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Expression of 25 kDa thiamine triphosphatase in rodent tissues using quantitative PCR and characterization of its mRNA

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Abstract

Thiamine triphosphate (ThTP) is found in most organisms, but its biological role remains unclear. In mammalian tissues, cellular ThTP concentrations remain low, probably because of hydrolysis by a specific 25 kDa thiamine triphosphatase (ThTPase). The aim of the present study was to use quantitative PCR, for comparing the 25 kDa ThTPase mRNA expression in various mouse tissues with its enzyme activities. ThTPase mRNA was expressed at only a few copies per cell. The highest amount of mRNA was found in testis, followed by lung and muscle, while the highest enzyme activities were found in liver and kidney. The poor correlation between mRNA levels and enzyme activities might result either from tissue-specific post-transcriptional regulation of mRNA processing and/or translation or from the regulation of enzyme activities by post-translational mechanisms. Purified recombinant human ThTPase was phosphorylated by casein kinase II, but this phosphorylation did not modify the enzyme activity. However, the characterization of the 3′-untranslated mRNA region revealed a unique, highly conserved, 200-nucleotide sequence that might be involved in translational control. In situ hybridization studies in testis suggest a predominant localization of ThTPase mRNA in poorly differentiated spermatogenic cells. This is the first study demonstrating a cell-specific 25 kDa ThTPase mRNA expression, suggesting that this enzyme might be related to the degree of differentiation or the metabolic state of the cell.

Keywords: Thiamine triphosphate; Thiamine triphosphatase; Quantitative PCR; Post-transcriptional control; Testis

Abbreviations: CKII, casein kinase II; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; HPRT, hypoxanthine phosphoribosyltranferase; NPP, *p*-nitrophenyl phosphate; PKA, protein kinase A; PKC, protein kinase C; ThDP, thiamine diphosphate; ThTP, thiamine triphosphate; ThTPase, thiamine triphosphatase; ThMP, thiamine monophosphate; UTR, untranslated region

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1. Introduction

Thiamine triphosphate (ThTP) is found at low concentrations in most organisms studied so far (Makarchikov et al., 2003). However, in contrast to the well-known cofactor thiamine diphosphate (ThDP) its role remains enigmatic. Our previous results have shown that ThTP can activate large-conductance chloride channels in excised inside-out patches of neuroblastoma cells, possibly by a phosphorylation-dependent mechanism (Bettendorff, Kolb, & Schoffeniels, 1993). More recently, it was shown that, in the electric organ of *T. marmorata*, ThTP phosphorylates rapsyn (Nghiêm, Bettendorff, & Changeux, 2000), a postsynaptic protein required for the clustering of acetylcholine receptors at the neuromuscular junction. ThTP also phosphorylates unidentified proteins in rodent brain (Nghiêm et al., 2000) and in *E. coli* extracts (Lakaye, Wirtzfeld, Wins, Grisar, & Bettendorff, 2004b). Furthermore, ThTP is required for the optimal growth of *E. coli* during amino acids starvation (Lakaye et al., 2004b). Thus, ThTP might be part of a new signaling cascade involving protein phosphorylation. This is an exciting hypothesis as, until now, ATP was the only known substrate for protein kinases in eukaryotic cells.

In bacteria and plants, ThTP levels rapidly increase in response to cellular stress (Makarchikov et al., 2003; Lakaye et al., 2004b) but, in mammalian cells, ThTP concentrations seem to be less variable and remain around 0.1–1 μM (Bettendorff, Weekers, Wins, & Schoffeniels, 1990), probably because of the existence of a specific thiamine triphosphatase (ThTPase) (Lakaye et al., 2002; Makarchikov & Chernikevich, 1992). This soluble ThTPase is a 25 kDa monomer that we have recently characterized (Lakaye et al., 2002). The enzyme is found only in mammalian tissues (Makarchikov et al., 2003) and is highly specific for ThTP (Lakaye et al., 2002; Makarchikov & Chernikevich, 1992; Makarchikov & Chernikevich, 1998; Penttinen & Uotila, 1981). The relatively low abundance of the protein is compensated by a high catalytic efficiency ($k_{cat}/K_m = 6 \times 10^6\,s^{-1}\,M^{-1}$). It has been suggested that the 25 kDa ThTPase defines, along with the bacterial CyaB-like adenylyl cyclases, the new CYTH superfamily of organic phosphate-binding proteins (Iyer & Aravind, 2002). ThTPase activity seems to be a relatively recent acquisition in this ancient superfamily. No other, even partially, homologous sequence is present in the human genome. Non-mammalian tissues also contain ThTPase activity but, so far, we do not know to what extent they are specific for ThTP (Makarchikov et al., 2003).

Preliminary results using a human multiple tissue expression array showed the highest mRNA expression in testis, prostate and uterus (Lakaye et al., 2002). The lowest expression was found in the ovaries, digestive tissues, transformed cell lines and the nervous system. These results are surprising, as ThTPase has mainly been studied in the brain, where the specific ThTPase activity is relatively high compared to other tissues. Here, using a quantitative PCR method, we show that 25 kDa ThTPase mRNA is expressed at relatively low levels in all mouse tissues tested. The mRNA levels are poorly correlated with ThTPase enzyme activities. The 3′ untranslated end of ThTPase mRNA contains a unique, highly conserved sequence, that might be involved in translational control.

2. Materials and methods

2.1. Determination of ThTPase and alkaline phosphatase activities

Mouse tissues were homogenized in a teflon–glass Potter–Elvehjem homogenizer in five volumes of 50 mM Tris–HCl buffer, pH 7.5, containing 150 mM KCl and 0.2 mM EDTA, then centrifuged at 27,000 × *g* for 30 min. The supernatant was used as enzyme extract. ThTPase activity was determined as described previously (Lakaye et al., 2002). The standard reaction medium contained 50 mM Bis–Tris-propane buffer (pH, 8.7), 5 mM $MgCl_2$, 100 μM ThTP (a three to four fold excess over K_m) and an aliquot of the enzyme preparation. After incubation during 10–30 min at 37 °C, the reaction was stopped by addition of trichloroacetic acid (final concentration 10%). The acid was extracted with diethyl ether and the ThDP formed was estimated by HPLC (Bettendorff, Peeters, Jouan, Wins, & Schoffeniels, 1991). ThTP was prepared as previously described (Bettendorff, Nghiêm, Wins, & Lakaye, 2003).

Alkaline phosphatase purified from bovine intestinal mucosa was obtained from Sigma–Aldrich (St. Louis, MO, USA). Hydrolysis of *p*-nitrophenyl phosphate (NPP), thiamine monophosphate (ThMP) and ThDP (all from Sigma–Aldrich) and ThTP was measured by estimation of P_i released by the method of Lanzetta, Alvarez, Reinach, and Candia (1979) as previously described (Lakaye et al., 2004a).

Protein concentrations were determined by the method of Peterson (Peterson, 1977).

2.2. Quantitative real time RT-PCR

Total RNA was isolated from mouse organs using Instapure reagent (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. In order to avoid contaminations by genomic DNA, extracts were treated by DNase I (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Ten microgram of RNA were reverse transcribed in a total volume of 40 μl using M-MLV (Invitrogen Life Technologies) and oligo-dT primers (Invitrogen Life Technologies). For quantitative PCR, 3 μl of cDNA were diluted 10 times and amplified with Quantitect SYBR® Green PCR master mix (Qiagen, Valencia, CA), following the manufacturer's protocol, using a Rotor Gene RG 3000 thermocycler (Westburg, Leusden, The Netherlands), by 1 step of 2 min at 50 °C, followed by 1 step of 15 min at 94 °C, and then by 45 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 45 s) and elongation (72 °C, 45 s). The primers F1 (5′-AGCATGCTTGGAGTGCCAG-3′) and R1 (5′-GCGAAGTCCGGAGACCTGATA-3′) were used at 0.5 μM for amplification. To avoid amplification of contaminating genomic DNA, F1 was chosen at the junction between the two exons.

For quantification, hypoxanthine phosphoribosyltransferase (HPRT) was used as endogenous control by amplification with the primers F2 (5′-TGACACGGCAAAACAATGCA-3′) and R2 (5′-GGTCCTTTTCACCAGCAAGCT-5′) at 2.5 μM (Vandesompele et al., 2002). Controls were made in the absence of reverse transcriptase both for HPRT and ThTPase, but no amplification was observed under these conditions.

The amounts of ThTPase and HPRT mRNA were calculated by means of standard curves obtained with known dilutions of the plasmids bearing the cDNA fragments as PCR template. All real time experiments were carried out in triplicate, on various organs from three mice.

2.3. Localization of ThTPase mRNA by in situ hybridization

A 644 bp cDNA fragment was amplified from rat brain mRNA by RT-PCR using forward (5′-CTTGAACTGCTGGGGTCTGG-3′) and reverse (5′-AATCAATGGAGGCTGCTTAGCG-3′) primers. It was cloned into pCRII-TOPO (Invitrogen Life Technologies, Carlsbad, CA, USA) and its sequence confirmed. To produce riboprobes, an *Sph*I/*Sac*I fragment of 430 bp encompassing 134 bp upstream and 224 bp downstream the stop codon was subcloned into pSP73. For the synthesis of sense and antisense non-radioactive riboprobes with the digoxigenin-RNA labeling kit (Roche Diagnostics, Vilvoorde, Belgium), the plasmid was linearized by *Eco*RI or *Hin*dIII, respectively.

Male Wistar rats were killed by cervical dislocation and the testis was removed immediately, frozen on dry ice and stored at −80 °C until used. Frozen, 20 μm thick tissue sections were cut with care to avoid RNase contamination of the cryostat knife blade. The sections were thaw-mounted onto Menzel Superfrost Plus glass slides (VWR, International, Leuven, Belgium), fixed with 4% paraformaldehyde and acetylated with 0.25% acetic anhydride. Hybridization was carried out at 42 °C with the digoxigenin-labeled riboprobes and the sections were washed in 0.1 × SSC at 55 °C (sodium saline citrate, SSC 20× = 3 M NaCl and 0.3 M Na-Citrate). Hybrids were detected by alkaline phosphatase-labeled anti-digoxigenin antibodies and NBT/BCIP (Roche Diagnostics) staining.

2.4. ThTPase 3′ end mRNA characterization

Mouse brain poly(A)+ RNA was isolated using the oligotex mRNA kit from Qiagen. For 3′ rapid amplification of cDNA end (RACE) experiments, we used the 5′/3′RACE kit from Roche Diagnostics. Bovine brain poly(A)+ RNA was from BD Biosciences Clontech (Palo Alto, CA, USA). Pig brain total RNA was isolated using Instapure reagent (Eurogentec). Bovine and pig ThTPase mRNA were

amplified following the manufacturer's instructions using Taq DNA polymerase and primers bF (5′-GAAGGACCCCCGACAGACAG-3′) and bR (5′-ACAGGAGGCACTCTACTCTCTAGG-3′) or primers pF (5′-ATGGCCCAGGGCTTGATTGAGGTGGAGCGA-3′) and pR (5′-CACGAGCGGAAATCAATGGAGG-3′), respectively. PCR fragments were cloned in plasmid pCRII by the TOPO cloning method (Invitrogen Life Technologies).

2.5. Phosphorylation of human recombinant ThTPase

Phosphorylation by casein kinase II (CKII) was carried out as previously described (Bontems et al., 2002) in Tris–HCl buffer (50 mM, pH 7.4), KCl (140 mM), $MgCl_2$ (10 mM) with 10 μg of human recombinant ThTPase purified as described earlier (Lakaye et al., 2002), 25, 50 or 100 U of casein kinase II (CKII, Sigma–Aldrich) and 10 μCi [γ-^{32}P]ATP (ICN Biomedicals, Inc, Costa Mesa, CA) in a final volume of 20 μl. The mixture was incubated for 30 min at 30 °C. The reaction was stopped with SDS–PAGE loading buffer and the proteins separated by electrophoresis. The gels were dried and submitted to autoradiography. The 45 kDa Varizella–Zoster virus protein IE63, a good substrate for CKII (Bontems et al., 2002), was produced in *E. coli* and used as a positive control.

Phosphorylation with the bovine heart catalytic subunit of protein kinase A (PKA, Calbiochem, San Diego, CA) was performed under the same conditions as for CKII. Phosphorylation assays with rat brain protein kinase C (PKC, Calbiochem) were performed as described by Woodgett et al. (Woodgett, Gould, & Hunter, 1986), using histone type III-S from calf thymus (Sigma–Aldrich) as positive control. For the determination of the effect of phosphorylation on ThTPase activity, the enzyme was phosphorylated under the same conditions, except that [γ-^{32}P]ATP was replaced by 0.1 mM unlabeled ATP. Then, ThTPase activity was determined as described earlier.

2.6. Data analysis

Statistical analyses were made using GraphPad Instat or GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. Comparison of ThTPase activities in various mouse tissues

In mouse tissues, specific ThTPase activities varied about 10-fold, with the highest values found in liver and kidney and the lowest in thymus and intestine (Fig. 1A). The determinations were made at a ThTP concentration of 100 μM which, taking into account a K_m of about 25 μM for mouse ThTPase (not shown), represents an 80% substrate saturation. Previous results suggest that the K_m values do not significantly differ from one tissue

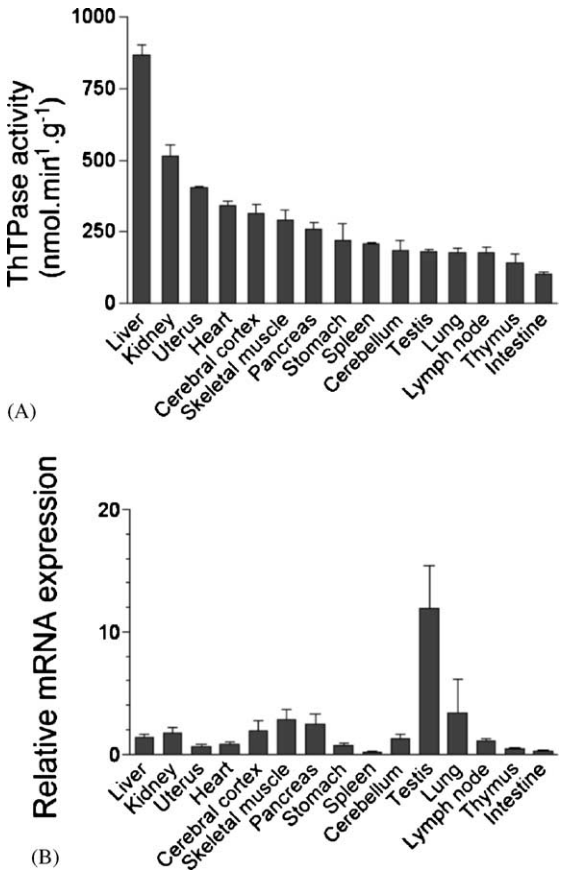

Fig. 1. ThTPase activities (A) and mRNA expression (B) in various mouse tissues. ThTPase mRNA expression was determined using quantitative real time PCR and the values were normalized relative to HPRT mRNA. All results are expressed as mean ± S.D. ($n = 3$); Kruskal–Wallis test $P < 0.0001$.

to another within the same species (Makarchikov et al., 2003). Our results agree with previous data on bovine tissues showing the highest ThTPase activity in kidney and liver, and the lowest in intestine (Makarchikov et al., 2003). They are also in agreement with the results of Penttinen and Uotila (Penttinen & Uotila, 1981), using rat tissues, the latter authors found a twice higher ThTPase activity in the liver than in the brain, while no significant activity was found in the intestine.

It could be argued that, for instance in liver or intestine, ThTP hydrolysis may be due to alkaline phosphatase rather than to the 25 kDa ThTPase. It was thus of interest to determine to what extent mammalian alkaline phosphatase can hydrolyze ThTP and other phosphorylated thiamine derivatives at pH 8.8. Using a commercial alkaline phosphatase preparation, we compared the rates of hydrolysis of NPP, ThTP, ThDP and ThMP. At a concentration of 100 μM (which is well below the K_m for all the substrates used), we found that the rate of ThTP hydrolysis was only about 3% of the rate of NPP hydrolysis. Thus, ThTP is a very poor substrate for intestinal alkaline phosphatase. The rates of ThDP and ThMP hydrolysis were 32 and 16% of the rate of NPP hydrolysis, respectively. Thus, if a significant part of ThTP hydrolysis is due to alkaline phosphatase, it can be predicted that the ThDP formed will be further hydrolyzed to ThMP, as ThDP is a much better substrate than ThTP for this enzyme. However, in all tissues tested, the amount of ThMP detected by HPLC was always less than 10% of the ThDP formed and, in most cases, it was undetectable. This strongly suggests that ThTP hydrolysis is essentially due to the specific 25 kDa ThTPase. Penttinen and Uotila (1981) also concluded that, in rat brain and liver, ThTP hydrolysis was almost exclusively due to the specific ThTPase.

3.2. Comparison of ThTPase mRNA expression in various mouse tissues and correlation with enzyme activities

ThTPase mRNA was amplified using quantitative PCR and the results were normalized with respect to HPRT to correct for errors during reverse transcription and subsequent amplification. HPRT, a housekeeping gene constitutively expressed at low levels, is generally considered as one of the best control genes (Vandesompele et al., 2002). The highest expression of ThTPase mRNA was found in testis (Fig. 1B), followed far behind by lung and skeletal muscle; the lowest expression was in spleen, intestine and thymus. These results are in good agreement with a semi-quantitative Northern blot analysis that we previously performed on a human multiple tissues expression array (Lakaye et al., 2002). ThTPase expression varied about 50-fold between various tissues and was highest in testis, uterus and prostate, followed by lung, bladder and kidney. The signal was relatively low in the liver and barely detectable in spleen and intestine. HPRT expression was much less variable (88000 ± 14000 copies/μg of total mRNA, mean ± S.E.M.), suggesting that it is suitable as control gene, as previously suggested (Vandesompele et al., 2002). In mammalian tissues, HPRT mRNA is of low abundance, expressed at less than 10 copies per cell (Pernas-Alonso, Morelli, di Porzio, & Perrone-Capano, 1999; Vandesompele et al., 2002). In many tissues, the ratio of ThTPase over HPRT mRNA was close to 1 (Fig. 1B), suggesting a similarly low abundance.

No significant correlation was obtained between ThTPase enzyme activity and mRNA expression in different tissues ($P = 0.73$). These results suggest that the enzyme activity may be controlled either by translational control or by post-translational modification of the protein.

3.3. Localization of ThTPase in rat testis using in situ hybridization

Our previous results showed that the highest expression of ThTPase mRNA was in mouse testis. It was therefore interesting to determine the cellular localization of ThTPase mRNA in this tissue. Preliminary experiments on rat tissues using RT-PCR suggested that in this animal 25 kDa-ThTPase mRNA has also the highest expression in testis (not shown). Therefore, in situ hybridization experiments were carried out on rat testis (Fig. 2). Fig. 2A shows that the highest labeling was observed in cells close to the basal lamina. The mRNA expression level decreased throughout the spermatogenic lineage, with the highest expression observed in poorly differentiated spermatogonies close to the basal lamina, and the lowest in differentiated spermatides towards the lumen of the

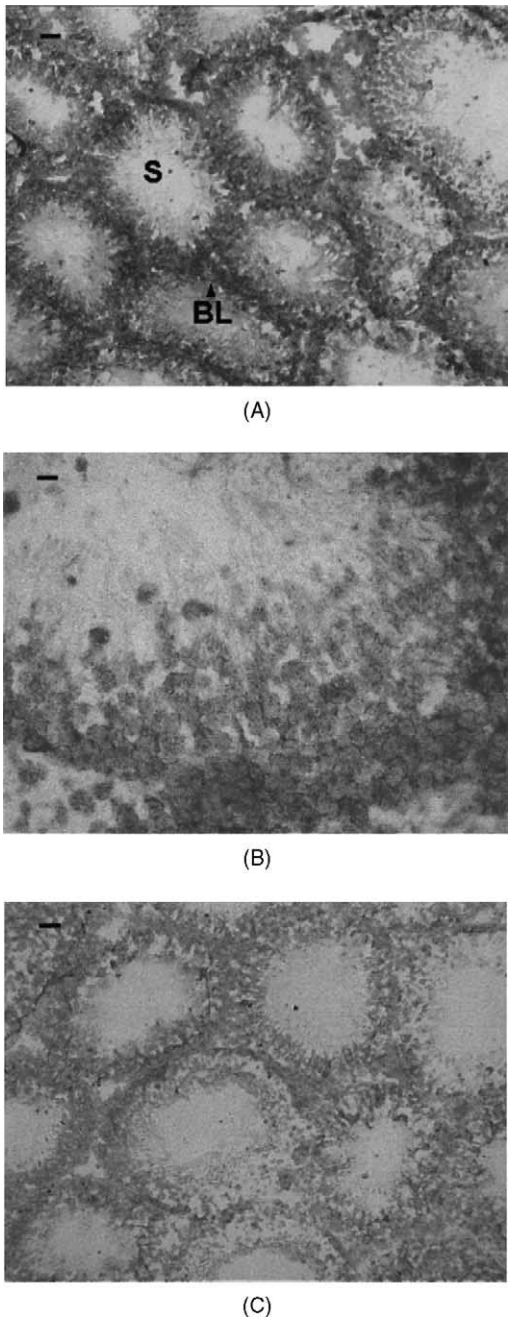

Fig. 2. Localization of 25 kDa ThTPase mRNA in rodent testis. mRNA distribution in rat testis seminiferous tubule, revealed by in situ hybridization at low and high magnification, respectively, is shown in (A) and (B). A control experiment using the sense probe is shown in (C). S, seminiferous tubule, BL, basal lamina. Scale bars: A and C = 200 μm; B = 50 μm.

seminiferous tubule. No signal was observed in late spermatides and spermatozoids.

3.4. Post-translational modification by phosphorylation

The sequence of hThTPase was checked for potential phosphorylation sites using PhosphoBase v. 2.0 (http://www.cbs.dtu.dk/databases/PhosphoBase/). Several potential sites were predicted with a high score (>0.95) for PKA (Ser-60, Thr-34, Thr-38), PKC (Thr-34) and CKII (Ser-60, Thr-34, Thr-38). The open reading frame for human ThTPase was cloned and overexpressed in *E. coli*, and the gene product was purified to homogeneity as previously described (Lakaye et al., 2004a). We then tested whether protein kinases are able to phosphorylate the recombinant protein in vitro. We did not observe any significant phosphorylation by PKC and PKA. However CKII phosphorylated human recombinant ThTPase in a dose-dependent manner (Fig. 3). Other phosphory-

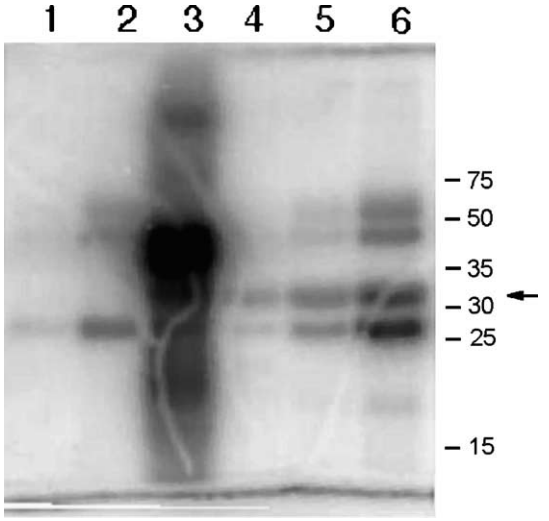

Fig. 3. CKII-dependent phosphorylation of human recombinant ThTPase. The proteins were separated by SDS–PAGE (12%). The gels were dried and submitted to autoradiography. Lane 1, 25 U CKII; lane 2, 50 U CKII; lane 3, IE63 + 25 U CKII; lane 4, 25 U CKII + 10 μg ThTPase; lane 5, 50 U CKII + 10 μg ThTPase; lane 6, 100 U CKII + 10 μg ThTPase. Molecular mass (in kDa) markers are indicated. The arrow indicates the location of human recombinant ThTPase. As previously observed (Lakaye et al., 2002; Makarchikov et al., 2003), ThTPase migrates at a higher apparent molecular mass than expected from its primary sequence.

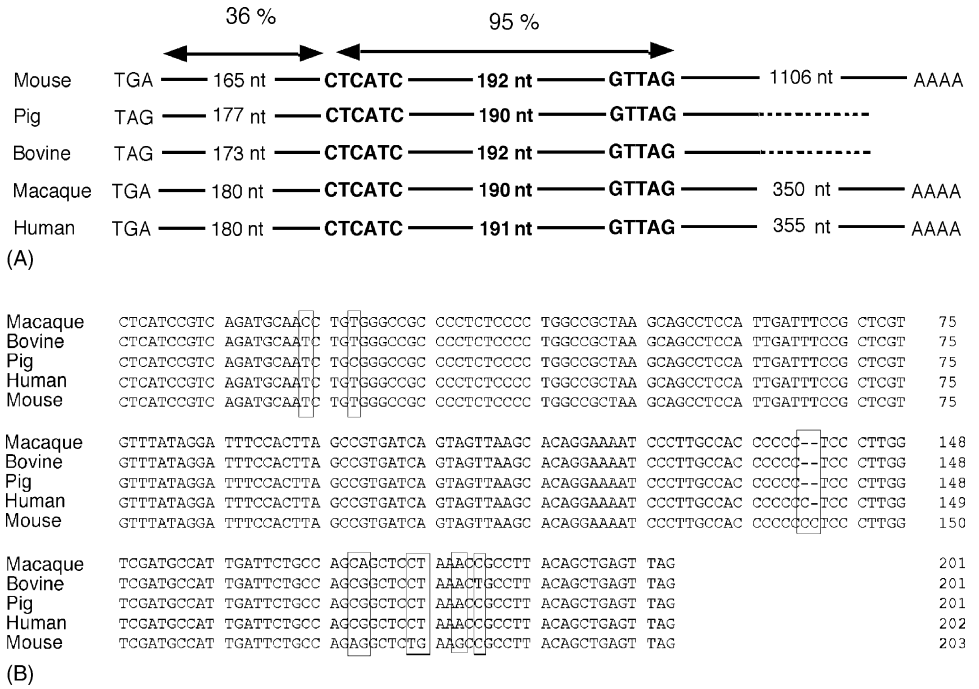

Fig. 4. Conserved 3′-UTR of ThTPase mRNA. (A) Schematic representation showing the length and identity level of different parts of 3′-UTR from mouse (AC no.: AF432864), pig (AC no.: AY442334), bovine (AC no.: AF432863), macaque (AC no.: AB055296) and human (AC no.: NM_024328) ThTPase. The conserved region is shown in bold (nt: nucleotide). (B) Alignment of the conserved region of the 3′-UTR from mouse, pig, bovine, macaque and human ThTPase mRNA. Non-conserved nucleotides are boxed.

lated proteins detected on the gel seem to be contaminations present in the commercial CKII preparation. The viral protein IE63 (Bontems et al., 2002) was used as a positive control. Phosphorylation by CKII did not significantly influence the kinetic parameters of human ThTPase (not shown).

3.5. Characterization of the 3′ end of ThTPase mRNA

Using 3′RACE, we characterized the 3′ end of the mouse ThTPase mRNA (Fig. 4). The 3′-untranslated region is 1474 nucleotides long and a conserved polyadenylation signal is present 13 nucleotides ahead of the polyA tail. By searching the GenBank database, we found numerous cDNA sequences corresponding to part of our mouse sequence. Among them, 49 expressed sequence tags (EST) from the mouse had the same 3′ end. By comparing the mouse sequence with the full length ThTPase mRNA from human and macaque, it appears that, although the 3′-UTRs are of variable length, there is a highly conserved region starting about 170 nucleotides after the stop codon. To extend the analysis, we compared this region with sequences from other species and found significant identity with several bovine and one pig EST. They were used to clone and sequence the 3′ end part of the bovine and pig ThTPase mRNA. As shown in Fig. 4, the 3′-UTR was highly conserved in these species.

4. Discussion

Among the enzymes involved in thiamine metabolism in mammals, only thiamine pyrophosphokinase and the 25 kDa ThTPase have been characterized at the molecular level. Because of the importance of ThDP in the cellular metabolism, thiamine pyrophosphokinase is expected to be present in all cell types. Its mRNA was expressed at a low level in most mouse tissues, but the expression was higher in liver and kidney (Nosaka et al., 1999). Surprisingly, in human tissues, expression of thiamine pyrophosphokinase was

highest in testis (Zhao, Gao, & Goldman, 2001), followed by kidney and small intestine, both involved in the absorption/reabsorption of thiamine.

It has long been suggested that ThTP might have a specific role in excitable tissues. Therefore, its metabolism has been mainly studied in nervous and muscular tissue. After the molecular characterization of a specific 25 kDa mammalian ThTPase, it became apparent that this enzyme is not specific of the nervous system. Moreover, using a human tissue expression array, we showed that its mRNA is present in lower amounts in brain than in many other tissues. A much higher expression was found in testis, uterus, prostate, lung, bladder and kidney.

It thus seemed interesting to compare ThTPase activity and mRNA expression in one model organism, i.e. the mouse. Our results show that all fifteen organs tested contain a measurable ThTPase activity, that is highest in liver and kidney and lowest in intestine. The activity varies by about an order of magnitude between liver and intestine.

ThTPase mRNA expression was highest in testis, and in situ hybridization experiments suggest that ThTPase expression is inversely correlated with the degree of differentiation of spermatogenic cells. In this respect, it is noteworthy that a histochemical study reported a similar localization for thiamine pyrophosphatase activity in human testicular biopsy tissue (Passia, Haider, & Hofman, 1985). It is known that many mRNAs are highly expressed in meiotic spermatogenic cells compared with somatic cells (Kleene, 2001). Moreover, they are sequestered in free-messenger ribonucleoprotein particles rendering their translation highly inefficient. This might be the case for ThTPase. On the other hand, a recent study (Fleming et al., 2003) shows that mice lacking the thiamine transporter Slc19a2 unexpectedly develop male infertility as germ cells do not develop beyond primary spermatocytes. This finding remains unexplained, but the absence of a functional thiamine transporter should lead to decrease in both ThDP and ThTP levels. Thus, an involvement of ThTP and ThTPase in the development of male infertility in these mice remains a possibility, and a high ThTPase mRNA expression in testis may not be fortuitous.

Comparison with the low abundance HPRT mRNA suggests that ThTPase mRNA is expressed only at a few copies per cell. This is in agreement with the low abundance of ThTPase protein, which represents only about 0.002% of the proteins present in a brain supernatant fraction (Lakaye et al., 2002).

A careful examination at the non-coding regions of ThTPase mRNA shows the presence of a highly conserved sequence of 200 nucleotides in the 3′-untranslated region (Fig. 4). This particular sequence has no equivalent in any vertebrate gene, including those involved in thiamine metabolism, as revealed by analysis of the mouse and human genome. Although the 25 kDa soluble ThTPase appears specific for mammals, ThTP is found in all organisms analyzed to date (Makarchikov et al., 2003). We therefore screened the genome of many other organisms including bacteria but did not find any homology. We also analyzed the sequence by the UTRscan on the UTRdb database (http://bighost.area.ba.cnr.it/BIG/UTRHome) (Pesole et al., 2002), but did not find any significant homology with any known motif.

The presence of a long and highly conserved sequence in a variable region, such as the 3′-UTR may have a particular significance. Usually, the main functional roles demonstrated for 5′- and 3′-UTR sequences are either the control of the cellular localization of the transcript, the control of mRNA stability, or the control of translational efficiency (Mazumder, Seshadri, & Fox, 2003). In the case of ThTPase mRNA, the precise role of the conserved sequence is not known, though it could be implicated in translational control. Recently, a conserved 3′-untranslated mRNA region was shown to be responsible for a complex post-transcriptional regulation of the expression of the human excitatory amino acid transporter (Kim, Chao, Choi, & Volsky, 2003). Further studies will be necessary to demonstrate that the 3′-UTR described here affects ThTPase protein expression, in order to understand the physiological significance of this phenomenon.

Previous results suggested that, at least in plants and bacteria, ThTP is synthesized in response to cellular stress (Makarchikov et al., 2003). This increase is transient and is followed by ThTP hydrolysis. The possibility of a rapid upregulation of ThTPase translation might be part of the cellular response, reducing ThTP levels just as phosphodiesterase decreases cAMP levels. It should be noted that intracellular ThTP concentrations, as intracellular cAMP concentrations, are in

the 10^{-7}–10^{-6} molar range. A three-fold increase in cAMP concentration is sufficient to mediate efficient signaling (Keely, Corbin, & Park, 1975). The 25 kDa ThTPase might thus play a role in the precise and timely control of intracellular ThTP levels.

ThTPase harbors several conserved consensus sequences for protein kinases, such as CKII, PKA and PKC. Among the three enzymes tested, only CKII was able to catalyze [γ-^{32}P]ATP-dependent phosphorylation of human recombinant ThTPase in vitro. Phosphorylation by CKII did not appear to modify the enzyme activity. However, we cannot exclude that CKII modifies other properties of the enzyme such as its stability or its cellular localization. Thus, in the case of the IE63 Varicella-zoster virus protein, phosphorylation catalyzed by CKII participates in the cellular relocalization of the protein (Bontems et al., 2002). The role of CKII in cell biology remains poorly understood. Recent evidence suggests that this kinase may play a role in cell survival signaling pathways (Ahmed, Gerber, & Cochet, 2002; Litchfield, 2003). Interestingly, we have recently shown that, in plant leaves and bacteria, ThTP is synthesized in response to cellular stress (Makarchikov et al., 2003; Lakaye et al., 2004b). In mammalian cells, ThTP concentrations are generally very low (Bettendorff et al., 1993; Makarchikov et al., 2002; Makarchikov et al., 2003), presumably due to the activity of the 25-ThTPase. Cellular stress might lead to down-regulation of ThTPase expression and thus an increase in ThTP concentration. Taken together, these observations point to a possible implication of the ThTP/ThTPase couple in some mechanisms of cell survival or differentiation.

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