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Molecular identification of the gene encoding porcine tristetraprolin (TTP)

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Abstract Tristetraprolin (TTP) is a CCCH tandem zinc finger protein that can bind to and destabilize certain mRNAs containing AU-rich element (ARE) binding sites. In this study, a novel porcine cDNA has been isolated by expressed sequence tag assembly and subsequently confirmed by RT-PCR analysis, and designated porcine TTP (poTTP). The open reading frame of the poTTP cDNA is 981 bp, encoding 326 amino acids. The *poTTP* gene is approximately 2.5 kb in size and contains a single intron. Southern blotting analysis demonstrated that it is a single copy gene. Real-time quantitative PCR analysis revealed that the *poTTP* gene is constitutively expressed in all detected tissues, and with the highest mRNA level in lymphoid tissues spleen and thymus. Recombinant His_6-tagged poTTP protein and its two zinc finger mutants (C146G and H127I) were efficiently expressed and purified from *Escherichia coli* BL21 (DE3), respectively. In vitro, RNA-electrophoretic mobility shift assay confirmed a direct interaction between poTTP protein and porcine TNF-α (poTNF-α) mRNA ARE probe; this interaction was eliminated when using either two zinc finger mutants of poTTP. Consistently, mutations within the ARE region prevented the binding interaction between recombinant poTTP protein and poTNF-α mRNA ARE probe. These results indicate that poTTP is an ARE-binding protein that might regulate the turnover of certain mRNAs in vivo.

Keywords ARE-binding protein · EST · Expression analysis · EMSA

Abbreviations

ARE	AU-rich element
EST	expressed sequence tag
ORF	open reading frame
BLAST	Basic Local Alignment and Search Tool
GAPDH	glyceraldehyde phosphate dehydrogenase
IPTG	isopropyl β-D-1-thiogalactopyranoside

Introduction

Tristetraprolin (TTP, also known as ZFP36, Nup475, TIS11, and G0S24) is a well-characterized tandem CCCH ($CX_8CX_5CX_3H$) zinc finger domain RNA-binding protein that can regulate the decay of many mRNAs [1–7]. Human TTP protein binds to the type Π AU-rich element (ARE) found in the 3′-untranslated region (3′-UTR) of target mRNA, leading to the removal of the poly(A) tail from that mRNA and increased rates of mRNA turnover [1, 2, 8, 9]. TTP can bind to the 3′-UTR AREs of certain clinically important mRNAs, such as those encoding tumor necrosis factor-α (TNF-α) [1, 3], granulocyte–macrophage colony-stimulating factor (GM-CSF) [2], interleukin-3 (IL-3) [9], cyclooxygenase 2 [10, 11], plasminogen activator inhibitor

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type 2 [12], *E2A*-encoded transcription factor E47 [13], IL-10 [14], and chemokine KC [15]. Mice deficient in TTP develop a severe inflammatory syndrome, including polyarticular arthritis, myeloid hyperplasia, autoimmunity, and cachexia. This syndrome seemed to be due predominantly to the increased stability of mRNAs for TNF-α and GM-CSF, and the resulting enhanced secretion of these proinflammatory cytokines [2, 3, 16].

The ARE is a loosely defined *cis*-element located in the 3′-UTR of many short-lived mRNAs. The study of additional short-lived mRNAs led to a classification scheme based on the number and spacing of a canonical AUUUA pentamer [17]. Class I AREs (e.g., c-fos) have a few scattered pentamers, class II AREs (e.g., TNF-α) have a cluster of 4–7 partially overlapping pentamers within a U-rich context, and class III AREs (e.g., c-jun) lack pentamers. The pentamer is critical to the function of class II AREs as point mutations within the pentamer prevent rapid mRNA decay [18]. Class II AREs typically have an extended UUAUUUAUU nonamer, and this motif is also the minimal sequence that can effectively induce mRNA decay in reporter systems [19]. The same nonamer binds to TTP with maximum affinity in vitro and is thus considered a canonical TTP binding site [20].

The pig (*Sus scrofa*) is a member of the artiodactyls, which are an evolutionary clade distinct from the primates and rodents. In pig proinflammatory, cytokines play an important role in the pathogenesis of bacterial infections, e.g., in respiratory diseases caused by bacterial and viral infections [21]. In this study, we describe for the first time the cDNA and the genomic DNA sequences, tissue expression pattern and specific ARE-binding activity of porcine TTP (poTTP), which might regulate the expression of certain proinflammatory cytokines.

Materials and methods

Expressed sequence tag database searches

The human TTP cDNA of 1745 bp (GenBank accession no. NM_003407) was used as a seed to search the NCBI expressed sequence tag (EST) database of pig (*S. scrofa*) (http://www.ncbi.nlm.nih.gov/blast). Retrieved five homologous ESTs (GenBank accession nos. CB288050, EW509567, CB286240, DY437561, CK460753) were constructed into a contig by CAP3 (http://pbil.univ-lyon1.fr/cap3.php). The open reading frame (ORF) of this contig sequence was determined by the program of NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequence and putative protein sequence were submitted to NCBI third party annotation databases, and deposited at GenBank.

Bioinformatics analysis

The searches for nucleotide and protein sequence similarities were conducted with Basic Local Alignment and Search Tool (BLAST) algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). In order to obtain the genomic DNA of *poTTP*, the publicly available pig genome database at the NCBI Porcine Genome Resources (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9823) was screened using the poTTP cDNA sequence as a query. A chromosome 14 clone CH242-159G20 (GenBank accession no. FP236263) which encompasses the entire *poTTP* gene was identified by BLASTGen analysis. For the exact localization of the exon–intron boundaries, the mRNA-to-genomic alignment program Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) was used. The putative promoter sequence was analyzed "*in silico*" with the program PROSCAN Version 1.7 from Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/). Primer pairs were designed manually based on Primer Express 2.0 software guidelines (Applied Biosystems).

Southern blotting analysis

Genomic DNA was extracted from porcine spleen using the DNeasy Tissue Kit (Qiagen, USA) following the manufacturer's instructions. The probe was a 566 bp fragment (nucleotide positions 469–1,034 bp in genomic DNA) which was DIG-labeled using the PCR DIG Probe Synthesis Kit (Roche, USA). Genomic DNA samples were digested using the restriction enzymes *Kpn* I, *Sph* I, and *Xho* I (Takara, Japan), respectively. The assay was performed as detailed by Shui et al. [22].

Real-time quantitative PCR

Tissue distribution of *TTP* mRNA in pig was studied by real-time quantitative PCR (qPCR). Large white pigs used in this assay were healthy, female with average weight between 30 and 40 kg from breed cultivation farm, Wuxi, China. Tissue from porcine heart, spleen, liver, lung, kidney, thymus, colon, and cecum was removed and processed for RNA isolation using TRIzol reagent (Gibco-BRL, USA). In brief, first-strand cDNAs were synthesized using Reverse Transcriptase XL (Takara, Japan) and then stored at −20°C for subsequent SYBR Green RT-PCR. A pair of poTTP primers, T1 (5′-TCCGACCACGGAGAGACTGAGTCCAG-3′) and T2 (5′-ACCAAGCTGGTGGAGCGACCAGGTAG-3′), was used to amplify a 125 bp product. The porcine *GAPDH* mRNA (GenBank accession no. DQ845173) was used as internal control to verify the

real-time qPCR reaction. Two primers G1 (5′-AGGCTG TGGGCAAGGTCATCCCTGAG-3′) and G2 (5′-GCAG GTCAGATCCACAACCGACAC-3′) were used to amplify a 98 bp fragment of glyceraldehyde phosphate dehydrogenase (GAPDH). DEPC–water for the replacement of cDNA template was used as negative control. The SYBR Green RT-PCR assay was carried out as previously described in detail [23]. The relative expression level of poTTP was represented by using the method of $2^{-\Delta\Delta Ct}$ [24, 25].

Productions of recombinant poTTP protein and its two mutants

The *poTTP* cDNA was amplified from total RNA of spleen by RT-PCR using primers T3 (5′-TCAGGACATATG GATCTCACCGCCATCTACGAG-3′) and T4 (5′-ACTG AGGAATTCTCACTCAGAAACAGAAATACGATTG-3′), which is introduced unique *Nde* I or *EcoR*I restriction site (underlined). The amplified product was subcloned into the pET-28a(+) expression plasmid (Novagen, USA), forming a sequence encoding a fusion protein of poTTP and a NH_2-terminal His_6 tag. After verification by sequencing, plasmids containing the poTTP cDNA were transformed into *Escherichia coli* BL21 (DE3) to express His_6-tagged poTTP protein. In brief, the bacteria were cultured in LB medium with vigorous shaking (240 rpm) at 37°C to the density of $OD_{600} \approx 0.6$. For production of soluble poTTP proteins, the induction scheme was established as follows: the concentration of isopropyl β-D-1-thiogalactopyranoside in culture medium is 0.2 mmol/l; the temperature is 18°C and the total induction time is 20 h (shaking at 200 rpm). After expression, soluble poTTP proteins were purified by affinity chromatography on a nickel chelate agarose column from *E. coli* as described previously [26]. Finally, protein fractions were concentrated, and imidazole was removed with an Amicon ultra device (Millipore, USA).

Site-directed mutagenesis was carried out using the QuikChange system (Stratagene, USA) to produce two poTTP mutant constructs, using primer pairs T5 (5′-GTA CAAGACGGAACTC**G**GCCACAAGTTCTACCT-3′) and T6 (5′-GTAGAACTTGTGGC**C**GAGTTCCGTCTTGT-3′), and T7 (5′-AAGTGCCAGTTTGCC**AT**TGGCCTGGGC GAG-3′) and T8 (5′-TCGCCCAGGCCA**AT**GGCAAAC TGGCACTTG-3′) (bold residues indicate mutated bases), that would change Cys-146 to Gly (C146G) or His-127 to Ile (H127I), both amino acids are predicted to be critical for RNA binding [3]. Both recombinant C146G and H127I mutant proteins were expressed in and purified from *E. coli* BL21(DE3) using the same strategy which was used for the production of wild-type poTTP.

RNA-electrophoretic mobility shift assay

For the RNA-binding activity assay, RNA-electrophoretic mobility shift assay was performed. In brief, single-stranded RNA oligonucleotides that represent the porcine TNF-α (poTNF-α, Genbank accession no. EU682384) mRNA ARE region (UUAUUUAUUUAUUUAUUUAUUAUU UAUU) as well as its mutant (UUAUUU**C**UUU**C**UU UAUUU**C**UUAUUU**C**UU) in which the binding sites have been mutated [27] were synthesized and their 3′-end were labeled with biotin (Invitrogen, Shanghai). Approximately, 2 μg (~50 pmol) of poTTP, or poTTP (C146G), or poTTP (H127I) was incubated with the ARE probe (100 fmol) at room temperature for 20 min in 1× RNA gel-shift buffer (10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 5% (v/v) glycerol, 0.5% (v/v) NP-40, and 2 mM DTT). Following addition of 50 μg of heparin and 1.2 μg of yeast tRNA and incubation at room temperature for 10 min, unprotected RNA was digested with 100 units of RNase T1 at 37°C for 15 min. For the competition experiments, preincubation was performed in the presence of unlabeled competitor RNA probe at a 10- to 100-fold molar excess. The reaction mixtures were separated at 250 V for 60–80 min through a 6% nondenaturing acrylamide gel in 0.4× TBE buffer (1×, 10.8% Tris, 5.5% boric acid, 20 mM EDTA, pH 8.0) and then transferred to a nylon membrane. The nylon membranes were cross-linked, and chemiluminescent detection was performed. Membranes were exposed to an X-ray film for 15 min before developing.

Results and discussion

cDNA and genomic DNA sequences of the *poTTP* gene

By EST assembly, a porcine contig (1,691 bp) which has high homology with human TTP cDNA was predicted to encode poTTP protein. By analysis of this contig, the predicted poTTP cDNA contains an ORF of 981 bp flanked by 31 and 679 bp of 5′- and 3′-UTR, respectively. Three instability motifs (ATTTA) were found included in 3′-UTR sequence. In order to verify the correctness of this sequence, two primers P1 (5′-ATGGATCTCACCGC CATCTACGAGAG-3′) and P2 (5′-TCACTCAGAAACA GAAATACGATTGAAG-3′) were used to clone the full-length ORF of poTTP cDNA. By RT-PCR and sequencing analysis, the DNA fragment of 981 bp was successfully isolated from porcine total RNA of spleen or other tissues (data not shown). Finally, this experimental confirmed poTTP cDNA sequence was deposited in GenBank under accession number GU066778.

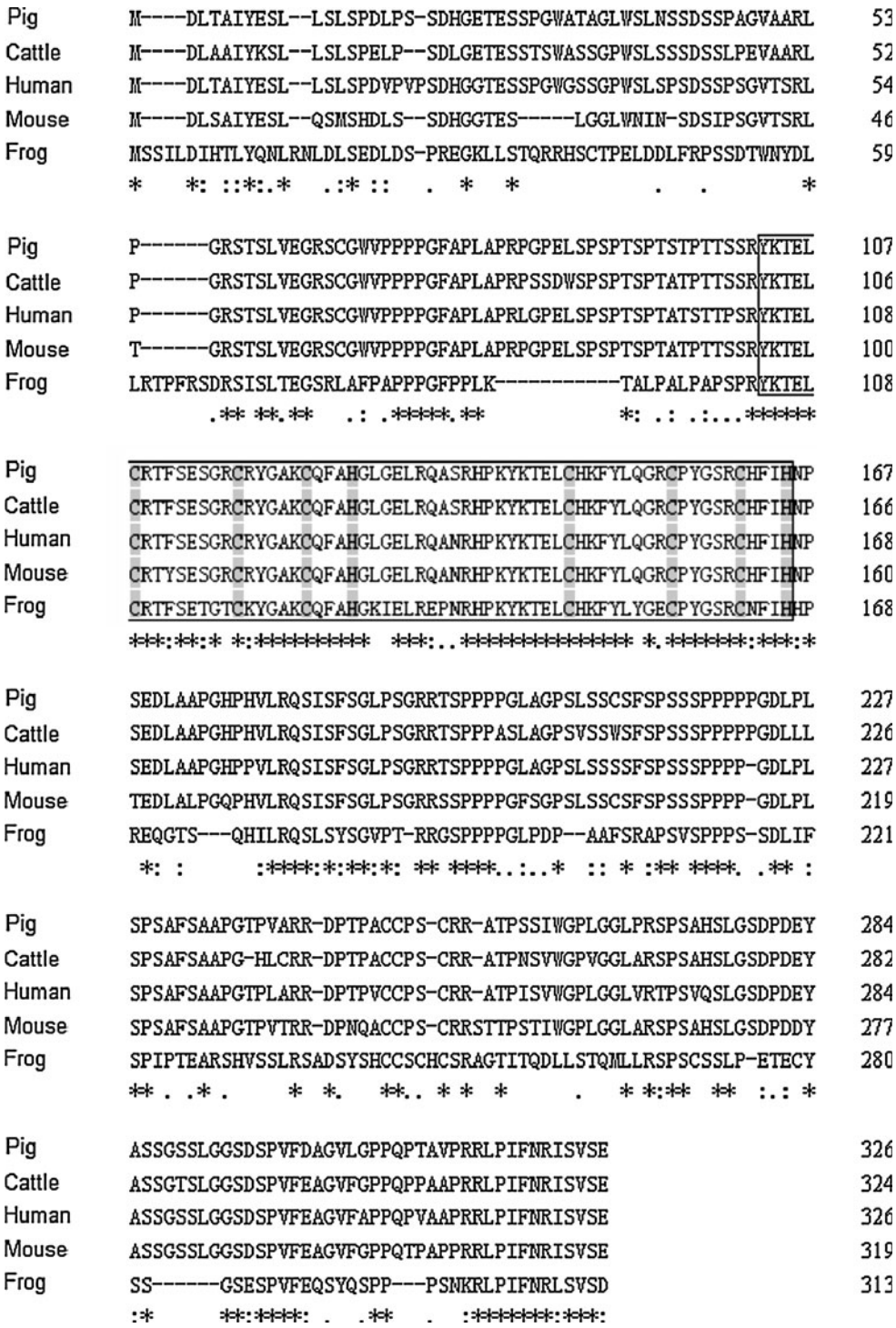

Fig. 1 Alignments of deduced amino acid sequences of poTTP (GenBank accession no. GU066778), cattle TTP (GenBank accession no. NM_174493), human TTP (GenBank accession no. NM_003407), mouse TTP (GenBank accession no. NM_011756), and frog TTP (GenBank accession no. AF061980). The symbol (*) indicates that the aligned residues are identical. Substitutions said to be conservative or semi-conservative by ClustalW are marked by (:) and (.), respectively. The tandem zinc finger (TZF) domain containing two $YKTELCX_8CX_5CX_3H$ motifs is *boxed*. Six cystine and two histine residues conserved in TZF domain are marked with a gray background

The *poTTP* gene (nucleotides 34,863–37,366 in clone CH242-159G20) is 2,504 bp in length and contains only one intron of 813 bp. The exon–intron splice junction sequences conform to the canonical GT–AG rule. The GenBank accession number for the *poTTP* genomic DNA is HM480487.

In order to investigate the 5′-flanking region of the *poTTP* gene, an approximately 2 kb fragment from the clone CH242-159G20, which includes the 5′-flanking region and the first exon of the *poTTP* gene, was analyzed. By computer-assisted analysis of the *poTTP* gene promoter region, it revealed motifs for several transcription factors, including sites for the TATA, Sp1, EGR-1, and AP2 binding proteins, highly conserved with human promoter sequence [28].

Sequence comparison analysis

Porcine TTP protein consists of 326 amino acids, within a 63-aa region containing $YKTELCX_8CX_5CX_3H$ tandem zinc finger (TZF) motifs. Sequence similarity at the amino acid level between TTP of pig and cattle, or human, or mouse, or frog is 94, 92, 89, and 47%, respectively, with 90, 85, 84, and 35% sequence identity respectively (Fig. 1).

Southern blotting analysis

The copy number of the *poTTP* gene was analyzed by southern blotting under stringent hybridization and washing conditions. The digests obtained with three different enzymes gave one band (Fig. 2). As the genomic sequence covered by the probe does not contain any cutting sites of all restriction enzymes used here, the results suggested that pig has only one copy of the *TTP* gene in its genome.

Tissue expression analysis

Indication about the physiological function of a protein might be obtained from its tissue distribution studies. The constitutive mRNA expression of porcine *TTP* in different tissues was analyzed by real-time qPCR normalized against *GAPDH* mRNA levels. A dissociation curve showing a single peak at the melting temperature expected for that amplicon suggested specific amplification. Expression was determined as fold increased $2^{-\Delta\Delta Ct}$ levels relative to the tissue with lowest expression (heart) set to 1. SYBR Green RT-PCR analysis revealed that the *poTTP* gene was constitutively expressed in all detected tissues, with highest mRNA levels observed in lymphoid tissues spleen (8.70-fold) and thymus (8.13-fold), followed by lung (6.32-fold), colon (5.97-fold), and cecum (5.93-fold). Lowest levels were detected in liver (2.96-fold), kidney (2.47-fold), and heart (the calibrator) (Fig. 3), in line with the reports on the expression profile analysis of human TTP [1, 5], indicating that TTP in pig as in human, might play a similar biological role in vivo.

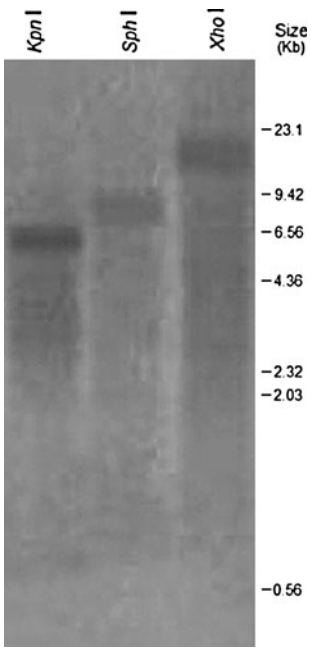

Fig. 2 Southern blotting analysis of genomic DNA isolated from porcine spleen digested with the indicated enzymes, and hybridized with a probe specific for the *poTTP* gene

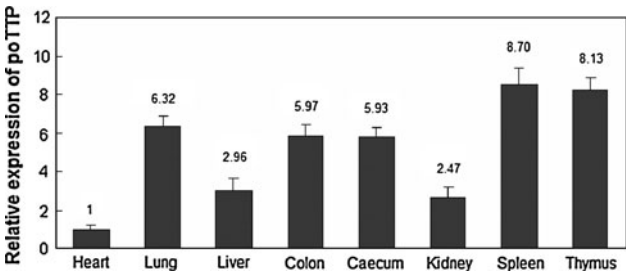

Fig. 3 Expression analysis of the *poTTP* gene in various tissues. Mean mRNA levels (including standard deviations) in eight tissues were analyzed by real-time qPCR. Data are $2^{-\Delta\Delta Ct}$ levels calculated relative to the tissue with the lowest expression (heart) set to 1, normalized against *GAPDH* mRNA levels

Porcine TTP protein specific binding to poTNF-α ARE probe

Since in vitro human TTP protein can bind to certain mRNAs (e.g., human TNF-α) containing ARE binding sites, the ability of recombinant poTTP to bind directly to poTNF-α ARE RNA probe was assessed using RNA gel shift assay. Porcine TTP and two separate zinc finger mutant forms of poTTP (C146G and H127I) were expressed in and purified from *E. coli* BL21 (DE3) (Fig. 4a). It shows that the recombinant proteins were quite pure based on SDS-PAGE. Purified proteins were recognized by a mouse anti-His_6 tag monoclonal antibody (Invitrogen, USA) and a rabbit anti-poTTP polyclonal antibody prepared in our laboratory, respectively (Fig. 4a).

When equal amounts of poTTP, poTTP (C146G), or poTTP (H127I) was allowed to bind the poTNF-α mRNA ARE probe, the probe was only shifted by the recombinant poTTP protein, resulting in a stable TTP–ARE complex, whereas the zinc finger mutants C146G and H127I caused no shifting of the probes (Fig. 4b). Consistently, poTNF-α ARE probe, which has mutations in the binding sites, could not be shifted into the gel by poTTP protein (Fig. 4b).

The specificity for poTTP was tested using competition assays with an excess of unlabeled poTNF-α ARE probes. It was demonstrated that the interaction was disrupted by competition with unlabeled poTNF-α ARE

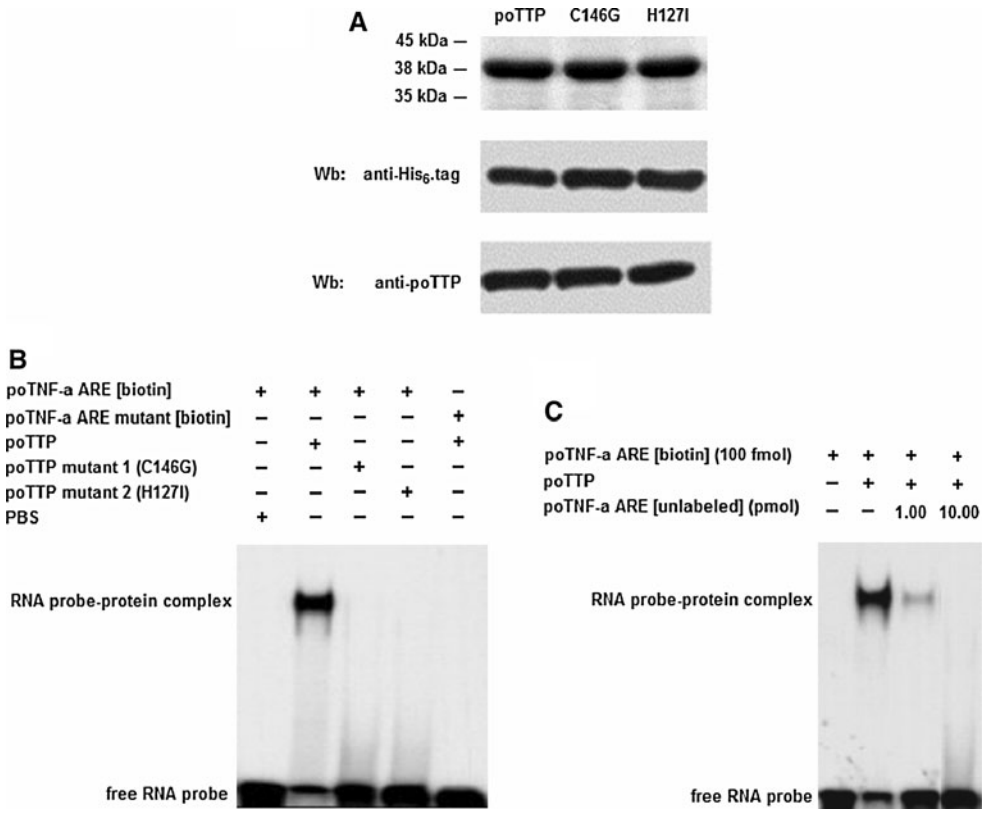

Fig. 4 Characterization of the poTTP protein. **a** A Coomassie blue-stained SDS-PAGE gel showing purified recombinant poTTP compared with recombinant poTTP zinc finger mutants (C146G and H127I). Molecular weight marker positions are indicated. *Wb* western blotting. **b** Gel shift of recombinant poTTP with ARE-containing RNA oligonucleotides. RNA probes (100 fmol each) representing the poTNF-α mRNA ARE region and its mutant in which the binding sites have been mutated were both labeled with biotin and incubated with purified poTTP, or mutant C146G, or mutant H127I, respectively (2 μg each). **c** Specificity was investigated by binding competition assays in the presence of 10- and 100-fold excess of unlabeled poTNF-α ARE probes. The position of the free probe at the bottom of each gel (**b**, **c**) is indicated

probes, indicating a specific interaction for poTTP with the poTNF-α ARE (Fig. 4c).

In summary, the cDNA and the genomic DNA of the *poTTP* gene were isolated in this study. Furthermore, the analysis of gene copy number, tissue expression profile and ARE-binding activity were also demonstrated. These data indicate that poTTP, like human TTP, is an ARE-binding protein that might regulate the turnover of certain mRNAs as a *trans*-acting factor. It will provide the basis for investigations on the role of poTTP in this important domestic species and an animal model for human diseases.

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