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Sarcomeric TPM3 α in developing chicken

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Abstract

Cloning and sequencing of various tropomyosin isoforms expressed in chickens have been described since the early 1980s. However, to the best of our knowledge, this is the first report on the molecular characterization and the expression of the sarcomeric isoform of the *TPM3* gene in cardiac and skeletal muscles from developing as well as adult chickens. Expression of TPM3 α was performed by conventional RT-PCR as well as qRT-PCR using relative expression (by C_T as well as ΔC_T methods) and by determining absolute copy number. The results employing all these methods show that the expression level of TPM3 α is maximum in embryonic (10-day/15-day old) skeletal muscle and can barely be detected in both cardiac and skeletal muscles from the adult chicken. Our various RT-PCR analyses suggest that the expression of high molecular weight TPM3 isoforms are regulated at the transcription level from the proximal promoter at the 5'-end of the TPM3 gene.

INTRODUCTION

An individual sarcomere, the fundamental functional unit in striated muscles, consists of many thin actin and thick myosin filaments. Tropomyosin (TPM), a dimeric coiled-coil actin-binding protein constitutes thin filaments in conjunction with several other proteins for example actin, troponins, tropomodulin, etc. Tropomyosin proteins that organize thin filaments are known as sarcomeric tropomyosin and play a critical function in calcium mediated striated muscle contraction. Four *TPM* genes designated as *TPM1*, *TPM2*, *TPM3*, and *TPM4* are present in vertebrates except for fish (Dube et al. 2017; Gunning et al. 2008; Lees-Miller and Helfman, 1991; Schvezov et al. 2011). Each TPM gene generates a multitude of isoforms (both sarcomeric and non-sarcomeric) via alternate splicing and/or using alternate promoters. The *TPM1* gene encodes two sarcomeric isoforms TPM1 α and TPM1 κ and at least fourteen non-sarcomeric isoforms (Denz et al. 2004; Rajan et al. 2010; Perry et al. 2001). Sarcomeric isoforms of TPM2, TPM3, and TPM4 are known as TPM2 α , TPM3 α , and TPM4 α , respectively (Lees-Miller and Helfman, 1991; Dube et al. 2014; Dube et al. 2014). Besides sarcomeric isoforms, each of these three TPM genes also generates a

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variety of non-sarcomeric isoforms (Lees-Miller, 1991; Dube et al., 2016; Schevzov et al. 2011). Each sarcomeric isoform consists of a protein with 284 amino acid residues whereas the non-sarcomeric isoforms of the four TPM genes comprise high molecular weight proteins with 284 amino acids and/or low molecular weight proteins with 248 amino acids (Lees-Miller and Helfman, 1991; Schevzov et al. 2011). Sarcomeric TPM transcripts consist of exons 1a, 2b or 2a (in case of TPM1 κ), 3, 4, 5, 6b, 7, 8, 9a/b (Denz et al. 2004; Dube et al. 2016, 2017).

The chicken is one of the earliest animal models studied for research in the field of developmental biology and myofibrillogenesis (Sanger et al. 2017; Wang et al. 2005; Wang et al. 2008) including the characterization of myofibrillar proteins, for example tropomyosin (Fleenor et al., 1992; Forry-Schaudies et al., 1990). Although, one of the low molecular weight TPM3 proteins from chicken skeletal muscle (TPM3 β as in Figure 1) was isolated, cloned, and sequenced in early 80's (McLeod, 1982), the molecular characterization of sarcomeric TPM3 from chicken striated muscle has still not been reported. To the best of our knowledge, this is the first report of cloning and sequencing of sarcomeric TPM3 (or TPM3 α in Figure 1) as well as its expression analyses by qRT-PCR using both relative quantification and determining absolute copy number in developing cardiac and skeletal muscles.

RESULTS

Determine the expression of TPM3 α in developing hearts and skeletal muscles by conventional RT-PCR

RT-PCR was carried out with cDNAs synthesized from the RNA of heart and skeletal muscle from various embryos, 15-day post hatch, and adult chickens. We amplified both TPM3 α and TPM3 β using isoform specific primer-pairs (Figure 1 & Table 1) designed from the sequences available in GenBank. For TPM3 α the template is the predicted TPM3 transcript variant X7 (GenBank accession number XM_015298570]. The GenBank accession number of TPM3 β is NM_001245927. The results depicted in Figure 2 show ethidium bromide staining of the amplified cDNA of TPM3 α in developing chicken heart and skeletal muscles (panel a). Results presented in panel b and c depict the Southern hybridization results with [32 P] labeled isoform specific oligonucleotide probe for longer and shorter exposures, respectively. The hybridization data show that TPM3 α expression is maximum in skeletal muscle of day 10 embryos (lane 2, Figure 2). The expression is also high in skeletal muscle from 15-day embryos (lane 3). However, expression is practically undetectable in 15-day post hatch (lane 4) and adult skeletal muscles (lane 5). TPM3 α expression is negligible in hearts from 7 days (lane 6), 10 days (lane 7), 15 days (lane 8) old embryos as well as in hearts from 15-day post hatch (lane 9) and adult (lane 10) animals. Lane 11 represents the negative DNA control. At the same time, the GAPDH expression in all samples from heart and skeletal muscles are comparable/equal (panel d, Figure 2). Besides Southern hybridization, the expression of TPM3 α in embryonic skeletal muscle was authenticated by determining the nucleotide sequences of the gel-purified amplified DNA from lane 1, 2, and 3 separately. The results strongly suggest that the expression level of TPM3 α is maximum in skeletal muscle from 10-day old embryonic chicken and is

insignificant in skeletal muscle from older animals. In hearts, however, the expression of TPM3 α is hardly detectable.

Cloning and sequencing of TPM3 α

The DNA extracted from the gel was used for direct sequencing and ligated into a T/A cloning vector. The appropriate clones were screened and plasmid DNA was isolated as stated above to determine the nucleotide sequences. Figure 3 depicts the nucleotide as well as the deduced amino acid sequences of TPM3 α , which are identical with the coding region of the predicted TPM3 sequence of variant X7 (XM_015298570.1).

Expression of low molecular weight TPM3 β (Chicken TPM3 variant 1) by conventional RT-PCR in developing chicken heart and skeletal muscle

TPM3 β was originally discovered in chicken skeletal muscle by MacLeod et al. in 1982 (NM_001245927.2). Using an isoform specific primer-pair (Figure 1 and Table 1) we have amplified TPM3 β with the cDNAs from embryonic, post embryonic and adult hearts and skeletal muscle and performed agarose gel electrophoresis followed by ethidium bromide staining (panel a, Figure 4). We have carried out Southern hybridization with a radio-labeled isoform specific oligonucleotide probe (Table 1). The results show that TPM3 β is expressed in embryonic skeletal muscles (lanes 1-3, panel b, Figure 4) and 15-day post hatch skeletal muscles (lane 4). The level of TPM3 β expression is not significant in adult skeletal muscles (lane 5). Similarly, the expression level is not significant in embryonic (lanes 6-8), post hatch (lane 9), and adult (lane 10) hearts, although the expression of GAPDH in all tissues is comparable (panel c, Figure 4).

Quantification of TPM3 α and TPM3 β transcripts in hearts and skeletal muscles from embryonic, 15-day post hatch, and adult chicken

In order to quantify transcript level in a given tissue one can determine both relative quantification and absolute quantification. Relative quantification is used to relate the amount of the transcripts of the gene of interest in equivalent amounts of different samples. However, the absolute quantification provides the copy number of the target gene present in the sample. Again, the relative expression can be determined by two methods, by C_t and by 2^{-C_t} . Both methods provide similar results.

As the nucleotide sequences of the coding regions of various TPM isoforms are very similar, we made the cDNA with gene specific oligonucleotide (or the negative primer) from the 3'-UTR (exon 9b) of TPM3 α and TPM3 β) which helps eliminate the amplification of the other tropomyosin gene products. We have used 18S rRNA as the reference gene in the present study. Again, TPM3 α and TPM3 β have the same 3'-UTR, we chose a positive primer for qRT-PCR amplification of TPM3 α from exon 2 (Table 1), which is absent from TPM3 β . For amplification of TPM3 β , we have designed the positive primer from exon 1b, which is not part of TPM3 α or any other high molecular weight TPM transcripts.

Figure 5a depicts the relative expression of TPM3 α in developing chicken heart and skeletal muscles using the C_T method. The expression level is always significantly higher in skeletal muscles compared to the corresponding heart tissues throughout embryonic

development and in post hatch as well as in adult chicken. The expression reaches its maximum in skeletal muscles from 15-day old embryonic heart and then decreases down. The relative expression in skeletal muscle is significantly lower in 15-day post hatch and adult chickens.

Using C_T method, the expression also starts in 7-day embryonic skeletal muscle and it reaches a maximum in 10-day old embryos (Figure 5b). The relative expression declines in 15-day post hatch animals. In adults, the expression is slightly higher compared to 15-day post hatch but is still ~25% of the level observed in 15-day old embryos.

We have also determined the absolute copy number of TPM3 α expressed in heart and skeletal muscles in embryonic, post hatch, and adult chicken. The standard curve using a dilution of the plasmid containing the TPM3 α dDNA is depicted in panel a, Figure 6. The results are in good agreement with the results on the relative expression data depicted in Figures 5a & 5b. The absolute copy number is maximum in 10-day old embryonic skeletal muscle. It starts declining from 15-day embryos. Absolute copy number is ~10-fold lower in post hatch animals. Again, expression of TPM3 α is slightly higher in adult chicken compared to 15-day post hatch chicken. In hearts, the expression remains lower consistently throughout development and also in adult chickens.

Detection of total high molecular weight TPM3 transcripts in developing chicken by conventional RT-PCR—High molecular TPM generally codes for TPM protein(s) with 284 amino acid residues and it contains exon 1a, 2, 3, 4, 5, 6, 7, 8, 9a/b or 9c/d. Although the expression of a high molecular weight TPM3 besides TPM3 α has not yet been reported, the predicted sequences as posted in GenBank do suggest the possibility of expression of several other high molecular weight TPM3 transcripts in chickens. Hence, we designed a TPM3-specific primer-pair for all high molecular weight isoforms from exon 1a and exon 3. We used a [32 P]-labeled oligonucleotide probe designed from exon 2 for Southern hybridization for the amplified product. The results presented in Figure 7a show ethidium staining of the amplified DNA. Southern hybridization shows the total TPM3 is expressed in skeletal muscle from 7-day embryo (lane 1), 10-day old embryo (lane 2), 15-day old embryo (lane 3), and slightly in 15-day post hatch animals (lane 4) (panels b and c, Figure 7). It is to be noted that panel b and c represent shorter and longer exposure respectively. Hardly, any signal can be detected in skeletal muscle from adult animals (lane 5, panels b and c in Figure 7). The conventional RT-PCR results strongly suggest that the transcripts of high molecular weight TPM3 isoforms are expressed in embryonic skeletal muscle. The expression level is high in both 10 and 15-day old embryos. One can detect the transcripts in skeletal muscle from the 15-day old animal but not in adult chicken. Transcripts can hardly be detected in embryonic and post embryonic chickens. It could be inferred that the expression of high molecular weight TPM3 isoforms in developing chicken is controlled at the transcription level in a tissue and ontologic specific manner.

DISCUSSION

Sarcomeric TPM3 isoform has long been known to play a critical role in muscle contraction in slow skeletal muscle in vertebrates. However, no sarcomeric TPM3 protein or its cDNA

clone has been reported until June 3, 2017 (variant 4; NM_001352041.1) (Ouyang et al. 2017), while we were submitting our TPM3 α sequence in GenBank (submission id:). Interestingly, the nucleotide as well as deduced amino acid sequences of a low molecular weight TPM3 isoform (TPM3 β) was reported by MacLeod et al. in 1982. Our TPM3 α nucleotide sequences presented in Figure 3 are identical with the predicted TPM3 sequence (variant #7) in GenBank (Accession # XM_015298570.1). Conventional as well as qRT-PCR results demonstrate that the expression of TPM3 α is distinctly higher in embryonic skeletal muscle compared to the corresponding cardiac muscles and also significantly higher than skeletal muscle from 15-days post hatch as well as the adult chicken. This is most likely the reason for our inability to clone the TPM3 α cDNA with RNA from multiple sources of adult chicken skeletal muscles. It is tempting to conclude that the expression of TPM3 α is mostly restricted to the embryonic skeletal muscle. It is worth mentioning at this point that we have also succeeded in amplifying TPM3 α cDNA with RNA from skeletal muscle from 15-day post hatch animal but not from adult chicken using nested RT-PCR only. The results alone suggest that embryonic skeletal muscles express higher TPM3 α transcripts than the 15-day post hatch animals and still higher than muscles from adult chicken. Our finding on the expression of TPM3 α transcripts in embryonic chicken skeletal muscles is in agreement with the findings of Ouyang et al. (2017). These authors performed proteomic analysis of chicken skeletal muscle during embryonic development and found the elevated amount of TPM3 expression in embryonic (E11) vs D1 or developed chicken.

The expression pattern of TPM3 α is comparable with the expression patterns of TPM1 α and TPM1 κ that we had reported earlier in developing chicken [Zajdel et al. 2003; Wong et al. 2007]. The dogma at that time was that chicken hearts only express the sarcomeric isoform of the TPM4 gene designated as TPM4 α but not TPM1 α or TPM2 α . This statement is however partly true as we found expression of other sarcomeric TPM isoforms for example TPM1 α and TPM1 κ , in embryonic chicken heart but they completely disappeared in adult cardiac tissues.

In order to understand the regulation of the developmental pattern of TPM3 α we have evaluated the expression of total high molecular weight TPM3 transcripts that includes TPM3 α using conventional RT-PCR with the total RNA of heart and skeletal muscles from developing chicken. Figure 7 depicts that the expression of total high molecular weight TPM3 transcripts are markedly higher in embryonic skeletal muscle compared to 15-day post hatch and adult animals, however, the expression of total TPM3 transcripts in embryonic and adult hearts is significantly lower (panel b, panel c in Figure 7). It is to be noted that each of the four TPM genes contains two promoters – one is at the 5' - end upstream to exon 1a and the other is in between exon 2 and exon 1b. The proximal promoter upstream of exon 1a is known to control the expression of transcripts of high molecular weight TPM isoforms with ~284 amino acid residues including sarcomeric isoforms. The internal promoter promotes the transcripts of low molecular weight TPM isoforms for example TPM3 β . The results presented in Figure 7 strongly suggest that the expression of total high molecular weight TPM3 including TPM3 α is regulated at the transcriptional level in a tissue specific manner.

TPM genes are thought to be like housekeeping genes which are expressed ubiquitously. The isoform diversity of TPM is believed widely to be controlled at the post transcriptional level for example, alternative splicing. It is also stated that the TPM isoform diversity is controlled by the use of alternate promoter (Lees-Miller and Helfman, 1991; Schevzov et al., 2011; Wiczroek 1988). However, transcriptional control is a scantily used term in the field of TPM isoform diversity with a few exceptions (Meinzel et al. 1989; Toutant et al. 1994; Pasquet et al. 2006). Meinzel et al. (1989) reported on the tissue specific control of TPM1 and TPM2 in developing chicken muscle. In pectoralis muscle, the TPM2 mRNA ceased accumulating after hatching through transcriptional control. In the same muscle, in parallel with disappearance of TPM2 transcripts, TPM1 transcript accumulation was augmented. In leg muscle, after hatching, TPM1 transcripts expression was increased moderately with the concomitant slight decrease of TPM2 transcripts [Meinzel et al. 1989]. Likewise, the results presented in this study strongly suggest that TPM3 α as well as total high molecular weight TPM3 transcripts are controlled at the transcriptional level by the proximal promoter at the 5'-end of exon 1a of the chicken TPM3 gene. Further studies such as identification and characterization of various cis- elements of the proximal promoter, is an essential pre-requisite for establishing the tissue specific transcriptional control of the *TPM3* gene in developing chicken. None the less, we believe this is by far the first report of the characterization and expression of the sarcomeric TPM3 α in developing chicken.

The technique of FRAP (Fluorescence Recovery After Photobleaching) has shown that the dynamic properties of sarcomeric proteins in skeletal muscle decrease as mature sarcomeres assemble from premyofibrils (Wang et al., 2005; 2007). However, the dynamics of actin and tropomyosin in cardiac muscle cells do not change when premyofibrils form into mature myofibrils (Wang et al., 2014; Sanger et al. 2017). The change in TPM3 isoform reported in this paper for adult skeletal muscle cells may be related to the need to suppress both actin and tropomyosin dynamics in adult muscle cells in order to exert increased forces on the thin actin filaments. In contrast, cardiac muscle cells are continually beating in both embryonic and adult stages. TPM4 may be the most useful isoform for the stability of mature myofibrils in both embryonic and adult use as the expression of both TPM1 α and TPM1 κ is turned off in adult chicken hearts (Zajdel et al. 2003).

4 | Materials and Methods

4.1 | Isolation of RNA from embryonic chicken hearts & skeletal muscles and adult chicken hearts

Fertile eggs (White Leghorn) were incubated at 37°C for 10–16 days. RNA was isolated from specific tissues, which were frozen in liquid nitrogen. The details have been described elsewhere (Zajdel et al., 2003; Dube et al. 2017). Tissues from adult chicken was procured from a local farm (Cobblestone Valley Farm, Preble, NY13141); also tissues from 15-day post hatch chickens were obtained from another local farm (Treasures of Joy Farm, Nedrow, NY 13120). RNA was isolated from hearts as described before (Zajdel et al., 2003; Dube et al. 2017). The RIN (RNA Integrity Number) of RNA samples of heart and skeletal muscles are between 8.90 to 9.2, suggesting the RNA we used were of good quality.

4.2 | Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNAs were made from various RNAs using oligo dT as the primer following our published protocols (Denz et al., 2004; Zajdel et al., 2003). Subsequently, PCR amplifications for various genes were carried out using gene-specific and/or isoform specific-primer-pairs as shown in Table 1. The PCR amplified DNAs were run in an agarose gel, stained with ethidium bromide and subsequently analyzed by for Southern hybridization using an appropriate, isoform-specific probe following the method as described earlier (Denz et al., 2011; Thurston et al., 2009). The sequences of various gene specific oligonucleotides are presented in Table 1 and also in the corresponding figure legend. (Figures 2, 4, and 7).

Also, the PCR amplified DNAs were run in an agarose gel and stained with ethidium bromide; the stained bands were excised from the gel from which DNA was extracted from using a MinElute Gel extraction kit from Qiagen (Valencia, CA) as stated earlier (Dube et al. 2017). Part of the DNA was used for sequencing and part of the gel extracted DNAs were ligated and cloned into the T/A cloning vector (Life Technologies) following our published protocol (Dube et al. 2017). Positive clones were identified by PCR as stated by Dube et al (2017). Positive clones were grown in appropriate media over-night and the DNA was extracted using Qiagen mini-prep kit (Valencia, CA). The isolated DNA was sequenced (Cornell University Life Science Core Laboratories Center, Ithaca, NY). Each clone was sequenced in both directions.

4.3 | Real-time quantitative RT-PCR

qRT-PCR was carried out using the LightCycler 480 Real-Time PCR System as described before (Dube et al. 2014; Nan et al., 2015; Dube et al. 2017) Reactions were carried out in a 384-well plate using the LightCycler 480 SYBR Green I Master kit (Roche). Relative quantification of qRT-PCR data was performed using the 2^{-CT} (sample CT minus 18S rRNA CT) and $2^{-\Delta CT}$ (sample CT minus comparator CT) methods (Livak & Schmittgen, 2001; Pfaffl, 2001; Yuan et al. 2006). A comparative value was calculated using the formula X^{-CT} , where X equals the efficiency of specific primer pairs. This is similar to the 2^{-CT} method but corrects for the assumption that the reaction is occurring with 100% efficiency. Efficiencies (X) were determined using dilution series of isoform-specific plasmid clones with respective isoform-specific primers pairs. The LightCycler 480 software plotted the CT at each concentration against the logarithm of the dilutions of the clone, generating a linear regression curve that calculated efficiency based on the formula $E = 10^{[-1/\text{slope}]}$. Efficiency of 18S rRNA was determined by serial dilution of zebrafish cDNAs generated with specific primers.

For determination of absolute copy number, the optical density of the plasmid DNA from a TPM3a clone was measured in a spectrophotometer at 260 nm. The copy number per volume of clone in solution was determined using the equation number of copies = (ng of plasmid DNA $\times 6.02 \times 10^{23}$)/(bp length of plasmid $\times 1 \times 10^9 \times 650$), 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 – 1×10^4 copies of template, which was used to create a standard curve after amplification (Figure 6) [Dube et al. 2017; Thomas et al., 2010; Thurston et al., 2009]. For better accuracy, each sample in the dilution series was run in triplicate.

Statistical Analyses—The means, standard deviations, and comparative analyses of each data set for statistical significance were done using paired Student's *t*-test.

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Figure 1. Alternative splicing pattern of the *TPM3* gene in chicken

Exon composition of the *TPM3* gene is derived from the published documents, the recently submitted data in Gen Bank (Accession# MF347378), and the predicted sequence available in Gen Bank (Accession # XM_015298570). Exons are shown in boxes.

Figure 2. Conventional RT-PCR amplification of TPM3a with RNA from developing chicken heart and skeletal muscle

Primer-pairs were designed from the predicted TPM3 variant #7 sequence (GenBank accession # XM_015298570)

Panel a. Ethidium bromide staining of the amplified products of TPM3a

Panel b. Long exposure. Hybridization with TPM3a probe, the sequences of which are in Table 1.

Panel c. Short exposure for the Southern hybridization.

Panel d. Ethidium bromide staining of the amplified products of GAPDH

cDNAs were made with oligo dT

Lane 1. Skeletal muscle from 7-day old embryos; Lane 2. Skeletal muscle from 10-day old embryos; Lane 3: Skeletal muscle from 15-day old embryos; Lane 4. Skeletal muscles from 15-day post hatch chicken; Lane 5. Skeletal muscle from adult chicken; Lane 6. Heart from 7-day old embryos; Lane 7. Heart from 10-day old embryos; Lane 8. Heart from 15-day old embryo; Lane 9. Heart from 15-day post hatch chicken; Lane 10. Heart from adult chicken; Lane 11. Primer control.

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1  agcccccgcagccatggaggccatcaagaagaagatgcagatgctgaagctggacaagga 60
   -----+-----+-----+-----+-----+-----+-----+-----+
      M E A I K K K M Q M L K L D K E -
61  gaacgccctggacagggcagagcaggcggaggccgagcagaagcaggcggaggagaggag 120
   -----+-----+-----+-----+-----+-----+-----+-----+
      N A L D R A E Q A E A E Q K Q A E E R S -
121 caaacagctggaggatgagctggccgccatgcagaagaagctgaaggggacggaggacga 180
   -----+-----+-----+-----+-----+-----+-----+-----+
      K Q L E D E L A A M Q K K L K G T E D E -
181 gctggacaaatactcggaggccctgaaggatgccaggagaagctggagctggcggagaa 240
   -----+-----+-----+-----+-----+-----+-----+-----+
      L D K Y S E A L K D A Q E K L E L A E K -
241 gaaggcggcggatgctgaggcagaggtggcatccctgaaccgcccatccagctggtaga 300
   -----+-----+-----+-----+-----+-----+-----+-----+
      K A A D A E A E V A S L N R R I Q L V E -
301 ggaggagctggaccgggcccaggagcgccctcgccaccgctctgcagaagctggaggaggc 360
   -----+-----+-----+-----+-----+-----+-----+-----+
      E E L D R A Q E R L A T A L Q K L E E A -
361 tgagaaggctgcggatgaaagcgaaagaggcatgaaagtcattgaaaacagagctctgaa 420
   -----+-----+-----+-----+-----+-----+-----+-----+
      E K A A D E S E R G M K V I E N R A L K -
421 ggatgaggagaagatggagctgcaggagatccagctgaaggaagccaagcacattgcaga 480
   -----+-----+-----+-----+-----+-----+-----+-----+
      D E E K M E L Q E I Q L K E A K H I A E -
481 ggaggcggacaggaagtatgaggaggtgtctcggaactggtgatcatagaggagacct 540
   -----+-----+-----+-----+-----+-----+-----+-----+
      E A D R K Y E E V A R K L V I I E G D L -
541 ggagcgtactgaggagagagctgagcttgcagagtctaaatgttccgagctggaggagga 600
   -----+-----+-----+-----+-----+-----+-----+-----+
      E R T E E R A E L A E S K C S E L E E E -
601 gctgaagaatgtcaccaacaatctcaagtctcttgaggctcaggctgagaagtactccca 660
   -----+-----+-----+-----+-----+-----+-----+-----+
      L K N V T N N L K S L E A Q A E K Y S Q -
661 aaaagaggataaatacgaagaggagatcaagatcctgactgataaactcaaagaggcgga 720
   -----+-----+-----+-----+-----+-----+-----+-----+
      K E D K Y E E E I K I L T D K L K E A E -
721 gacccgtgctgagttcgctgagcgctctgtagccaagctggagaagaccatcgatgattt 780
   -----+-----+-----+-----+-----+-----+-----+-----+
      T R A E F A E R S V A K L E K T I D D L -
781 ggaagatgagctctatgccagaaactgaagtacaaagcaattagtgaggaactggacca 840
   -----+-----+-----+-----+-----+-----+-----+-----+
      E D E L Y A Q K L K Y K A I S E E L D H -
841 cgccctgaatgacatgacttccatataaactgaaatccacc 881
   -----+-----+-----+-----+-----+-----+-----+-----+
      A L N D M T S I * -

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Figure 3. Nucleotide and deduced amino acid sequence of TPM3a

The nucleotide sequence has been submitted in the GenBank (Submission Id. 2024828 MF347378)

Figure 4. Conventional RT-PCR amplification of TPM3 β with RNA from developing chicken heart and skeletal muscle

Panel a. Ethidium bromide staining of the amplified products of TPM3 β

Panel b. Hybridization with TPM3 β probe, the sequences of which are in Table 1.

Panel c. Ethidium bromide staining of the amplified products of GAPDH.

cDNAs were made with oligo dT

Lane 1. Skeletal muscle from 7-day old embryos; Lane 2. Skeletal muscle from 10-day old embryos; Lane 3: Skeletal muscle from 15-day old embryos; Lane 4. Skeletal muscles from 15-day post hatch chicken; Lane 5. Skeletal muscle from adult chicken; Lane 6. Heart from 7-day old embryos; Lane 7. Heart from 10-day old embryos; Lane 8. Heart from 15-day old embryos; Lane 9. Heart from 15-day post hatch chicken; Lane 10. Heart from adult chicken; Lane 11. Primer control.

Figure 5. Relative expression of TPM3α in heart and skeletal muscle from developing, 15-day post hatch, and adult chicken
a. Relative expression using C_T method
b. Relative expression using C_T method
E7: 7-day old embryos; E10: 10-day old embryos; E15: 15-day old embryos; P15: 15 day post hatch; Adult.
cDNA was synthesized using gene-specific oligonucleotide Chi.TPM3-9b(-).qRT-PCR was carried out in triplicate with isoform specific primer-pair (Chi.TPM3α.qRT (F)/

Chi.TPM3 α .qRT(R) sequences of which are given in Table 1. The primer used for 18S rRNA is shown in Table 1.

c. Melt curves of qRT-PCR of chicken TPM3 α show a single melting point. The multiple curves represent the products from multiple replicates of the RT-PCR assay.

d. PCR products separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. The results demonstrate amplification of a single product for TPM3 α .

Lane 1. Skeletal muscle from 7-day old embryos; Lane 2. Skeletal muscle from 10-day old embryos; Lane 3: Skeletal muscle from 15-day old embryos; Lane 4. Skeletal muscles from 15-day post hatch chicken; Lane 5. Skeletal muscle from adult chicken; Lane 6. Heart from 7-day old embryos; Lane 7. Heart from 10-day old embryos; Lane 8. Heart from 15-day old embryo; Lane 9. Heart from 15-day post hatch chicken; Lane 10. Heart from adult chicken; Lane 11. Primer control.

Figure 6. Quantitative RT-PCR of chicken TPM3 α by determining absolute copy number
a. Standard curve derived from amplification of chicken TPM3 α plasmid clone DNA after serial dilution. Copy number of TPM3 α was calculated per microgram of total RNA from heart and skeletal muscle from developing, post hatch, and adult chicken.
b. Number of copies of TPM3 α transcripts per μ g of total RNA from heart and skeletal muscle from embryonic, post hatch, and adult chicken.
E7: 7-day old embryos; E10: 10-day old embryos; E15: 15-day old embryos; P15: 15 day post hatch; Adult.

Figure 7. Conventional RT-PCR amplification of total high molecular weight TPM3 transcripts with RNA from developing chicken heart and skeletal muscle

The positive and negative primer pairs were designed from exon 1a and exon 3 respectively. The hybridization was carried out with a oligonucleotide from exon 3 of the TPM3 gene. The nucleotide sequences of the primer-pair are given in Table 1. cDNA was made with oligo dT.

Panel a. Ethidium bromide staining of the amplified total high molecular weight TPM3 transcripts.

Panel b. Short exposure of hybridization with total high molecular weight TPM3 probe, the sequences of which are available in Table 1.

Panel c. Long exposure for the Southern hybridization.

Lane 1. Skeletal muscle from 7-day old embryos; Lane 2. Skeletal muscle from 10-day old embryos; Lane 3: Skeletal muscle from 15-day old embryos; Lane 4. Skeletal muscles from 15-day post hatch chicken; Lane 5. Skeletal muscle from adult chicken; Lane 6. Heart from 7-day old embryos; Lane 7. Heart from 10-day old embryos; Lane 8. Heart from 15-day old embryos; Lane 9. Heart from 15-day post hatch chicken; Lane 10. Heart from adult chicken; Lane 11. Primer control.

<i>TPM3</i>		Type of amplification		Nucleotide sequence
Isoform TPM3 α	Used for	Con RT-PCR	qRT-PCR	
Chi.TPM3-1a(+) Chi.TPM3-9a/b(–) Chi.TPM3-1a.2 nd (+) Chi.TPM3 -3 Probe Chi.TPM3-9b(–) Chi.TPM3a.qRT (F) Chi.TPM3.qRT(R) Chi.TPM3.HM.Total (+) Chi.TPM3.HM.Total (–) Chi.TPM3.Ex2	Full length Ampli Full length Ampli Probing TPM3 α (br/)Making cDNA qRT-PCR forward qRT-PCR reverse Amp.total HMW.TPM3 Amp.total HMW.TPM3 Probe for total HMW TPM3	X X X X X X 	- X X 	5'-GGAGCAAACAGCTGGAGGATGAGC-3' 5'-TTTCAGTTTATATGGAAGT-3' 5'-AGCCCCCGCAGCCATGGAGGCC-3' 5'-GTGGCGAGGCGCTCCTGGGC-3' 5'-AGGTCTAGCAGGGTCTGGT-3'' 5'-ATGGAGGCCATCAAGAAGAA-3' 5'-GTGGCGAGGCGCTCCTGGGC-3' 5'-AGCCCCCGCAGCCATGGAGGCC-3' 5'-GTGGCGAGGCGCTCCTGGGC-3' 5'-GAAGGCGGCGGATGCTGAGGCA-3'
TPM3 β				
Chi.TPM3-1b(+) Chi.TPM3-9a/b(–) Chi.TPM3 -3 Probe	Full length Amlifi Full length Amlifi Probing full length TPM3 β	X X 		5'-CGCGGAGCGGAGGGACCATG-3'(BR)/5'-TTTCAGTTTATATGGAAGT-3' 5'-GTGGCGAGGCGCTCCTGGGC-3'
18S rRNA				
Chi.18S rRNA Plus CHI.18S rRNA Minus		X X 		5'-ATGCAATGAATAGAGAGGTCA-3' 5'-GAACGCCACTTGTCCTCTTA-3'
GAPDH				
Chi. GAPDH F+ Chi. GAPDH R-		X X 		5'-TGACTTCAATGGTGACAGC-3' 5'-AGAAGTGGCCTCTCACTGCA-3'