR using 2^DCt and 2^(-DDCt) methods

We performed qRT-PCR for determining the relative expression of TPM4α and TPM4ε. We analyzed our results by both 2^DCt (Top, **Figure 5**) and 2^(-DDCt) (Bottom, **Figure 5**) methods using 18S rRNA as the reference gene. The data derived from both methods demonstrate that TPM4α transcript expression is about 5-fold higher in bovine hearts compared to skeletal muscle.

In contrast, the expression of the newly discovered TPM4ε transcripts is about 3-fold higher in bovine skeletal compared to cardiac muscles using both DCt (Top, **Figure 6**) and DDCt methods (Bottom, **Figure 6**).

Relative expression of TPM1α, TPM2α, and TPM3α in bovine heart and skeletal muscle as determined by qRT-PCR using 2^(-DDCt) method

The results depicted in **Figure 7** show that the expression of TPM1α is significantly lower in bovine skeletal muscle compared to cardiac muscle. On the contrary, the expression level of TPM2α transcript is 2.6 fold higher in skeletal muscle compared to cardiac muscle. However, the expression level of TPM3α in bovine heart and skeletal muscles is comparable.

A

```
818 aaggaggacaaatatgaagaagaaattaaacttctgtccgacaaactgaa 867
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
636 AAGGAGGACAAATATGAAGAAGAAATTAAACTTCTGTCCGACAAACTGAA 685

868 agaggctgagacccgtgccgaatttgcagagagaacagttgcaaaactgg 917
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
686 AGAGGCTGAGACCCGTGCCGAATTTGCAGAGAGAACAGTTGCAAAACTGG 735

918 aaaagacaattgatgacctgga.............................. 939
    ||||||||||||||||||||||
736 AAAAGACAATTGATGACCTGGAAGCCCCTGTACCTCTTCTGAACTCTCTC 785
```

←----Part of Intron between

End Exon 8 | New Splice Junction

```
940 .....................agatgagctatacactcagacactcacata 969
                         ||||||||||||||||||||||||||||||
786 TTTGGGGACCTCTGCGCTCTAGATGAGCTATACACTCAGACACTCACATA 835
```

exon 8 and exon 9a→ | ←-----Exon 9a

```
970 caaagctaccagcgaggagctggcccatgctttcaccgacacgacctctc 1019
    ||  ||||||||||||||||||||||||||||||||||||||||||||||||
836 CAGAGCTACCAGCGAGGAGCTGGCCCATGCTTTCACCGACACGACCTCTC 885

1020 tctgaggtggctggggtgccaccctct 1046
     |||||||||||||||||||||||||||
 886 TCTGAGGTGGCTGGGGTGCCACCCTCT 912
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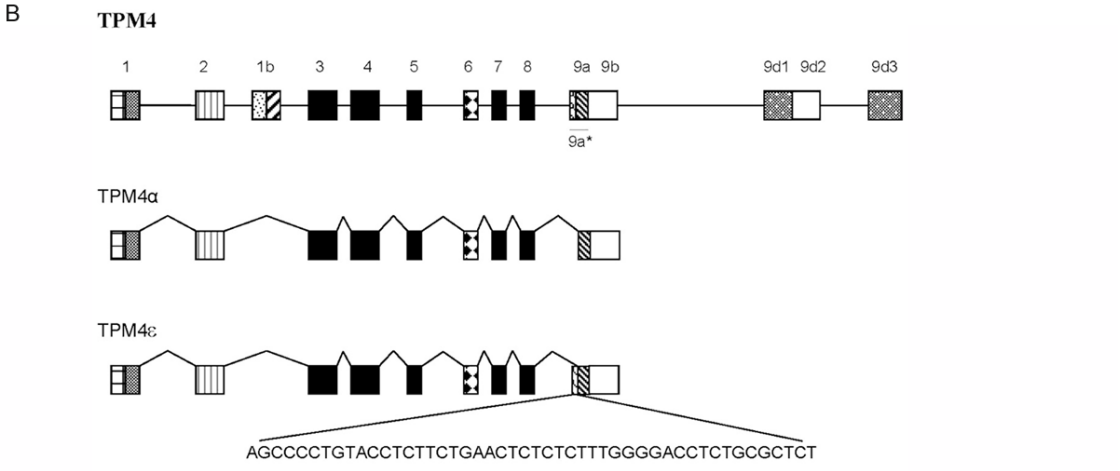
B



9a* has a part of intron along with 9a

C

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Bos taurus [1]          AGCCCCTGTACCTCTTCTGAACTCTCTCCTTGGGGACCTCTGCGCTCTAGAT
Ovis canadensis[2]      .C.....................................C.......C...C...C
Human TPM4[3]           GT.........---..........G.....C.........C..C.........
```

Figure 4. Novel splice junction in TPM4ε isoform. A. Depicts the new splice junction that include 48 nucleotides from the intron between exon 8 to exon 9a. B. Diagram exhibiting the known splice patterns for TPM4α and TPM4ε. In TPM4v 48 nucleotides are inserted joining exon 8 and exon 9a. C. Alignment of the nucleotide sequences of the intron preceding exon 9a and following exon 8 of the TPM4 gene of various organisms. [1]Bos taurus breed Hereford chromosome 7, Bos_taurus_UMD_3.1.1 Sequence ID: AC_000164.1 Length: 112638659 Number of Matches: 1 Range 1: 7927965 to 7928012. [2]Ovis canadensis canadensis isolate 43U chromosome 5 sequence. Sequence ID: CP011890.1 Length: 108026203 Number of Matches: 1. Range 1: 6659099 to 6660090. [3]Homo sapiens chromosome 19, GRCh38.p7 Primary Assembly. Sequence ID: NC_000019.10 Length: 58617616 Number of Matches: 1 Range 1: 16094557 to 16095265.

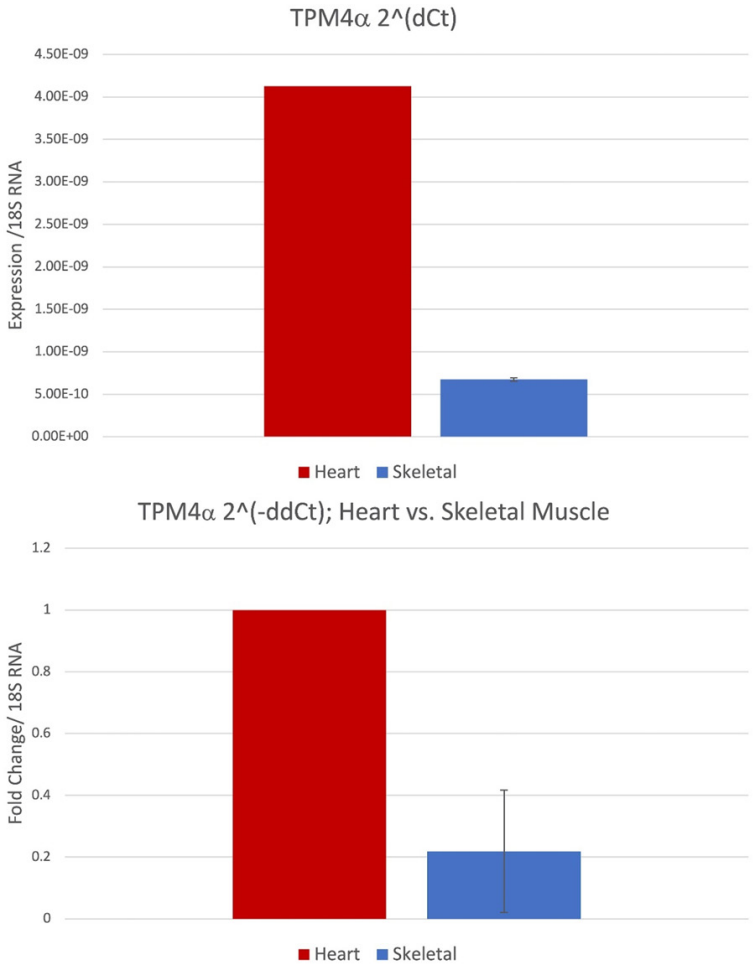

Figure 5. Relative expression of sarcomeric TPM4α transcripts in adult bovine heart and skeletal muscle using 2^{DCt} and 2^{-DDCt} methods. Top: $2^{\Delta Ct}$ method. 18S rRNA was used for normalization. Bottom: 2^{-DDCt} method. Fold changes in adult heart versus skeletal where 18S rRNA is the reference gene. Red bar: Heart and Blue bar: skeletal muscle. The results for the heart were considered to equal one. TPM4α (+)/TPM4α (-) and 18S rRNA (+)/18S rRNA (-) primer-pairs were used for amplification of TPM4α and 18S rRNA, respectively. The nucleotide sequences of each of the primers are given in **Table 1**.

Determination of absolute copy number of various sarcomeric TPM isoforms in bovine heart and skeletal muscle

The quantification of copy number of different TPM isoforms was carried out using our published protocol [26, 27]. The standard curves for different isoforms are not shown. The results presented in **Table 2** show the absolute copy number for each isoform in heart and skeletal muscle. The copy number of TPM1α per mg of total RNA is ~100 fold higher in bovine heart compared to bovine skeletal muscle. The copy number data agrees with the relative expression results depicted in **Figure 7**. Also, the copy numbers of TPM3α and TPM4α expressed in bovine heart are slightly higher than in skeletal muscles. In contrast, the expression levels TPM2α and TPM4ε are higher in bovine skeletal muscle. Most importantly, qRT-PCR data obtained by absolute copy number determination are consistent with the relative expression analyses using both 2^DCt and 2^(-DDCt) methods. Within the heart sample the rank order of expression of the various sarcomeric isoforms were TPM1α>TPM3α>TPM2α>TPM4α>TPM4ε. Within the skeletal muscle sample, the order was TPM2α>TPM3α>TPM4α>TPM4ε>TPM1α.

2D Western blot analyses of bovine heart protein extract with CH1 monoclonal antibody that reacts with all sarcomeric TPMs and subsequent mass spectroscopy

Our two-dimensional Western blot analysis was carried out with commercially available adult bovine heart extract. Comparing the Coomassie

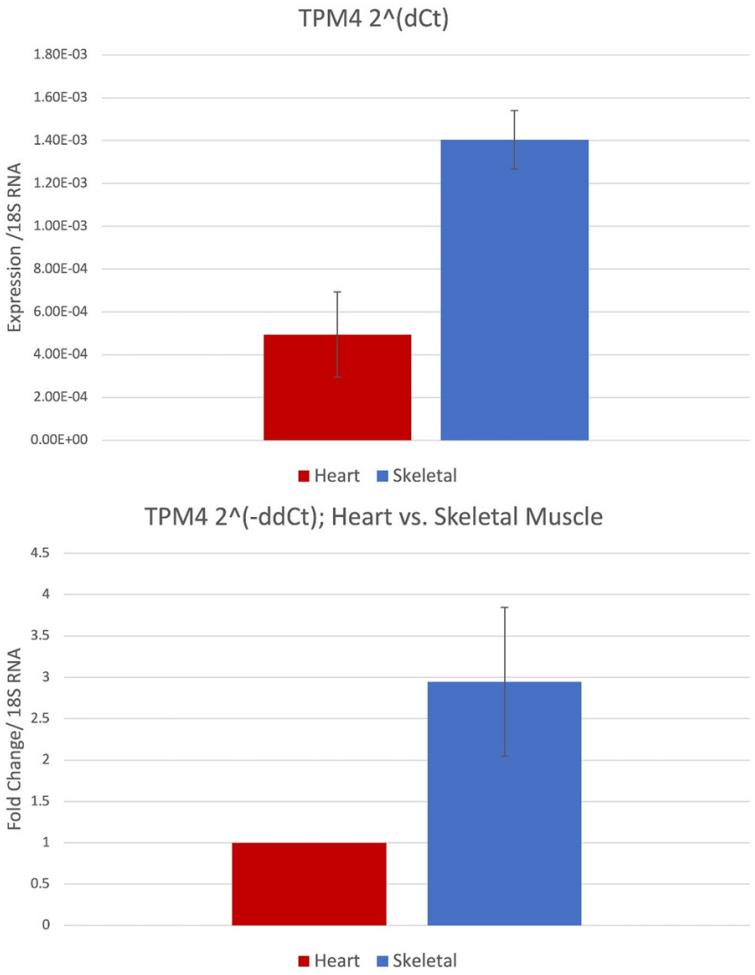

Figure 6. Relative expression of TPM4ε transcripts in adult bovine heart and skeletal muscle using and 2^{DDCt} and 2^{DCt} methods. Top: 2^{DCt} method. 18S rRNA was used for normalization. Bottom: 2^{-DDCt} method. Fold changes in adult heart versus skeletal where 18S rRNA is the reference gene. Red bar: Heart and Blue bar: skeletal muscle. The results for the heart were considered to equal one. For amplification of TPM4ε and 18S rRNA, TPM4ε (+)/TPM4ε (-) and 18S rRNA (+)/18S rRNA (-) primer pair was used respectively. The sequences of primers are furnished in **Table 1**.

stained blot (Top **Figure 8**) with the developed x-ray film (Bottom of **Figure 8**), three spots were identified which are CH-1 positive and marked as A, B, and C, each of which represent high molecular weight TPM. The results suggest that there would be more than one CH1-positive, high molecular weight tropomyosins expressed in bovine heart. Each of the protein spots that were separated by 2D PAGE were processed for LC-MS/MS analyses.

In spot A, the presence of TPM1 was indicated by a total of 8 peptides, of which 2 peptides were unique to TPM1α and the remaining peptides were common to various known high molecular weight TPM isoforms (TPM1, TPM2, TPM3, and TPM4), which have the potential to react with CH1 monoclonal antibody. None of the peptides were unique to TPM2, TPM3, or TPM4 (**Table 3**). The peptides which matched with bovine TPM1α sequences are shown in red color in **Table 5**.

In Spot B, 61 peptides were found to match with TPM1 proteins and six are unique to TPM1.The alignment of these peptides with TPM1α are exhibited in **Table 5**. None of these TPM peptides are unique to either TPM2, TPM3, and TPM4 (**Table 3**). Hence, there was no evidence for the presence of TPM2, TPM3, and TPM4 protein in Spot B.

In Spot C, however, we found that 3 peptides out of fifteen are unique to TPM1 and only one peptide out of 8 is unique to TPM2 (**Table 3**). Hence, mass spectra analyses revealed the presence of TPM1α and TPM2α proteins in this spot (**Tables 3** and **5**). There was no evidence for TPM3 or TPM4 protein. On the basis of gene specific peptide(s) found in all three spots, we have calculated that 91.66% of tropomyosin protein in this bovine heart extract is TPM1 and 8.33% is TPM2 (**Table 3**).

2D Western blot analyses of bovine skeletal muscle protein extract with CH1 monoclonal antibody followed by LC-MS/MS analysis

Figure 9 depicts the 2D Western blot analysis of the protein extracts from adult bovine skeletal muscle where Top of **Figure 9** represents the Coomassie stained skeletal muscle proteins across the gel and Bottom of **Figure 9** represents the developed X-ray film after ECLing

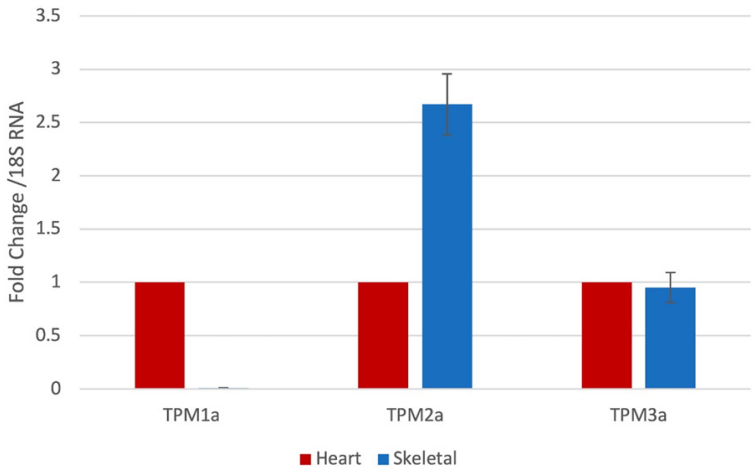

Figure 7. Relative expression of TPM1α, TPM2α and TPM3α transcripts in adult bovine heart and skeletal muscle using 2^{-DDCt} method. Heart in Red bar was considered as 1 and Blue bars are for skeletal muscle. 18S rRNA was assessed as reference gene. Primer-pairs used for amplification of various TPMs are as follows: TPM1α (+)/TPM1α (-) for TPM1α; TPM2α (+)/TPM2α (-) for TPM2α; TPM3α (+)/TPM3α (-) for TPM3α. The nucleotide sequences of primer-pairs are provided in **Table 1**.

Table 2. Absolute copy number of various TPM isoforms expressed in bovine heart and skeletal muscle

Isoform	Copy number per mg total RNA		Ratio H/S
	Heart (H)	Sk muscle (S)	
TPM1α	$1.12 \times 10^6 \pm 9.64 \times 10^3$	$1.02 \times 10^4 \pm 1.08 \times 10^2$	101
TPM2α	$8.5 \times 10^5 \pm 4.4 \times 10^4$	$1.50 \times 10^6 \pm 7.8 \times 10^4$	0.566
TPM3α	$1.02 \times 10^6 \pm 4.15 \times 10^4$	$7.8 \times 10^5 \pm 7.35 \times 10^4$	1.3
TPM4α	$1.79 \times 10^5 \pm 6.3 \times 10^2$	$1.49 \times 10^5 \pm 7.87 \times 10^2$	1.2
TPM4ε	$0.86 \times 10^4 \pm 0.33 \times 10^2$	$1.59 \times 10^4 \pm 0.79 \times 10^2$	0.54

the PVDF filter onto which the gel proteins were transferred. Comparing these two figures, five spots were identified and marked as A, B, C, D, and E. The results suggest the presence of several CH-1 positive high molecular TPMs in bovine skeletal muscle. The extracted proteins from each of these spots were subjected to mass spectra analyses as described under method section.

In Spot A, 19 TPM1 peptides were detected. However, one peptide was unique to TPM1α and the rest of the peptides were common to other known TPMs. Four out of 31 TPM2 peptides were unique to TPM2α. No unique peptide for TPM3 and TPM4 was identified in spot A (**Table 4**). The peptides which matched TPM1α and TPM2α are shown in red color in **Table 6**.

In Spot B, 37 peptides were matched with TPM1 proteins and four of them were unique to TPM1 (**Tables 4** and **6**). On the other hand, although 14 peptides in this spot matched with TPM2 protein (as shown in **Tables 4** and **6**), and only one was unique for TPM2 (**Table 4**). No unique peptide for either TPM3 or TPM4 was detected in Spot B (**Table 4**).

In Spot C, three out of 24 peptides (**Tables 4** and **6**) that matched TPM1 were unique to TPM1 protein whereas one out of 10 peptides (**Tables 4** and **6**) was found to be unique to TPM2 (**Table 4**). No unique peptide was detected for either TPM3 or TPM4 protein.

Although, we initially identified five CH-1 positive spots by comparing the Coomassie stained gel with the developed x-ray film, extracted peptides from Spots D and E upon LC-MS/MS analyses, yielded no TPM specific peptides. One plausible explanation for this anomaly is that Spots D & E were very close to the other three CH-1 positive spots (A, B, and C) (**Figure 9**). As a result, Spots D and E were under the halo of the strong ECL signals from Spots A, B, and C, which misled the identification of Spots D & E as CH-1 positive. Alternatively, one may argue that CH-1 monoclonal antibody is recognizing some other protein(s) in Spot D and E. It is to be noted that CH-1 is well established anti-sarcomeric TPM antibody that recognizes the epitope present in exon 9a of all TPM genes (Developmental Studies Hybridoma Bank, dhsb.biology.uiowa.edu/CH1). We have performed similar analyses many times with different systems, for example chicken [15], humans [27], zebrafish [3], horse [28], and we have never found that CH-1 interacts with any protein(s) other than TPM. Hence, there were probably only two high molecular weight TPMs in the skeletal muscle. In the skeletal muscle

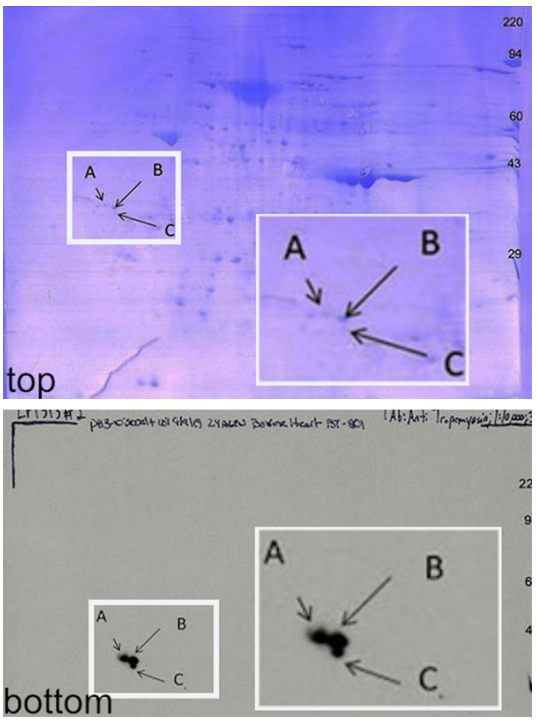

Figure 8. 2D western blot analyses with extracts from adult bovine heart. Top. The Coomassie stained bovine adult cardiac muscle protein across the gel. Bottom. The PVDF filter was stained with CH1 monoclonal antibody followed by treatment with secondary antibody as stated under materials and methods, and subsequently treated with ECL and exposed to x-ray film. Developed X-ray film was superimposed on the top of the Coomassie stained second gel as well as on the Coomassie stained PVDF filter. Three spots A, B, and C were marked, excised and were used for extraction of protein for subsequent mass spectrometric analyses. The box at the left has been zoomed and inserted at the right side.

sample 57.1% of the unique peptides were TPM1α and 42.9% were TPM2α.

Discussion

Cardiac muscles function solely for pumping blood away from the heart towards the lungs and throughout the body. As in skeletal muscles, myofibrils, the contractile apparatus of myocardium, consist of several proteins. There is not much information in the literature about expression pattern(s) of various myofibrillar proteins, for example tropomyosin, in bovine cardiac muscles. This is probably the only report about tropomyosin expression in bovine cardiac tissues. In this study, we detected and characterized the expression of sarcomeric TPM transcripts, one each from TPM1, TPM2 and TPM3 and two from TPM4. Sequencing identified these as TPM1α, TPM2α, TPM3α, TPM4α, and the novel transcript TPM4ε. However, we have failed to detect the expression of another sarcomeric TPM1 known as TPM1κ.

The expression level of each of the five TPM transcripts are given in **Table 2**. The expression of TPM1α, TPM3α and TPM4α transcripts is higher in bovine hearts, whereas, TPM2α and TPM4ε expression is higher in skeletal muscle. As far as protein expression in the bovine cardiac muscle is concerned, we have found that 91.66% of total TPM protein is TPM1α and the rest 8.33% is TPM2α. Our results are in good agreement with the published literature in mammals including humans [29] with the exception of the absence of TPM1κ protein in bovine cardiac muscle. Interestingly, although we have detected TPM3α, TPM4α, and a new variant of TPM4 transcripts in bovine cardiac muscles, we were unable to detect any translational product of any of these RNAs.

In addition to their importance in body movement bovine skeletal muscles are one of the major sources of meat for humans. In order to better understand the relationship between meat quality, the relationship between muscle type and the ratio of various TPM isoforms, Oe et al. [12], using conventional RT-PCR, determined the percentage of different TPM transcripts in various bovine skeletal muscles types for example *masseter* (MS), *diaphragm* (DP), *psoas major* (PM), *longissimus thoracis* (LT) and *semitendinosus* (ST). They detected dissimilar percentages of each TPM transcript in different skeletal muscles. For example, the percentage of TPM1 in MS is zero but it is highest in ST (31.7%). The percentage of TPM2 in all tissues was around 50%. TPM3 transcripts on the other hand varied in different skeletal muscles. These authors did not look for TPM4 transcripts in any skeletal muscle type.

Oe et al. [13] studied the distribution of TPM isoforms in different types of single fibers in bovine skeletal muscle extensively. It is to be noted that isoforms of myosin heavy chain (MyHC) are major myofibrillar proteins, and are in control of different fiber types [11]. Oe et al. [13] reported that in some slow muscle (for example MS) fibers, where slow-MyHC is

Table 3. Tropomyosin peptides identified in bovine heart

SPOT #	TPM1			TPM2			TPM3			TPM4		
	Total	Unique	Isoform	Total	Unique	Isoform	Total	Unique	Isoform	Total	Unique	Isoform
A	8	2	TPM1α	5	0	0	5	0	0	5	0	0
B	61	6	TPM1α	32	0	0	26	0	0	17	0	0
C	15	3	TPM1α	8	1	TPM2α	5	0	0	4	0	0
Overall Unique Peptides		11	-	-	1		-	-			-	
% TPM in whole heart		91.66			8.33			0		0		

Table 4. Tropomyosin peptides identified in bovine skeletal muscle

SPOT #	TPM1			TPM2			TPM3			TPM4		
	Total	Unique	Isoform	Total	Unique	Isoform	Total	Unique	Isoform	Total	Unique	Isoform
A	19	1	TPM1α	31	4	TPM2α	14	0	0	13	0	0
B	37	4	TPM1α	20	1	TPM2α	14	0	0	8	0	0
C	24	3	TPM1α	10	1	TPM2α	9	0	0	8	0	0
Overall Unique Peptides		8	-	-	6		-	-			-	
% TPM in whole heart		57.14			42.87			0		0		

Table 5. Identification of amino acid sequences from the peptides extracted from Spots A, B, and C after 2D western blot analyses of adult bovine heart protein with CH1 monoclonal antibody

Spot #	TPM Isoform and peptide Sequences identified
A	TPM1α (Accession # gI|57281697) (17.2% identified)
	1 MDAIKKKMQM LKLDKENALD RAEQAEADKK AAEDRSKQLE DELVSLQKKL KATEDELDKY
	61 SEALKDAQEK LELAEKKATD AEADVASLNR RIQLVEEELD RAQERLATAL QKLEEAEKAA
	121 DESERGMKVI ESRAQKDEEK MEIQEIQLKE AKHIAEDADR KYEEVARKLV IIESDLERAE
	181 ERAELSEGKC AELEEELKTV TNNLKSLEAQ AEKYSQKEDK YEEEIKVLSD KLKEAETRAE
	241 FAERSVTKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI
B	TPM1α (Accession # gI|57281697) (46.1% sequences identified)
	1 MDAIKKKMQM LKLDKENALD RAEQAEADKK AAEDRSKQLE DELVSLQKKL KATEDELDKY
	61 SEALKDAQEK LELAEKKATD AEADVASLNR RIQLVEEELD RAQERLATAL QKLEEAEKAA
	121 DESERGMKVI ESRAQKDEEK MEIQEIQLKE AKHIAEDADR KYEEVARKLV IIESDLERAE
	181 ERAELSEGKC AELEEELKTV TNNLKSLEAQ AEKYSQKEDK YEEEIKVLSD KLKEAETRAE
	241 FAERSVTKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI
C	TPM1α ((Accession # gI|57281697) (28.1% sequences identified)
	1 MDAIKKKMQM LKLDKENALD RAEQAEADKK AAEDRSKQLE DELVSLQKKL KATEDELDKY
	61 SEALKDAQEK LELAEKKATD AEADVASLNR RIQLVEEELD RAQERLATAL QKLEEAEKAA
	121 DESERGMKVI ESRAQKDEEK MEIQEIQLKE AKHIAEDADR KYEEVARKLV IIESDLERAE
	181 ERAELSEGKC AELEEELKTV TNNLKSLEAQ AEKYSQKEDK YEEEIKVLSD KLKEAETRAE
	241 FAERSVTKLE KSIDDLEDEL YAQKLKYKAI SEELDHALND MTSI
	TPM2α (Accession # gi|57281699) (21.47% sequences identified)
	1 MDAIKKKMQM LKLDKENAID RAEQAEADKK QAEDRCKQLE EEQQALQKKL KGTEDEVEKY
	61 SESVKDAQEK LEQAEKKATD AEADVASLNR RIQLVEEELD RAQERLATAL QKLEEAEKAA
	121 DESERGMKVI ENRAMKDEEK MELQEMQLKE AKHIAEDSDR KYEEVARKLV ILEGELERSE
	181 ERAEVAESKC GDLEEELKIV TNNLKSLEAQ ADKYSTKEDK YEEEIKLLEE KLKEAETRAE
	241 FAERSVAKLE KTIDDLEDEV YAQKMKYKAI SEELDNALND ITSL

Red color letters indicate peptide sequences identified by mass spectra.

expressed, sarcomeric TPM2 and TPM3 (~71% and 29%) were the major TPM isoforms present, while TPM1 expression was practically absent. On the contrary, in ST, where 2a-MyHC

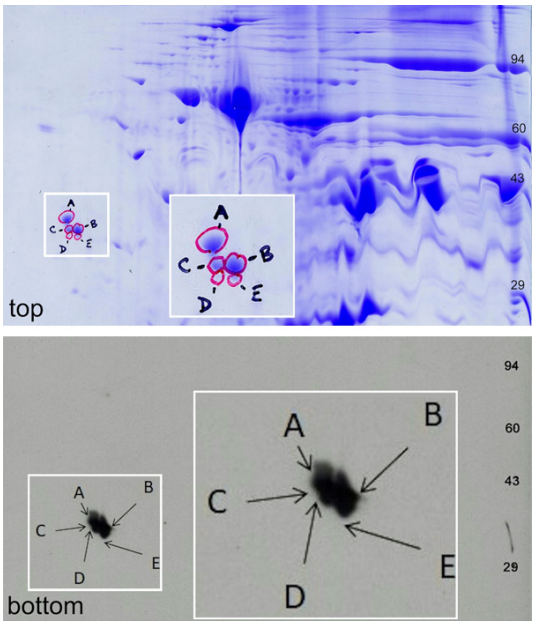

Figure 9. 2D western blot analyses with extracts from adult bovine skeletal muscle. Top. The Coomassie stained bovine adult skeletal muscle protein across the gel. The box at the left has been zoomed and inserted at the right side. Bottom. The PVDF filter was stained with CH1 monoclonal antibody followed by treatment with secondary antibody as stated under materials and methods, and subsequently treated with ECL and exposed to x-ray film. Developed X-ray film was superimposed on the top of the Coomassie stained second gel as well as on the Coomassie stained PVDF filter. Five spots A-E were marked, excised and were used for extraction of protein for subsequent mass spectrometric analyses. The box at the left has been zoomed and inserted at the right side.

and 2x-MyHC were mostly expressed, sarcomeric TPM2 expression is ~50-60%) followed by the expression of sarcomeric TPM1 (~40%), while TPM3 expression was negligible/insignificant. These authors concluded that the translated protein product of any isoform of either *TPM* gene or MyHC gene is proportional to the corresponding mRNAs.

As far as TPM isoform protein expression in bovine skeletal muscle is concerned, our 2D western blot followed by mass spectrometric analyses show that TPM1α is 57.14% and TPM2α is 42.87% in the sample studied. Although several TPM3 peptides common to other TPM are present in various spots, we have not detected a unique TPM3-specific peptide in our bovine skeletal muscle extract. Similarly, due to the lack of a unique peptide, we have assumed TPM4 protein is either absent or very low in this sample of bovine skeletal muscle (**Table 4**). We would like to point out that Oe et al. [12] analyzed the relative amounts of TPM1, TPM2 and TPM3 proteins in six different skeletal muscle types (M, MS, DP, PM, LT, and ST) by scanning the image of the 2DE gel stained with Ruby Gel stain. They found TPM2 protein to be about 50% in muscle types whereas the content of TPM1 and TPM3 proteins vary in DP, PM, LT, an ST. In MS only TPM3 but not TPM1 protein is expressed along with ~50% TPM2.

Oe et al. [13] determined, by qRT-PCR, the percent of transcripts of TPM-1, TPM-2, and TPM-3, compared to total TPM transcripts in different types of single fibers expressing slow MyHC or 2a MyHC or 2x MyHC from three types of bovine skeletal muscles, for example, MS, Semispinalis, and ST. They reported the co-expression of TPM-1 and TPM-2 in 2a & 2x type fibers, whereas TPM-3 and TPM-2 were in slow type fibers. In brief, they found the expression of TPM-2 and TPM-3 in slow type muscle fibers where there was hardly any expression of TPM-1 transcripts. In addition, these authors concluded that TPM-3 is specific for slow muscles only. Broadly, their results, as well as interpretation, implies the transcriptional control of different TPM isoforms in bovine skeletal muscles, which is rather a unique/novel claim.

In this study, we found that transcripts of TPM1α, TPM2α, TPM3α, TPM4α, and the novel TPM4ε are expressed in bovine skeletal muscle (**Table 2**). To the best of our knowledge, this is the first report of the expression of TPM4α and TPM4ε in bovine skeletal muscle. As far as TPM protein expression is concerned, we found the presence of TPM1α and TPM2α in bovine skeletal muscle and we failed to detect the TPM3α, TPM4α, and TPM4ε protein in skeletal muscles. It should be noted that we used skeletal muscle RNA (for qRT-PCR) and skeletal muscle protein extract (for 2D Western with CH1 antibody and subsequent LC-MS/MS analysis) from different commercial sources and from different animals. Also, we do not know what type of skeletal muscle samples were used for either protein or RNA extraction.

Table 6. Identification of amino acid sequences from the peptides extracted from Spots A, B, and C after 2D western blot analyses of bovine skeletal muscle protein with CH1 monoclonal antibody

Spot #	TPM isoform and peptide sequence identified
A	TPM1α (Accession # gI|57281697) (27.81% identified)
	1 mdaikkkmqmlkldkenaldraeqaeadkkaaedrskqledelvslqkklkatedeldky
	61 sealkdaqeklelaekkatdaeadvaslnrriqlveeeldraqerlatalqkleeaekaa
	121 desergmkviesraqkdeekmeiqeiqlkeakhiaedadrkyeevarklviiesdlerae
	181 eraelsegkcaeleeelktvtnnlksleaqaekysqkedkyeeeikvlsdklkeaetraE
	241 faersvtkleksiddledelyaqklkykaiseeldhalndmtsi
	TPM2α (Accession # gI|57281699) (42.6% identified)
	1 MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCKQLEEEQQALQKKLKGTEDEVEKY
	61 sesvkdaqekleqaekkatdaeadvaslnrriqlveeeldraqerlatalqkleeaekaa
	121 desergmkvienramkdeekmelqemqlkeakhiaedsdrkyeevarklvilegelerse
	181 eraevaeskcgdleeelkivtnnlksleaqadkystkedkyeeeiklleeklkeaetrae
	241 FAERSVAKLEKTIDDLEDEVYAQKMKYKAISEELDNALNDITSL
B	TPM1α (Accession # gI|57281697) (33.45% identified)
	1 MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKATEDELDKY
	61 sealkdaqeklelaekkatdaeadvaslnrriqlveeeldraqerlatalqkleeaekaa
	121 desergmkviesraqkdeekmeiqeiqlkeakhiaedadrkyeevarklviiesdlerae
	181 eraelsegkcaeleeelktvtnnlksleaqaekysqkedkyeeeikvlsdklkeaetraE
	241 faersvtkleksiddledelyaqklkykaiseeldhalndmtsi
	TPM2α (Accession # gi|57281699) (21.47% identified)
	1 mdaikkkmqmlkldkenaidraeqaeadkkqaedrckqleeeqqalqkklkgtedeveky
	61 sesvkdaqekleqaekkatdaeadvaslnrriqlveeeldraqerlatalqkleeaekaa
	121 desergmkvienramkdeekmelqemqlkeakhiaedsdrkyeevarklvilegelerse
	181 eraevaeskcgdleeelkivtnnlksleaqadkystkedkyeeeiklleeklkeaetrae
	241 faersvaklektiddledevyaqkmkykaiseeldnalnditsl
C	TPM1α (Accession # gI|57281697) (19.36% identified)
	1 mdaikkkmqmlkldkenaldraeqaeadkkaaedrskqledelvslqkklkatedeldky
	61 sealkdaqeklelaekkatdaeadvaslnrriqlveeeldraqerlatalqkleeaekaa
	121 desergmkviesraqkdeekmeiqeiqlkeakhiaedadrkyeevarklviiesdlerae
	181 eraelsegkcaeleeelktvtnnlksleaqaekysqkedkyeeeikvlsdklkeaetraE
	241 faersvtkleksiddledelyaqklkykaiseeldhalndmtsi
	TPM2α (Accession # gi|57281699) (18.3% identified)
	1 MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCKQLEEEQQALQKKLKGTEDEVEKY
	61 sesvkdaqekleqaekkatdaeadvaslnrriqlveeeldraqerlatalqkleeaekaa
	121 desergmkvienramkdeekmelqemqlkeakhiaedsdrkyeevarklvilegelerse
	181 eraevaeskcgdleeelkivtnnlksleaqadkystkedkyeeeiklleeklkeaetrae
	241 faersvaklektiddledevyaqkmkykaiseeldnalnditsl

Red color letters indicate peptide sequences identified by mass spectra.

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Disclosure of conflict of interest

None.

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