Tissue Specific Splicing of Pre-mRNA Porcine Mitochondrial Transcription Factor A

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ABSTRACT


Mitochondrial transcription factor A plays, due to its role in replication and transcription of the mitochondrial DNA, an important role in mitochondrial biogenesis and regulation of its activity. In different mammalian species, mitochondrial transcription factor A is present in two different splicing forms. The aim of the study was to verify the presence of both splicing forms and to estimate their ratio in different tissues. The expression levels of both splicing forms in two skeletal muscles (m. semispinalis capitis and m. semimembranosus), heart muscle, brain, spleen, and liver in commercial hybrids and in the autochthonous Krsko polje pig breed are reported. In all analyzed tissues the expression of the long form of the mitochondrial transcription factor A was about one order of magnitude higher compared to the short form, lacking almost the entire exon 4 region. Taking into account the expression ratio of both splicing forms and biological function of the mitochondrial transcription factor A, the possible explanation for the persistence of both forms in different mammalian species is provided.

Keywords: pig; mitochondrion; mitochondrial biogenesis; splicing forms; expression

Mitochondrial transcription factor A (TFAM) is an integral part of the basal mitochondrial transcription machinery in mammals (Gaspari et al. 2004). TFAM is, together with the mitochondrial transcription factors B1 (TFB1M), B2 (TFB2M), and mitochondrial RNA polymerase (POLRMT), required for the transcription of mammalian mitochondrial DNA (mtDNA) (Falkenberg et al. 2007). In addition to its transcription factor function, TFAM is also involved in the regulation of mtDNA copy number and mtDNA stability (Larson et al. 1998) through its binding to mtDNA on its entire length. In our previous research, we reported the full length of porcine TFAM complementary DNA (cDNA), its location at SSC14 and genetic variation in the region of intron 1 for 252 animals belonging to 12 different pig breeds (Kunej et al. 2009). In addition, the existence of two different splicing forms of porcine TFAM in the adipose tissue was reported. The long form represented...
the full length transcript, and was predominant in the adipose tissue, whereas the short form, missing the considerable part of the exon 4, was present in a much smaller amount (Kunej et al. 2009). However, alternative splicing of TFAM has been demonstrated also in other mammalian species, where different regions of the gene were affected, in human, mouse, and rat the exon 5 and in pig the exon 4 were reported to be subject of alternative splicing (D’Errico et al. 2005; Kunej et al. 2009).

In spite of the fact that alternative splicing of TFAM transcripts has already been reported in several mammalian species, there is no information about the tissue specificity and quantitative proportion of both splicing forms available. The general purpose of this study was to study the distribution of differentially spliced long and short forms of TFAM transcripts in tissues with different metabolic activity. TFAM expression has been analyzed in two skeletal muscles (m. semispinalis capitis (SSC) and m. semimembranosus (SM)), as well as in the heart, liver, spleen, and brain tissue and correlated with mitochondrial activity in SSC and SM. The structure and biological role of the short form of TFAM has been proposed.

MATERIAL AND METHODS

**Pig tissue samples.** Tissue samples were obtained from six commercial hybrid males (Large White × Swedish Landrace females × Pietrain males, designated as crossbreed line 1244) and six autochthonous Krsko polje pig breed males, and were collected immediately after slaughter in a commercial slaughterhouse. Sample collection and animal treatment were performed in accordance with the National Animal Protection Law (ULRS 43:18.5.2007, ISSN 1318-0576). Commercial hybrids were kept under standard conditions on a commercial farm and slaughtered at 120 kg of body weight and 6 months of age, whereas Krsko polje pigs were kept under extensive conditions on a small family farm and slaughtered at the same body weight as commercial hybrids at 8 months of age, due to their slower growth performance. Samples of skeletal muscles (m. semispinalis capitis and m. semimembranosus), heart muscle, brain, spleen, and liver were collected, one aliquot was immediately frozen in liquid nitrogen for later isolation of mitochondria, the other aliquot was stored in RNALater™ (Thermo Fisher Scientific, USA), at –25°C, for subsequent RNA isolation. Samples were homogenized using a tissue homogenizer Precellys® (Bertin Technologies, France). The homogenization of samples and RNA isolation were performed in TRIzol® Reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions. Genomic DNA was eliminated from samples prior to reverse transcription using RQ1 RNase-Free DNase Kit (Promega, USA). All RNA samples were tested with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and reached integrity number (RIN) between 7 and 9. Reverse transcription of total RNA was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The assay for fast quantification of absolute amounts of both splicing forms of TFAM has been developed.

**Primer design.** The TaqMan® assay and SYBR® Green method were used for qPCR. Primers for the TaqMan® assay (Applied Biosystems) were designed to detect alternatively spliced TFAM transcripts using Primer Express (Applied Biosystems). TaqMan® probe was designed to span over the site of alternative splicing (Table 1). In the case of SYBR® Green method, the forward primer was designed to span over a junction between exons 3 and 4 and reverse primer was situated within the exon 4 sequence. Primers for amplification of reference genes beta actin (ACTB), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L4 (RPL4), and TATA binding protein (TBP) were used for normalization of sample concentration. The specificity of amplified fragments was confirmed by sequencing.

**DNA sequencing.** DNA sequence of primary transcripts of TFAM was determined using Sanger DNA sequencing of reverse transcribed mRNA. The cDNA products were amplified and cloned into pUC19 vector and transfected into competent E. coli JM105 cells using Ca-phosphate transfection method. Twenty recombinant plasmid clones were sequenced and two different types of cDNA sequences were obtained. DNA sequences were aligned using Clustal W and DNA sequence was translated into amino acid sequence according to appropriate reading frame.

**Expression analysis using qPCR.** qPCR was performed using a ViiA 7 qPCR apparatus (Applied Biosystems). For each form of TFAM gene, serial dilutions (from 10^2 to 10^6) were performed in order
to generate a standard curve. The the SYBR® Green method with Power SYBR® Green PCR Master Mix (Applied Biosystems) and Custom TaqMan® gene expression assay (Applied Biosystems) were used for quantification of mRNA expression levels of two splice forms of TFAM. The sample concentration was normalized using reference genes ACTB, HPRT1, RPL4, and TBP as endogenous controls, according to Nygard et al. (2010). The qPCR was performed in a total volume of 10 µl containing: 5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.5 µl of custom TaqMan® assay (Applied Biosystems), and 1.5 µl H₂O for the short form of TFAM mRNA and 5 µl of Power SYBR® Green PCR master mix, 1.2 µl of forward and reverse primers, and 1.8 µl of H₂O for the long form of TFAM mRNA. To all reactions 3 µl (100 ng/µl) of cDNA template were added. The cycling protocol for TaqMan® and SYBR® Green methods was the same, consisting of hold stage (2 min at 50°C and 10 min at 95°C) and cycling phase (40 cycles; 95°C for 15 s, and annealing temperature of 60°C for 60 s). For the SYBR® Green method one more stage was added at the end – melt curve stage (continuous 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s). All reactions were assayed in triplicates and no template controls were included with each run, according to MIQE guidelines (Bustin et al. 2009). The absolute quantification of the long and short form of TFAM mRNA was performed using gBlock® Gene Fragments (Integrated DNA Technologies, Inc., USA), which are double-stranded synthetic genomic blocks. Two different gBlock® Gene Fragments were designed according to the sequence of TFAM on the site where SYBR® Green primers and TaqMan® probe anneal. The number of copies in gBlock® Gene Fragments was calculated by multiplying moles of synthetic DNA with the Avogadro constant.

Isolation of mitochondria and cytochrome C oxidase activity in SM and SSC muscles. Mitochondria were isolated using a standardized protocol (Dimaro et al. 2012), which was originally optimized for a skeletal muscle tissue. For homogenization of samples, the Precellys® homogenizer (Bertin Technologies, France) was used. After homogenization, samples were kept on ice for 30 min, vortexed and centrifuged at 800 g for 15 min. Mitochondria were isolated from supernatant after centrifugation at 11 000 g for 10 min. The supernatant was discarded and the pellet was re-suspended in 200 µl STM buffer (270 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂) and centrifuged at 11 000 g for 10 min. Mitochondrial pellet was re-suspended in 100 µl SOL buffer (50 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.5% Triton-X-100, protease and phosphatase inhibitors). Finally, the mitochondria were sonicated on ice for 10 s. For cytochrome C oxidase activity measurement, we used Cytochrome Oxidase Activity Colorimetric Assay Kit (BioVision, USA). The activity of the enzyme was determined colorimetrically, using

Table 1. Primers used for quantification of TFAM mRNA splice variants and reference genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer code</th>
<th>Primer sequence (5’—3’)</th>
<th>Accession No.</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFAM (TaqMan® assay)</td>
<td>TFAM-F</td>
<td>AGACTGGCAGGTGTACAAAGAAG</td>
<td>NM_001130211</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>TFAM-R</td>
<td>GCTGATCGAGGTCTTTTTTGGTTTT</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TFAM-probe</td>
<td>AGGAATTAACACAGCTTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFAM (SYBR® Green assay)</td>
<td>TFAM-sybr-F</td>
<td>GGAACCTTTCTGATTCCAGAGAAAAAGATA</td>
<td>NM_001130211</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>TFAM-sybr-R</td>
<td>CAAAAGATACCATTTGCATGGAGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>ACTB-F</td>
<td>GGACCTGAGCGACTCCTCA</td>
<td>ENSSCG00000007585.2</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>ACTB-R</td>
<td>GCACAGCTTTCTCTTTGTATGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT1</td>
<td>HPRT1-F</td>
<td>GGGCAAAAACTCGAAGACCTTT</td>
<td>ENSSCG00000027175.1</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>HPRT1-R</td>
<td>ACATTCGAGGGGTCTTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL4</td>
<td>RPL4-F</td>
<td>CTTGGCGTAAGGCTGCCCTCA</td>
<td>ENSSCG00000004945.2</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>RPL4-R</td>
<td>GGAGGGGTCTCTGGTAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBP</td>
<td>TBP-F</td>
<td>ACGTGCTTTAGTGGTAGCTGAG</td>
<td>ENSSCG00000022683.1</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>TBP-R</td>
<td>CAGGAAGCCTTTGGAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the BioVision's kit, based on the decrease of absorption at 550 nm caused by the oxidation of reduced Cytochrome C. Because the oxidation of cytochrome C by cytochrome C oxidase is a biphasic reaction with an initial burst followed by a slower activity, the rate of the reaction is calculated in the linear range. One unit oxidizes 1 µmol reduced cytochrome C per min at pH 7.2 at 25°C. Correlation between the mitochondrial activity and the amount of TFAM mRNA was calculated from cytochrome C oxidase activity data and amount of TFAM mRNA determined by gBlock® Gene Fragments assay using the RStudio software (Version 0.98.1091).

**Data and statistical analysis.** Data analysis was performed using Viia™ 7 software (Version 1.2.2., Applied Biosystems). The baseline was set automatically and threshold was manually adjusted for determination of Ct. The standard curve for absolute quantification of the alternatively spliced and the long form TFAM was constructed for a known synthetically designed double stranded DNA by gBlock® Gene Fragments (Integrated DNA Technologies, Inc.). gBlock® standards for absolute quantification were diluted 10 fold in order to adapt the standard concentration to the TFAM mRNA concentration in the samples. For statistical analysis RStudio software (Version 0.98.1091) was used. A ratio between wild type and alternatively spliced form of TFAM (quantified by qPCR) was calculated and ANOVA was performed using the GLM procedure. \( P \)-value of < 0.05 was considered statistically significant.

**Statistical model:**

\[
y_{ijk} = \mu + T_i + B_j + e_{ijk}
\]

where:

\[
y_{ijk} = \text{ratio} \\
\mu = \text{mean}
\]

\( T_i \) = tissue (m. semispinalis capitis, m. semimembranosus, heart, spleen, liver, and brain) \( B_j \) = breed (commercial hybrid or Krsko polje breed) \( e_{ijk} \) = residual variance

Due to the different conditions in which the commercial pig line and Krsko polje pigs were kept, the possible environmental effects are added to the breed effect in our model. However, it seems not very likely that the environment would have an effect on the splicing regime.

**RESULTS**

Molecular characterization of the long and the short form of the TFAM transcript was performed using cDNA sequencing. The alignment of both transcripts with its translation to amino acid sequence is shown in Figure 1.

**Quantification of TFAM.** In the present study the qPCR for quantification of the long and short form of TFAM mRNA has been established. The presence of both TFAM mRNA forms was confirmed in all six tissues (skeletal muscles SSC and SM, heart muscle, liver, brain, and spleen) in both analyzed genotypes (commercial hybrid and Krsko polje pig). However, the ratios of the long form compared to the short one differed considerably between tissues and breeds.

As shown in Figure 2, the qPCR thresholds for the long and short form show significant prevalence of the long form in all tissues. The overall difference between long and short form exceeds one order of magnitude and on average the short form represents less than 10% of the long form.

**Relative ratio between the long and the short TFAM mRNA forms in different tissues.** The ratio

\[
g_{i} = \frac{n_{i}^{s}}{n_{i}^{l}}
\]

where \( n_{i}^{s} \) and \( n_{i}^{l} \) represent the amount of short and long form TFAM mRNA respectively in tissue \( T_i \).
between the long and the short form (long form/\(\Delta\)Ex4 form) was strongly in favour of the long form in all six tissues used in this study. The highest ratio of the long form vs short form (16.38) was found in the brain tissue of the commercial hybrid pig line. On the other hand, the lowest proportion of the long form vs short form (4.59) was found in SM of the commercial hybrid line (Figure 3). The ratio of the long form vs short form was in all other tissues between these two values. In all tissues, with the only exception of the brain tissue, the proportion of the short form was higher in the Krsko polje pig compared to the commercial hybrid. The average ratio of the short form in Krsko polje pig was 7.64% and in the commercial hybrid 9.83%. According to the statistical model used in this study, the effect of breed was highly significant for all tissues (\(P < 0.001\)). Because the rearing conditions were different among breeds, the environmental factors could potentially contribute to the breed effect and therefore the breed

Figure 2. qPCR thresholds for the long and short form of mitochondrial transcription factor A (TFAM) mRNA in different tissues. Two clusters of samples represent amplifications of the long (mean Ct 20.31, left cluster) and the short (mean Ct 29.15, right cluster) form

Figure 3. Expression ratios between tissues and breeds (SM = \textit{m. semimembranosus}, SSC = \textit{m. semispinalis capitis}, heart, liver, brain, and spleen). The gray colour represents commercial hybrid pigs and black colour represents Krsko polje pig. The ratio is calculated for the long form mitochondrial transcription factor A (TFAM) vs alternatively spliced short form –\(\Delta\)Ex4 TFAM. Asterisk indicates a significant difference in the ratio between breeds in SM
The effect of tissue was statistically significant ($P < 0.05$) with the only exception of the liver tissue, where the effect was not significant ($P > 0.05$).

**Correlation between mitochondrial activity and absolute amount of TFAM mRNA in SSC and SM.** The cytochrome C oxidase activity is positively correlated with the amount of TFAM mRNA in SSC and SM. Due to the low number of samples analyzed and a considerable variation in mitochondrial activity and the amount of TFAM mRNA, only a small portion of variation in cytochrome C oxidase activity can be attributed to the different amount of TFAM mRNA in SSC ($R^2 = 0.11372$) and SM ($R^2 = 0.16589$). Correlation between cytochrome C oxidase activity and the absolute level of TFAM mRNA in SSC and SM is presented in Figure 4.

**Effect of alternative splicing on the structure of TFAM protein.** Using the 3D modelling (Arnold et al. 2006) of the effect of the loss of one high mobility group (HMG) box, encoded by the mRNA sequence which is a subject of alternative splicing, is visualized in Figure 5. The long form of TFAM is presented on the left hand panel (A) and the short form, which is the consequence of partially skipped exon 4, is shown on the right hand panel (B). The deletion of one HMG box might have consequences for TFAM binding affinity for mtDNA.

**DISCUSSION**

TFAM is one of the most important proteins regulating mitochondrial biogenesis. Its transcription factor activity is necessary for replication and transcription of mtDNA. In addition to its function in replication and transcription, the presence of TFAM is necessary for mtDNA maintenance. In this study we have shown that
alternatively spliced TFAM transcripts, missing part of exon 4, are present in all examined tissues in both pig breeds included, although at low concentrations. Taking into account our previous observations (Kunej et al. 2009), we can assume that both forms, the long (full length) and the short form of TFAM (ΔEx4), are present in different pig breeds. The ratios between alternatively spliced TFAM and full length TFAM differ significantly among tissues. Tissue has a statistically significant effect with the only exception of the liver tissue. The highest relative amount of the –ΔEx4 form was found in both muscles (SM and SSC) and the lowest in the brain tissue. These findings support the hypothesis that the high energy demand of the cell favours the long form of TFAM. In addition, the effect of the breed was highly significant, showing lower proportion of –ΔEx4 form in the Krsko polje pig, which might also reflect the association with the higher energy demand in Krsko polje pig. Since the TFAM is a 25-kDa protein that binds to DNA (Falkenberg et al. 2007) and contains two high mobility group box domains (HMG box 1 and 2) responsible for DNA binding, the TFAM has an important role in mtDNA packaging (Alam 2003), due to its ability to bend DNA with its high mobility group box (HMG) domains (Fisher et al. 1992). DNA binding plays an important role in the maintenance of mtDNA genome, mainly through prevention of depletion of mtDNA through harmful damages like deletions (Wang et al. 2013). Alternative splicing of TFAM transcripts was also found in other species, although in the majority of them the skipping of exon 5 was reported in humans, mouse, and rats (Rantanen and Larsson 2000; Bruno et al. 2007). The product of alternative splicing leading to the –ΔEx5 TFAM variant in man is characterized by the deletion of the half of the HMG box 2 (Rantanen and Larsson 2000). However, in our case the product of alternative splicing affecting the larger part of the exon 4 resulted in deletion of the HMG box 1. From the comparison of secondary structure models for both TFAM protein variants it can be concluded that in pig the larger part of the helical structure is abolished and probably the DNA binding affinity of TFAM is affected. Since the ΔEx4 mutation most likely leads to the TFAM protein with reduced binding affinity, the persistence of the shorter form in all investigated tissues and in both breeds raises the question why natural and artificial selection did not eliminate this splicing variant. The quantity of the spliced variant is relatively low, but expression ratios vary between tissues and breeds, as shown in Figure 4. The higher ratios in some tissues could be linked with high metabolic rate (brain and liver) (Fatouros et al. 1995; Belanger et al. 2011; Rui 2014). The large difference in ratios between SSC and SM in commercial hybrid pigs may reflect the difference between predominant metabolic types in these two muscles. In both muscles the ratio of the long form of TFAM is significantly higher in Krsko polje pig compared to the commercial hybrids, however the difference between both muscles is much more pronounced in the commercial hybrids which might be the consequence of breeding for meat traits in the modern crossbreed line, whereas in the less rigorously selected Krsko polje pig the oxidative metabolism type seems to prevail.

CONCLUSION

The presence of alternative splicing of TFAM mRNA has been confirmed in all examined porcine tissues. The effects of tissue on the ratio between the long (WT) and the short (alternatively spliced) transcript were statistically significant in all tissues, except for the liver tissue. On the other hand, the effect of the breed (which, according to the model used, was combined with environmental effects) was only significant for the SM muscle. The oxidative potential of SM and SSC muscles was positively correlated with the absolute TFAM mRNA quantity. The proportion of alternatively spliced TFAM was low in all tissues, suggesting that alternative splicing is a rare event and not detrimental for the survival of the cell. Assuming that the short form of TFAM may have impaired binding to mtDNA, due to the lack of one helical domain, it is surprising that the appearance of the short form seems to be conserved across different species and across different pig breeds.

Acknowledgement. The authors thank Andrej Kastelic, MSc, for his help in getting Krsko polje pig samples.

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Received: 2017–03–22
Accepted after corrections: 2017–10–24