Characterization of cDNA for murine tripeptidyl-peptidase II reveals alternative splicing

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Tripeptidyl-peptidase II (TPP II) is a cytosolic high-M_r exopeptidase with an active site of the subtilisin type. This paper describes cloning of cDNA encoding murine TPP II. Four clones were isolated from a murine mastocytoma cDNA library and the 5′-end was isolated by use of 5′-RACE (rapid amplification of cDNA ends). A total of 4611 bp were isolated, including the complete coding region. The deduced amino acid sequence shows a 96% overall identity when compared with the previously cloned human TPP II. The remarkably high identity indicates that not only the catalytic domain, but almost the entire subunit, must be of functional importance. Alignment with subtilisin-like serine peptidases identified Asp^{44}, His^{264} and Ser^{449} as the catalytic triad, thus defining an extra domain of ~200 amino acids between the catalytic Asp and His in TPP II as compared with other subtilases. In addition, it was demonstrated that different polyadenylation signals can be utilized, since two different clones with untranslated 3′-ends of 155 bp and 781 bp respectively have been isolated. Finally, one of the isolated clones contains an extra 39 bp insert encoding 13 amino acids, which implies alternative splicing of the mRNA.

INTRODUCTION

Tripeptidyl-peptidase II (TPP II) (EC 3.4.14.10) is a large intracellular exopeptidase [1], with a broad tissue and species distribution [2,3]. TPP II has been purified from rat liver and human erythrocytes [2], and the cDNA encoding the human enzyme has been cloned and sequenced [4,5].

The enzyme has a rather broad substrate specificity, i.e. it removes tripeptides with little apparent similarity from a free N-terminus of larger peptides [1,2]. In addition, the peptidase appears to be structurally conserved both immunologically [3] and at the DNA level [4]. The physiological role of TPP II has yet to be established, but these facts, taken together with its broad distribution, would indicate a general role in intracellular proteolysis.

One remarkable property of the enzyme is its large size. The native enzyme occurs in a complex ($M_r > 10^6$ [1,2]) which is built up of subunits with an M_r of 138000. The oligomeric structure has been shown to be a prerequisite for enzymic activity [6]. Human TPP II has a catalytic domain which shows 56% similarity to that of bacterial serine peptidases of the subtilisin-type [4]. The function of the remainder of this large subunit is not known. Since identification of domains which are conserved between species would localize parts of the subunit which are functionally important, the cloning and sequencing of murine TPP II was undertaken.

MATERIALS AND METHODS

Screening of a cDNA library

A cDNA library containing 4×10^6 independent clones was constructed in the λgt 11 vector with mRNA from murine mastocytoma [7]. A 3.9 kbp *Kpn*I fragment corresponding to the complete coding sequence of human TPP II cDNA [8] or the 1260 bp 5′-end of the fragment was labelled to high specific radioactivity with [α-^{32}P]dCTP by random priming [9] and used as a probe in the screening of the library. Hybridization was carried out overnight at 42 °C in the low-stringency hybridization solution used previously to detect murine genomic DNA [4]. The filters were washed twice for 5 min with 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate buffer, pH 7.0)/0.2% SDS at room temperature and twice for 25 min at 58 °C with 0.7 × SSC/0.5% SDS. Positive clones were plaque-purified and λ-phages were then prepared by the liquid-culture method [10]. The DNA was extracted by standard methods [10]. Fragments obtained after digestion of positive clones with *Eco*RI were subcloned into the plasmid vector pUC 19 [11].

Nucleotide sequence determination

The nucleotide sequence was determined in alkali-denatured pUC 19 using the dideoxy-chain-termination reaction with [α-^{35}S]dATP and the modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH, U.S.A.) [12]. Specific or universal oligonucleotide primers (binding on either side of the pUC 19 polylinker) were used. Sequence determination was made on both strands in at least one clone and on at least one strand in all clones.

5′-RACE experiments

5′-RACE (rapid amplification of cDNA ends) was performed according to the manufacturer's recommendations [13], using the 5′AmpliFINDER RACE kit (Clontech, Palo Alto, CA, U.S.A.). Briefly, first-strand cDNA was synthesized using polyadenylated [poly(A)$^+$] RNA from murine mastocytoma cells (2 μg) as template and a gene-specific oligonucleotide primer BT24 (GCC ACA CCA AGC AGT CAT ATA CAG G). The RNA was thereafter hydrolysed and the cDNA purified. After ligation of an oligonucleotide anchor to the 3′-ends of the cDNA, an aliquot (2 μl of a 1:40 dilution) of the anchor-ligated cDNA was used as a template in a PCR in 10 mM Tris buffer, pH 8.3, containing 50 mM KCl, 0.01% gelatin, 2 mM $MgCl_2$, 0.2 mM of each

Abbreviations used: RACE, rapid amplification of cDNA ends; TPP II, tripeptidyl-peptidase II; poly(A)$^+$, polyadenylated.

The nucleotide sequence data have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession no. X81323.

dNTP and 2.5 units of *Taq* DNA polymerase in a total volume of 50 μl. After heating for 1 min at 82 °C, the anchor primer and a nested gene-specific primer (BT 25, TTA GGA TCC TTC CGT TCT TTC TGT ATC CTT TCC) were added to a final concentration of 0.2 μM. Amplification was started immediately and continued for 40 cycles with a temperature-cycling programme (94 °C for 45 s; 60 °C for 45 s and 72 °C for 2 min) in a Gene-ATAQ Controller (Pharmacia–LKB, Uppsala, Sweden). An aliquot (20%) of the reaction mixture was analysed by agarose gel-electrophoresis and found to contain a dominant 500 bp band (results not shown). The rest of the mixture (from two identical samples) was digested with *Eco*RI (site included in the anchor sequence) and *Bam*HI (site included in BT 25). The 500 bp fragment was purified from an agarose gel by use of the Wizard PCR-preps (Promega, Madison, WI, U.S.A.) and subcloned into pUC 19. A total of nine individual clones were sequenced and shown to start at bp 1, 10, 20, 30, 43 (two clones), 47, 53 and 88 respectively.

For the characterization of the 5′-end of the human transcript, 5′-RACE-Ready cDNA from human placenta was obtained from Clontech. Using 2 μl of this anchor-ligated cDNA as template, PCR (30 cycles) was carried out as described above, with BT 24 and the anchor primer. A 2 μl portion of a 1:10 dilution of the reaction mixture was used as template in a secondary PCR with BT 25 and the anchor primer. Part of the reaction mixture was analysed by agarose-gel electrophoresis. Several bands between 200 and 500 bp could be detected. The remainder of the mixture was digested with *Eco*RI and *Bam*HI, and the 500 bp fragment was purified and subcloned as described above. A total of five individual clones were sequenced and shown to start at bp 1 (two clones), 17 and 20 (two clones) respectively.

Northern-blot analysis

Multiple tissue Northern blots with 2 μg of poly(A)$^+$ RNA from different murine and human tissues were purchased from Clontech. A 3.8 kbp cDNA fragment, corresponding to the murine N10 clone, or a 956 bp cDNA fragment, corresponding to the 5′-end of the human A2 clone [4], were labelled by random priming [9] and used as probes. Hybridization was carried out according to the manufacturer's recommendations. The Northern blots were also hybridized with β-actin to ensure equal loading of RNA on the blots.

PCR experiments

First-strand cDNA synthesis, using 2 μg of poly(A)$^+$ RNA from murine mastocytoma cells and random hexamers, was performed according to the manufacturer's recommendations, using the 5′AmpliFINDER RACE kit. The murine cDNA was used without purification. 5′-RACE-Ready cDNA from human placenta (2 μl) or 7% of the preparation of murine cDNA was used as template in the PCR. For the detection of the N10 extra insert, oligonucleotides BT 17 (AGA TTA TAC AAT TCG ACT AC) and BT 6 (TGT CAT CCA CTG AAT TTT AA) were used. The primers were expected to give rise to two fragments of 490 and 451 bp respectively, depending on whether or not the extra insert was present. For the detection of the N17 extra insert, a degenerated antisense oligonucleotide corresponding to bp 3430–3411 [4] and BT 7 (CTA CTT AAT ACC TCC ACC AA) were used. These oligonucleotide primers were expected to give rise to products of 545 and 375 bp respectively.

For the PCR, DNA template was mixed with oligonucleotide primers (1 μM each), dNTP (200 μM each) and 1.3 units of *Taq* DNA polymerase in 10 mM Tris buffer, pH 9.0, containing 50 mM KCl, 0.1% Triton X-100 and 1.5 mM $MgCl_2$, in a total volume of 50 μl. The reaction was run for 35 cycles, including denaturing for 45 s at 95 °C, annealing for 45 s at 45 °C and extension for 2 min at 72 °C. After PCR, the samples for detection of the N10 extra insert were precipitated with ethanol, dissolved, and digested with *Pst*I.

RESULTS AND DISCUSSION

Isolation and characterization of cDNA clones encoding murine TPP II

Six independent clones were isolated when 1×10^6 clones from a murine mastocytoma cDNA library [7] were screened with a full-length human TPP II cDNA probe [8] as described in the Materials and methods section. An additional four clones were identified when the library (2×10^6 clones) was rescreened using as a probe a 1260 bp fragment corresponding to the 5′-end of the human cDNA. The relationship between the four largest clones, designated K1, K9, N10 and N17, and the previously cloned human TPP II cDNA [4,5] is outlined in Figure 1.

Since the first base-pair of the murine sequence corresponded to bp 118 in the human sequence, experiments using RACE were performed. A PCR product of about 500 bp was obtained and subcloned into pUC 19. A total of nine individual clones obtained through RACE were sequenced. Together the four clones and the 5′-RACE products encode 4611 bp, consisting of 83 bp of the 5′-untranslated region, the complete coding sequence and 781 bp of the 3′-untranslated region (Figure 2). The murine cDNA is extended 67 bp toward the 5′-end as compared with the previously isolated human cDNA [5]. Therefore 5′-RACE was used also for the isolation of the human 5′-end. The longest isolated human clone contained an untranslated 5′-region of 53 bp (Figure 2). No substantially longer PCR products could be amplified, using either murine or human cDNA. Since the leader sequences of most of the vertebrate mRNAs fall in the range of 20–100 nucleotides [15], the obtained 5′-ends could represent the true starting point for transcription. However, the variable starting points of individual clones (cf. the Materials and methods section) may indicate the presence of secondary structures in the transcripts. Therefore the final identification of the transcription starting points must await the characterization of the genes encoding TPP II.

The 3′-untranslated region includes a possible polyadenylation signal starting at bp 4552, but no poly(A) tail. Since the polyadenylation signal is usually 10–35 nucleotides upstream from the poly(A) [16], it is possible that this signal is not utilized and that the last part of the non-coding 3′-end is missing. This is corroborated by the comparison with human TPP II cDNA, since the last base-pair in the murine cDNA corresponds to human bp 4621, which is 35 bp upstream of the poly(A) in the human cDNA (results not shown).

Two of the isolated clones, N10 and N17, contain extra inserts of 39 bp and 170 bp respectively (Figure 1), which will be discussed below.

Comparison of murine and human TPP II

The nucleotide sequences for the murine and human TPP II-cDNAs [4] show 90% identity between the coding parts (Figure 2) and 92 and 74% identity between the untranslated 5′- and 3′-ends respectively. One gap was introduced in the human untranslated 5′-end (Figure 2), and several gaps in the untranslated 3′-ends (results not shown). A number of the base substitutions within the coding region are 'silent', making the similarity between the deduced amino acid sequences even higher. On the amino acid level, an overall identity of 96%, and a similarity of

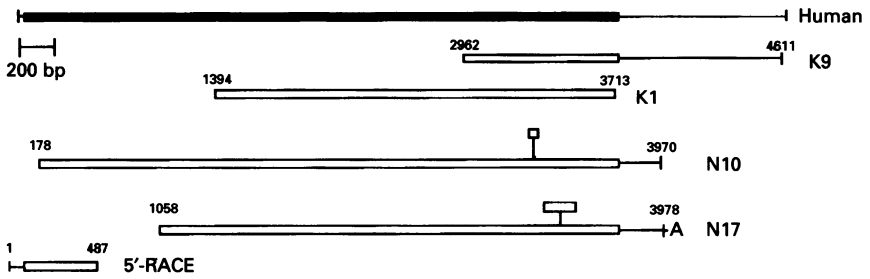


Figure 1 Relationship beween the four murine clones, the 5'-RACE product and human TPP II cDNA

Bars and lines represent coding and non-coding regions respectively. The numbers refer to start and finish of the clone compared with the assembled sequence presented in Figure 2. The N10 and N17 clones contain extra inserts of 39 bp and 170 bp respectively, which are indicated by the boxes. A indicates the poly(A) tail.

98 % are obtained, since a number of the amino acid substitutions are conservative (Figure 2). This high degree of sequence similarity is remarkable for such a large protein. As a comparison it can be mentioned that murine furin (a subtilisin-like serine peptidase of 793 amino acid residues) is 94 % identical with its human counterpart [17], and that murine blood-coagulation Factor VIII (2320 amino acid residues) shows 74 % identity with the human enzyme, even though functionally important domains are more conserved (84–93 % identity) [18].

Interestingly, there is one small part near the C-terminus, residues 1140–1183, where 13 out of 44 amino acids (i.e. 30 %) are different in the two sequences. The reason for this hypervariability is not understood, but will be the subject of further investigations.

It was expected that the catalytic domain would be well conserved. However, evidently the entire subunit of the enzyme is conserved with the exception of the small domain close to the C-terminus, thus implying that practically all parts of the subunit are of importance for the function of the enzyme.

Comparison with other proteins

Murine TPP II was also compared with other proteins in the Swiss database (release 27.0, containing 33329 sequences) by use of the program BLAST [19]. Out of the 65 highest scoring proteins, 44 were serine proteinases of the subtilisin-type (subtilases). The alignment identified Asp^{44} as the aspartic residue of the catalytic triad (Figure 3) and not Asp^{211}, as previously suggested [4]. Thus in TPP II there are 219 amino acids between the Asp and His of the catalytic triad, compared with 31 in subtilisin BPN' (Figure 3). This large insert makes it virtually impossible to obtain an unambiguous alignment to the corresponding area in other subtilases by use of the GAP command of the GCG program [14]. Large inserts in the catalytic domain have previously been reported for other large subtilases, e.g. the cell-wall proteinases from *Lactococcus lactis* have inserts of 151 amino acid residues in one of the variable regions between the His and the Ser residues of the catalytic triad [20]. However, the position of the large insert in TPP II makes it unique among the subtilases. Since TPP II is also the only hitherto-identified exopeptidase belonging to this peptidase class [20], I speculate that the extra domain may be of importance for substrate recognition, e.g. for the observed dependence of a free N-terminus [1,2].

The majority of the other similar sequences which were identified by the BLAST program recognized similarities between charged areas of TPP II (e.g. residues 999–1029, where 20 out of 30 amino acids are charged) and other proteins such as calreticulin [27] and nucleolin [28]. The function of the charged domains in the latter proteins is not known, but it has been speculated that they could be of importance for Ca^{2+} binding [27] or histone interactions [28]. However, acidic and/or basic domains can be found in a number of different proteins, and the functional significance of the observed similarity will have to be investigated experimentally.

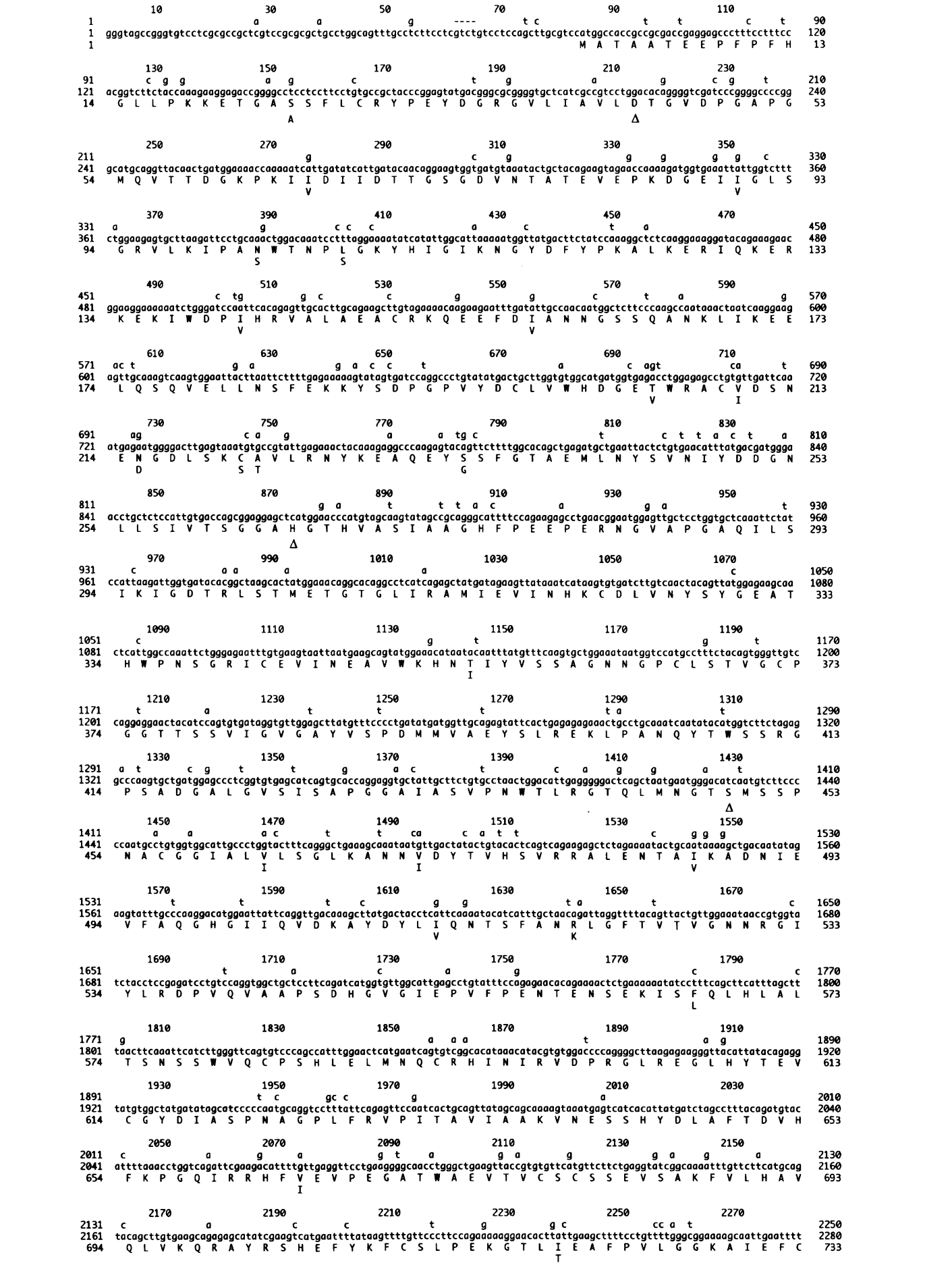
TPP II utilizes two different polyadenylation signals

A Northern-blot analysis shows that both murine and human TPP II are encoded by two different mRNAs, differing about 0.7 kb in size (Figure 4). When this was observed previously for the human enzyme, it was assumed to be due to the utilization of two different polyadenylation signals [4]. This has now been shown for the murine enzyme. Thus, in addition to the K9 clone, which contains a 781 bp untranslated 3'-end, one of the clones, N17, has a 155 bp untranslated 3'-end containing a short poly(A) tail (6 bp), starting at bp 3980, and a possible polyadenylation signal, ATTAAA [16], starting at bp 3955 (Figure 2). The polyadenylation signal is conserved between the human and the murine cDNA and was previously proposed to be utilized in the short human mRNA [4].

The broad tissue distribution reported previously [2] is confirmed by the Northern-blot analysis (Figure 4). Even though no reaction can be seen with pancreatic RNA in Figure 4, bands of the expected size can be detected after overexposure of the blot (results not shown). The weak reaction in pancreas could be due to the fact that a substantial fraction of the mRNA from pancreas represents organ-specific transcripts. It is also evident from Figure 4 that both mRNAs are present in about equimolar amounts in most of the tissues analysed. However, while the larger mRNA species dominates in brain, testis contains more of the short mRNA, thus implying a functional difference between the two mRNAs.

Investigation of extra inserts and evidence for alternative splicing

The N17 clone contains an extra insert of 170 bp between bp 3294 and 3295 (results not shown). This insert is tentatively considered as a cloning artefact, since (i) it does not contain an open reading frame, (ii) it does not have the characteristics of an intron sequence [29] and (iii) the expected 545 bp product cannot be demonstrated by PCR experiments in either murine or human



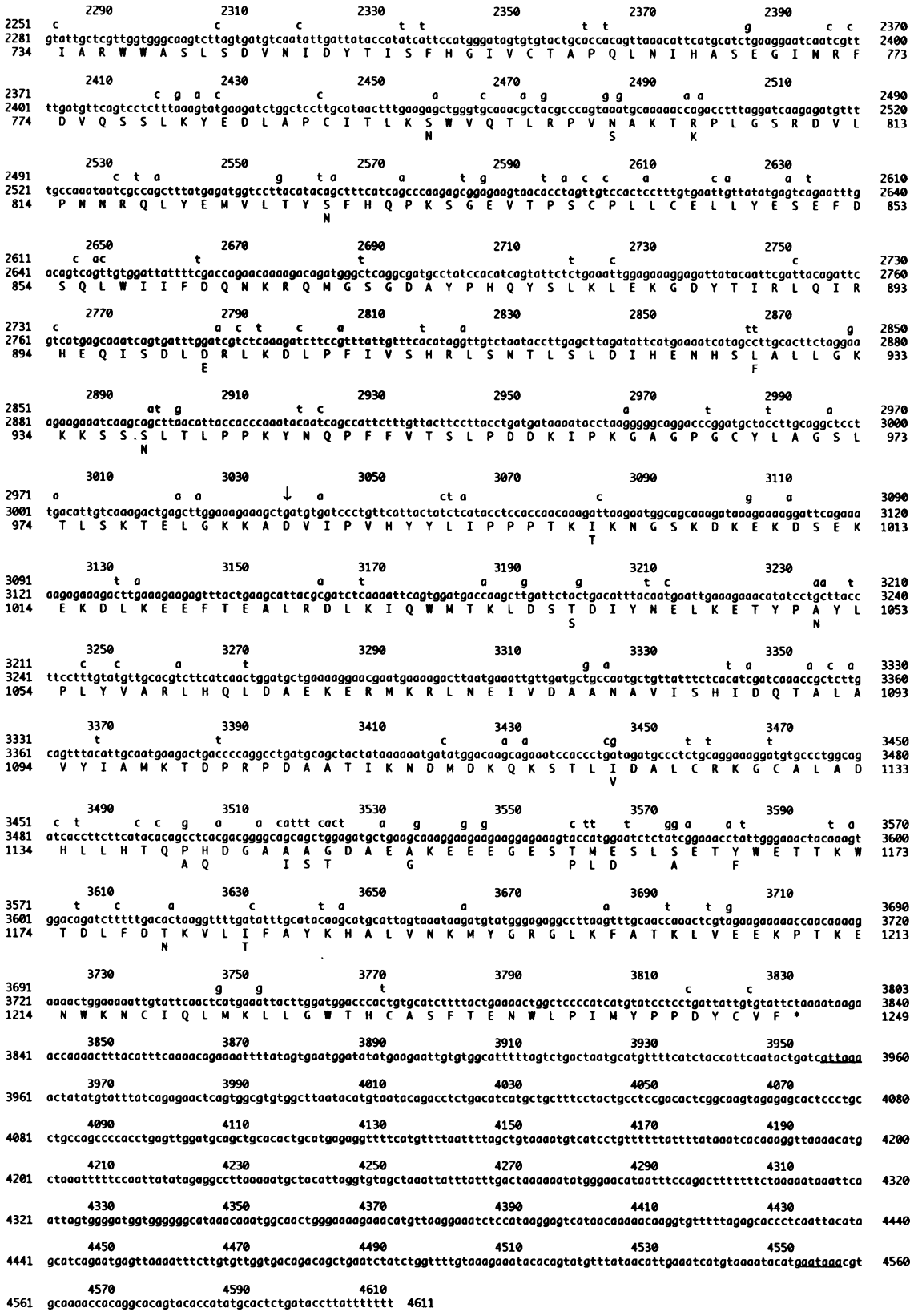


Figure 2 Nucleotide sequence and predicted amino acid sequence of murine TPP II and comparison with human TPP II

The nucleotide sequence and the deduced amino acid sequence of murine and human TPP II [4,5] were compared by use of the command GAP of the GCG program [14]. Nucleotide differences in the human untranslated 5′-region and coding sequence are indicated above the murine nucleotide sequence. A gap introduced in the human nucleotide sequence is represented by '—'. Amino acid differences in the human sequence are indicated below the murine amino acid sequence. Asp, His and Ser of the catalytic triad are marked by Δ. Two possible polyadenylation signals are underlined. The asterisk indicates termination codon. The arrow indicates the extra insert in clone N10 (cf. Figure 5).

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                    *
TPP II   32  EYDGRGVLIA VLDTGVDPGA PGMQVTTDGK PKIID  66
Bacs9   133  TSGGGGINIA VLDTGVNTNH PDLRNNVEQC KDFTV 167
Isp1     38  GVKGKNIKVA VLDTGCDTSH PDLKNQIIGG KNFSD  72
LLP     205  KYKGEGTVVS VIDSGIDPTH KDMRLSDDKD VKLTK 239
BPN'    127  GYTGSNVKVA VIDSGIDSSH PDLKVAGGAS MVPSE 161
ISP      37  QTRGRGVKVA VLDTGCDADH PDLKARIIGG RNFTD  71
MPC3    143  GLTGRGVVIS ILDDGIEKDH PDLWANYDPL ASYDF 177

                    *
TPP II  250  DDGNLLSIVI SGGAHGTHVA SIAAGHF--P EEPER-NGVA PGAQILSIKI 296
Bacs9   168  GTTYTNNSCI DRQGHGTHVA GSALADG--G TGNGV-YGVA PDADLWAYKV 214
Isp1     73  DDGGKEDAIS DYNGHGTHVA GTIAAN---D SNGGI-AGVA PEASLLIVKV 118
LLP     267  DNNDTITDDT VDEQHGMHVA GIIGANGTGD DPAKSVVGVA PEAQLLAMKV 316
BPN'    157  MVPSETNPFQ DNNSHGTHVA GTVAAL---N NSIGV-LGVA PSASLYAVKV 202
ISP      72  DDEGDPEIFK DYNGHGTHVA GTIAAT---E NENGV-VGVA PEADLLIIKV 117
MPC3    182  PDPQPRYTPN DENRHGTRCA GEVSATA--N NGFCG-AGVA FNARIGGVRM 228

                    *
TPP II  422  VSISAPGGAI ASVPNWTLRG IQLMNGTSMS SPNACGGIAL VLS 464
Bacs9   335  VEISAPGAAI YSTWF--DGG YATISGTSMA SPHAAGLAAK IWA 375
Isp1    221  IDLVAPGENI LSTLP--NKK YGKLTGTSMA APHVSGALAL IKS 261
LLP     595  PDITAPGGNI WSTQN--NNG YTNMSGTSMA SPFIAGSQAL LKQ 635
BPN'    303  LDVMAPGVSI QSTLP--GNK YGAYNGTSMA SPHVAGAAAL ILS 343
ISP     219  VDLVAPGEDI LSTVP--GGK YATFSGTSMA TPHVAGALAL IKQ 259
MPC3    344  SSGVVTDPQI VTTDLH-HQC IDKHTGTSAS APLAAGMIAL ALE 385
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Figure 3 Sequence similarities at the active site between murine TPP II and other subtilases

Sequences were aligned by use of the BLAST program [19]. Gaps (indicated by '–') are introduced in variable regions as described by Siezen et al. [20]. Amino acids identical with those in the TPP II sequence are underlined. Abbreviations: Bacs9, subtilisin from *Bacillus TA39* [21]; Isp1, major intracellular serine proteinase [22]; LLP, cell-wall-associated proteinase from *Lactococcus lactis* [23]; BPN′, subtilisin BPN′ [24]; ISP, intracellular serine proteinase from *B. polymyxa* [25]; MPC3, murine prohormone convertase 3 [26]. Asp, His and Ser of the catalytic triad are marked with asterisks.

cDNA (Figure 5b, lanes 1 and 4), even after hybridization of the PCR products with murine cDNA (results not shown).

The N10 clone contains an extra insert of 39 bp at bp 3036 (Figure 5a), compared with the other clones (Figure 1). Using PCR, it could be demonstrated that this extra exon is present in mRNA from both murine mastocytoma and human placenta, since both a 451 bp and a 490 bp fragment could be amplified (Figure 5b). The true identities of the PCR products were

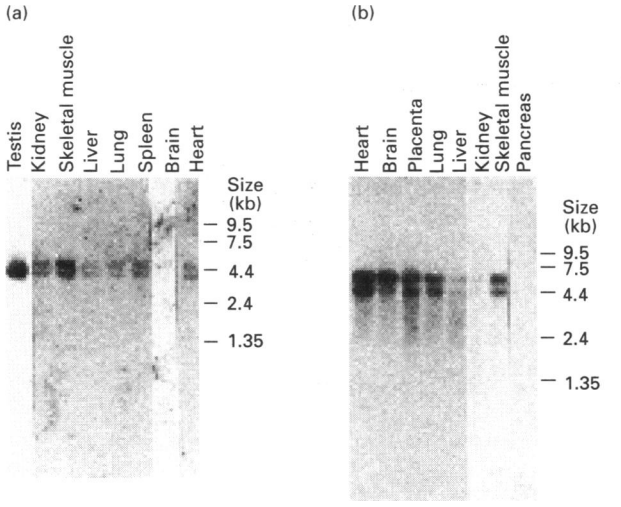

Figure 4 Northern-blot analysis of mRNA from different murine (a) or human (b) tissues

Northern blots were hybridized with a 3.8 kbp murine cDNA fragment (**a**) or a 956 bp human cDNA fragment (**b**), as described in the Materials and methods section. The sizes of the RNA ladder markers are shown in kb.

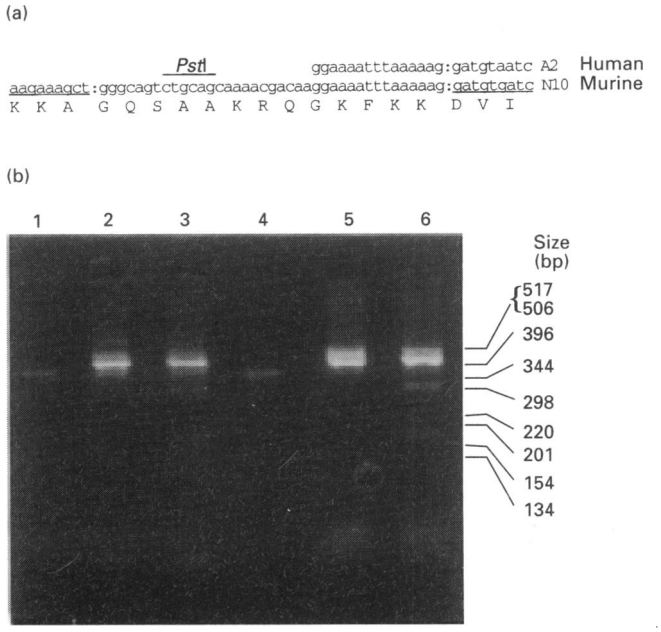

Figure 5 Investigation of extra inserts in clones N10 and N17

(**a**) Nucleotide sequence and deduced amino acid sequence of the extra insert in clone N10. The underlined nucleotides are also present in the other clones. The comparison with the human A2 clone [4] is shown above the nucleotide sequence. The *Pst*I site used for the identification of PCR fragments containing the extra insert is indicated above the sequence. (**b**) PCR experiments for the investigation of the extra inserts. Murine (lanes 1–3) or human (lanes 4–6) cDNA was used as template in PCR experiments with primers binding on either side of the N17 extra insert (lanes 1 and 4) or the N10 extra insert (lanes 2, 3, 5 and 6). The experiment was performed as described in the Materials and methods section. The PCR products formed were precipitated with ethanol, dissolved and digested with *Pst*I (lanes 3 and 6).

confirmed, not only by their size but also by hybridization to murine cDNA (results not shown). In addition, the larger product could also be identified by the presence of a *Pst*I site (Figure 5b, lanes 3 and 6), dividing the 490 bp product into two fragments of 156 bp and 334 bp. It is noteworthy that when the human cDNA was cloned, one of the isolated clones (A2) contained a 'divergent' 5′-end [4], but it could not at that time be determined whether this was due to alternative splicing or an intron sequence left by incorrect splicing [4]. It is now evident that the last 15 bp of the extra insert in the N10 clone is identical with that divergent end (Figure 5a). Taken together, these results demonstrate that the extra insert is due to alternative splicing. The extra exon represents an open reading frame encoding 13 amino acids. The physiological importance of this extra domain is not known and will be the subject of future investigations.

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