FULL PAPER *Toxicology*

Cloning of Full-Length Preproendothelin-2 cDNA and Its Expression in Dog

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ABSTRACT. Endothelin-2 (ET2) is a member of the endothelin family of 21-amino acid peptides with vasoconstrictive activity. We report here the molecular cloning of the canine full-length cDNA of the precursor form of ET2, prepro-ET2 (PPET2), from intestinal tissue by means of reverse transcription-polymerase chain reaction (RT-PCR) in conjunction with 5'- and 3'-rapid amplification of cDNA ends (RACE). Aside from the poly (A) tail the cDNA was found to be 1195 bp and included an open reading frame of 534 bp encoding a PPET2 polypeptide of 178 residues, in which the regions corresponding to bioactive mature ET2 peptide, an intermediate form big- ET2, and endothelin-like peptide are found. The organ distributions of PPET2 mRNA and a splicing variant were analyzed by RT-PCR. PPET2 transcript was detected in duodenum, colon, stomach, lung, liver, uterus, ovary, testis and kidney, but not in spleen. A splicing variant was found in none of the organs. Thus, based on the cloned cDNA sequence, we established a quantitative assay for dog P PET2 mRNA level using a real-time PCR system. Quantitative analysis by this method in various organs of the dog demonstrated that the dominant gene expression occurs in the intestine, with higher expression in large intestine than in small intestine.

KEY WORDS: canine, cloning, gene expression, preproendothelin-2 cDNA, real-time PCR.

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The endothelin family of peptides consists of four isoforms: endothelin-1 (ET1), vasoactive intestinal contractor (VIC), endothelin-2 (ET2) and endothelin-3. These isopeptides are composed of 21-amino acid residues and have a variety of biological roles, including involvement in vasoconstriction [26], smooth muscle contraction [8, 12], cardiomyocyte contractility [20] and cell growth [10, 11].

ET2 originally was identified in human as a peptide showing strong vasoconstrictive and pressor effects [7], and the murine counterpart [1], VIC, has been characterized as a potent intestine-contracting compound [8]. The full-length cDNA sequences of the precursor forms of ET2 and VIC, prepro-ET2 (PPET2) and prepro-VIC (PPVIC), have been cloned in human [13], and in mouse [18] and rat [3], respectively. In dog, however, only a partial PPET2 cDNA sequence, corresponding to part of a coding region, has been reported [9]. Based on comparative analysis of this limited, deduced amino acid sequence for dog with sequences for these other animals, it has been suggested that dog PPET2 is considerably shorter than the prepro forms for the other animals, missing the c-terminal portion of the polypeptide [9]. While it is possible that the reported sequence may be a spliced orthologue, as has been observed for human [14], the reliability of this sequence is arguable because the reported sequence is only part of a coding region and insufficient evidence was shown to prove that it is from a true cDNA [9].

To determine the true nature of the canine PPET2 sequence, we cloned and sequenced the full-length canine PPET2 cDNA, compared the nucleotide and deduced amino acid sequences to those previously reported, and searched for a possible splice variant in dog various organs by RT-PCR. Having established a quantitative method for determining mRNA level by real-time PCR, we performed quantitative expression analysis of the gene in various organs.

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MATERIALS AND METHODS

Preparation of mRNA: Canine tissues (duodenum, colon, stomach, lung, liver, uterus, ovary, testis, kidney and spleen) were obtained from 10-year-old male and female beagles, and they were preserved at –80°C after immediately freezing in liquid nitrogen. Total RNA was prepared from dog intestinal (100 mg of the tissue) homogenate using Isogen solution (Nippon Gene, Japan) according to the manufacturer's protocol. mRNA was obtained from total RNA by oligo (dT)-cellulose chromatography (Amersham Pharmacia Biotech, UK).

RACE-PCR: To determine the full-length nucleotide sequence of dog PPET2 mRNA, we cloned the cDNA by reverse transcription-polymerase chain reaction (RT-PCR) and 5'- and 3'-rapid amplification of cDNA ends (RACE) using a commercially available kit (SMART™ RACE cDNA Amplification Kit, Clontech, U.S.A.). The first-strand cDNA was synthesized with mRNA from dog intestine as a template using Moloney murine leukemia virus (MMLV) reverse transcriptase (Clontech, U.S.A.) according to the protocol given by Clontech. Briefly, the first-strand cDNA for 5'-RACE PCR was synthesized with an oligo (dT) primer and adapter oligonucleotide, which are provided with the kit as the 5'-cDNA synthesis (5'-CDS) primer and the SMART II oligo, respectively (Table 1). The

Table 1. Primers and oligonucleotides used for cloning and cDNA amplification of dog PPET2 and GAPDH

Name	Nucleotide sequences	used
SMART II	5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3'	cDNA synthesis
5'-CDS	5'-$(T)_{25}$(A,G, or C) (A,C,G, or T)-3'	cDNA synthesis
3'-CDS	5'-AAGCAGTGGTAACAACGCAGAGTAC$(T)_{30}$(A,G, or C)(A,C,G, or T)-3'	cDNA synthesis
Universal Primer Mix	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT-3' 5'-CTAATACGACTCACTATAGGGC-3'	cDNA synthesis
5'-GSP	5'-AGGCC(G/A)TA(G/A)GGAGCTGTCTG-3' (antisense)	5'RACE PCR
3'-GSP	5'-CCCGAGGCTCCCACCTGCGGACTCGACGTT-3' (sense)	3'RACE PCR
5'-NGSP	5'-ACCCAGATAATGGCCA(A/G)GTG-3' (antisense)	5'RACE PCR
3'-NGSP	5'-TTGCTCCTGCAGCTCCTGGCTCGACAAG-3' (sense)	3'RACE PCR
PPET2-dF	5'-TTGCTCCTGCAGCTCCTGGCT-3' (sense)	PCR
PPET2-dR	5'-TCTCTTCCTCCGCCTTGGGTGT-3' (antisense)	PCR
GAPDH-F	5'-CTTCACCACCATGGAGAAGGC-3' (sense)	PCR
GAPDH-R	5'-GGCATGGACTGTGGTCATGAG-3' (antisense)	PCR
PPET2-rF	5'-TGGCGGGTTTCCCAGG-3' (sense)	Real-Time PCR
PPET2-rR	5'-CGACAAGGAGTGCGTCTACTTCT-3' (antisense)	Real-Time PCR
TaqMan probe	5'-FAM-AACACTCCCGGACAGACAGCTCCTTACG-TAMRA-3'	Real-Time PCR

first-strand cDNA synthesis for 3'-RACE PCR was also done with an oligo (dT) primer having an adapter portion at its 5' end, which is provided with the kit as the 3'-cDNA synthesis (3'-CDS) primer (Table 1). For amplification of first-strand cDNA gene specific primers (GSP) and nested gene specific primers (NGSP) were designed to produce an overlapping region between the 5'- and 3'- RACE-PCR fragments, based on the nucleotide sequences conserved among human [13], mouse [18] and rat [3] (Table1). 5'-RACE PCR was performed with 5'-GSP or 5'-NGSP and Universal Primer Mix (provided with the kit and primes with the adapter oligonucleotide, the SMART II oligo) using AmpliTaq Gold DNA polymerase (Applied Biosystems, U.S.A.) under the following conditions: 1 cycle of 95°C for 10 min, 35 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 3 min, and 1 cycle of 72°C for 30 sec. 3'-RACE PCR was performed with 3'-GSP or 3'-NGSP and Universal Primer Mix, which primes with the adapter portion of the 3'-CDS primer under the same conditions as in 5'-RACE PCR. After agarose gel electrophoresis of the PCR products, a fragment of predicted size was purified from the gel and inserted into a plasmid vector, pCR2.1 (Invitrogen, U.S.A.).

DNA sequencing and analysis: DNA sequencing was performed on both strands with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, U.S.A.) by an automated DNA sequence analyzer (Model 310, Applied Biosystems, U.S.A.). To avoid possible sequencing errors due to RACE artifacts, the sequence analysis was performed on 30 independent clones derived from each RACE. The full-length cDNA sequence was obtained by combining the overlapping regions. The complete nucleotide and deduced amino acid sequences were analyzed using commercial software (GENETYX-MAC Ver. 8, Software Development, Japan).

Conventional PCR: Oligonucleotide primers specific for the dog PPET2 gene (PPET2/dF and PPET2/dR) were designed based on the full-length cDNA sequence obtained by cloning (Table 1 and Fig. 2). Primers specific for the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH/F and GAPDH/R), which is known as a housekeeping gene, were also designed (Table 1). cDNAs were synthesized with avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Japan) from total RNAs obtained from duodenum, colon, stomach, lung, liver, uterus, ovary, testis, kidney and spleen as templates. PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, U.S.A.) under the following conditions: 1 cycle of 95°C for 10 min, 35 (PPET2) or 25 (GAPDH) cycles of 95°C for 1 min, 65°C for 2 min and 72°C for 3 min, and 1 cycle of 72°C for 30 sec.

Real-time PCR: A quantitative method for analysis of canine PPET2 mRNA level by real-time PCR was established as in a previous report [22]. Briefly, oligonucleotide primers and a detection probe (TaqMan Probe, Applied Biosystems, U.S.A.) were designed (Table 1 and Fig. 2) and dog PPET2 cDNA inserted in plasmid was prepared as a quantitative standard to estimate PPET2 gene expression level. Amplification was carried out with the TaqMan PCR kit (Applied Biosystems, U.S.A.). Template was mixed with 1 × buffer, 3.5 mM $MgCl_2$, 200 μM dATP, dCTP and dGTP, 400 μM dUTP, 0.025 U/μl AmpliTaq Gold polymerase, 100 nM TaqMan Probe, and 200 nM of each primer. All reactions were performed in the Model 7700 Sequence Detector (Applied Biosystems, U.S.A.). Reaction conditions were 95°C for 10 min followed by 50 cycles of the amplification step (95°C for 15 sec and 60°C for 1 min). For quantification of the amount of PPET2 mRNA a standard curve was determined with ten-fold serially diluted standard templates by plotting the starting amount of standard template against the number of threshold cycles. The 18S ribosomal RNA level, assessed by a commercial kit (TaqMan

Ribosomal RNA Control Reagents Kit, Applied Biosystems, U.S.A.), was used to normalize the PPET2 mRNA level as an internal control.

RESULTS

Characterization of dog PPET2 cDNA and polypeptide: The full-length cDNA sequence of dog PPET2 was determined by combining sequence information from all of the 5'- and 3'-RACE PCR clones. Figure 1 shows the nucleotide and deduced amino acid sequences. Aside from the poly (A) tail, the full-length canine PPET2 cDNA is 1195 bp. The ATG and TGA triplets corresponding to the predicted initiation and stop codons are located at 113–115 and 647–649 of the cDNA, respectively. The open reading frame consisting of 534 bp encodes a putative polypeptide of 178 residues. In the 3'-non coding region the consensus polyadenylation signal AATAAA [15] is observed at position 1177–1182 bp. Two copies of ATTTA, which is the consensus sequence of mRNA instability [2, 5, 6], are present at positions 1121–1125 bp and 1153–1157 bp. Regions corresponding to a bioactive mature ET2 peptide (C^{49} - W^{69}), an intermediate form known as big-ET2 (C^{49} - R^{86}) and an endothelin-like (ET-like) peptide (C^{96} - H^{111}) are found on the deduced polypeptide as they are in human [13], mouse [18] and rat [3]. Dibasic amino acid pairs flank both the amino and carboxyl terminals of the big-ET2 and ET-like peptides.

Homology analysis of nucleotide and amino acid sequences: Homology analysis of nucleotide and amino acid sequences between dog and other mammals (human, mouse and rat) was carried out (Table 2). For the full-length cDNA the homology of dog with human, mouse and rat is 64.2, 59.3 and 60.9%, respectively, whereas in the coding region the homology with human, mouse and rat is 81.3, 75.9 and 75.3%, respectively. The similarity of the dog amino acid sequence with that of human, mouse and rat is 69.1, 68.5 and 67.3%, respectively.

Comparison of amino acid and nucleotide sequences: Figure 2 shows the alignment of dog PPET2 cDNA and the deduced amino acid sequences obtained here along with sequences reported previously [9]. A putative exon organization, predicted based on mouse PPVIC gene structure [16], is also shown. Focusing on the region corresponding to exon 2, the amino acid sequence we present completely matches that reported by Itoh *et al.* However we noticed the following discrepancies between the two sequences: (1) While the cDNA we present encodes a 178-amino acid peptide showing the same structural characteristics as observed in human [13], mouse [18] and rat [3], the cDNA by Itoh *et al.* encodes a smaller (77-amino acid) PPET2 which lacks the c-terminal portion because of the presence of a stop codon at the start region of exon 3; (2) the amino acid region corresponding to exon 1 shows little homology between the two sequences. The nucleotide sequences corresponding to exon 1 and exon 3 by Itoh *et al.* are more homologous to mouse intron 1 and intron 2 than to our exon 1 and exon 3 sequences.

Analysis of dog PPET2 transcripts in various organs: Organ distributions of PPET2 mRNA and a splicing variant were analyzed by conventional PCR. The primers for this experiment were designed to span introns and to generate a 231-bp fragment (Fig. 2), based both on our cDNA sequence and on the reported mouse PPVIC gene structure [16]. Figure 3A shows the expression of PPET2 and GAPDH mRNAs. PPET2 gene expression was detected in duodenum, colon, stomach, lung, liver, uterus, ovary, testis and kidney, but not in spleen. Whereas one would expect to observe additional bands generated as a result of amplification from spliced variant cDNAs, we observed no band other than the 231-bp fragment from the intact cDNA, indicating the absence of a splicing variant. Subsequently by cloning and sequencing the PCR fragments, we confirmed the PCR fragments as those from dog PPET2 cDNA (Data not shown).

Quantitative analysis of dog PPET2 mRNA levels in various organs: For quantitative analysis of dog PPET2 mRNA levels a real-time PCR assay was established by designing specific primer pairs to produce an amplicon of 91 bp and designing a TaqMan probe (Table 1 and Fig. 2). To examine the reproducibility of this assay system, we calculated the intra- and inter-assay coefficients of variation (CVs) (Table 3). The intra-assay CVs for PPET2 gene expression level, which were determined by testing two different samples from a single assay five times, were 25.9 and 32.0%, respectively (CVs for the threshold cycle: 0.61 and 0.81%, respectively). The inter-assay CVs, determined by testing two different samples in five assays over 2 weeks, were 42.6 and 50.2%, respectively (CVs for the threshold cycle: 1.15 and 1.29%, respectively).

Using this assay system, quantitative gene expression analysis of dog PPET2 was performed in various organs (Fig. 3B). The gene expression levels found in the intestinal tracts, duodenum and colon, were roughly ten- to one thousand-fold higher than in other organs. Furthermore, the level in colon was about two times higher than that in duodenum. An expression pattern showing the dominant expression occurring in the intestine, with a higher level of expression in the large intestine than in the small intestine, mirrors that of the VIC gene in rat [4, 21]. The expression level in spleen was very low (about five hundred times lower than in duodenum), supporting the finding, illustrated in Fig. 3A, that the PCR product is undetectable in spleen.

DISCUSSION

We isolated and sequenced about 1.2 Kbp of dog PPET2 cDNA, which is similar in size to the cDNAs of human PPET2 [13] and mouse PPVIC [18]. In the 3' non-coding region dog PPET2 cDNA contains two copies of the ATTTA motif (Fig 1), which also is found in human PPET2 and mouse PPVIC cDNAs, suggesting a short life span for the mRNA because the motif is proposed to mediate selective translation-dependent destabilization of mRNA as was

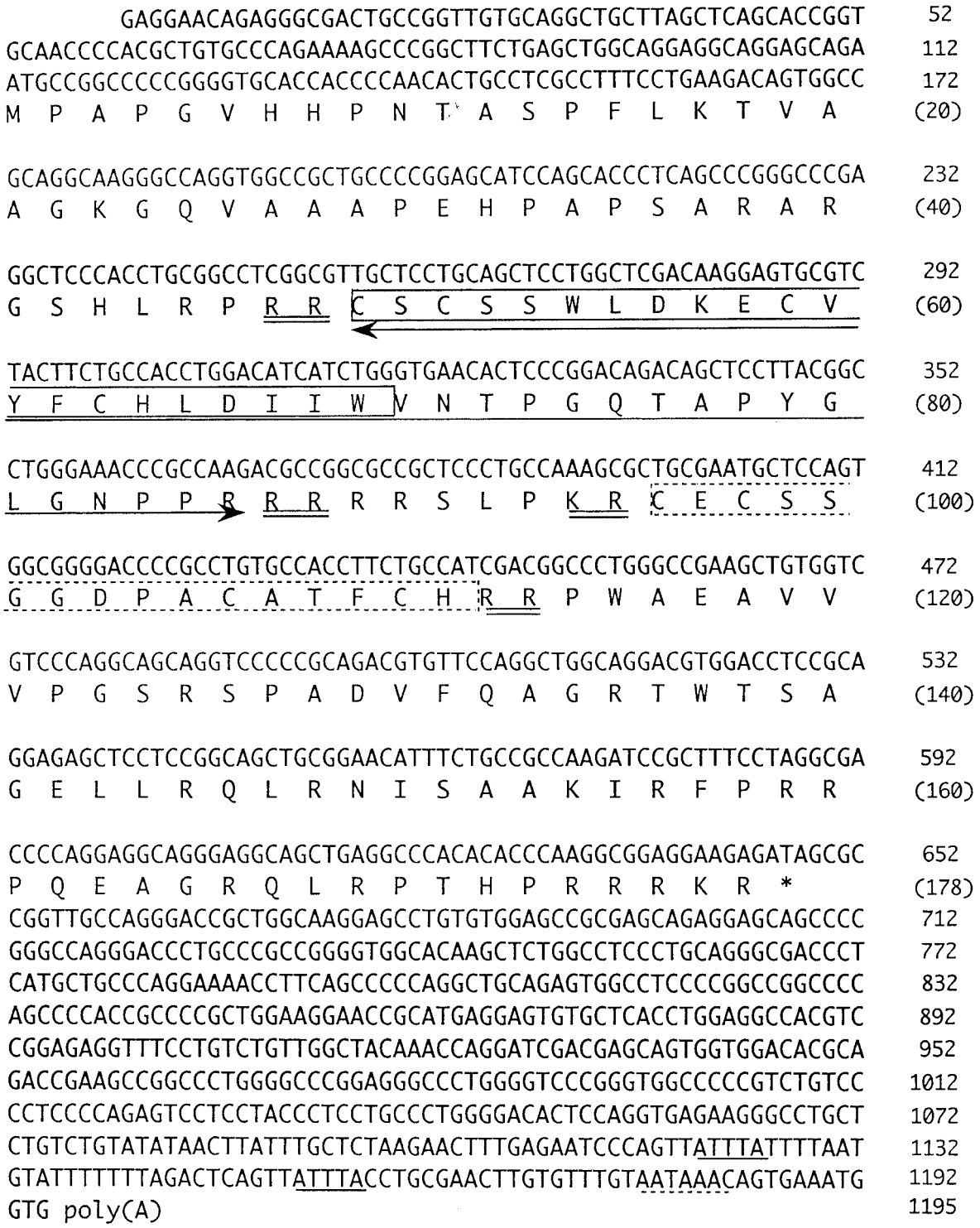

Fig. 1. Nucleotide and deduced amino acid sequences of dog PPET2. The full-length dog PPET2 cDNA is 1195 bp, excluding the poly (A) tail. A 534-nucleotide open reading frame encoding 178 amino acids of dog PPET2 follows the ATG codon closest to the 5' end. The stop codon is indicated by an asterisk. Mature, big and like peptides are indicated by solid box, arrow and dashed box, respectively. Dibasic amino acid pairs, which are recognized by processing endopeptidase, are double underlined. The consensus sequences of mRNA instability and polyadenylation signal in the 3' non-coding region are indicated by solid and dashed underlines, respectively. Nucleotide and amino acid sequences are numbered to the right with and without parentheses, respectively.

Table 2. Homology analysis of nucleotide and amino acid sequences between dog and other mammals

Species (gene)	Homology (%)			
	Nucleotide		Amino acid	DDBJ/EMBL/GenBank
	Full-length	ORF		accession number
Human (PPET2)	64.2	81.3	69.1	M65199
Mouse (PPVIC)	59.3	75.9	68.5	X59556
Rat (PPVIC)	60.9	75.3	67.3	NM012549

The full-length sequences were obtained from the EMBL/Genebank and DDBJ Libraries.

Table 3. Intra-assay and interassay coefficient of variation of gene expression level

Assay	PPET2	
	Sample 1	Sample 2
Intra-assay	25.9%	32.0%
Inter-assay	42.6%	50.2%

shown for cytokine genes [5, 6].

Through conceptual analysis of the domain structure of dog PPET2 we found that the regions corresponding to big-ET2 and ET-like peptide are present in dog PPET2, flanking dibasic amino acid pairs at both their amino and carboxyl terminals, as in human PPET2, mouse PPVIC and rat PPVIC. Additionally, in dog big-ET2, a proteolytic target site by endothelin converting enzyme is found between W and V as it is in the human, mouse and rat big forms. These findings suggest that dog bioactive ET2 is generated through the same proteolytic processing steps as have been proposed for human, mouse and rat [3, 13, 18]. The putative biosynthesis pathway of dog ET2 is summarized in Fig. 4A. Internal homology analysis demonstrates that the ET-like peptide observed within PPET2 is structurally related to the 16 animo-terminal residues of mature ET2, sharing with nine amino acids of ET2 including four cysteine residues at the 1, 3, 11 and 15 positions (Fig. 4B). Considering that the ET-like peptide is closely flanked by dibasic amino acid pairs at both the N- and C-terminals, the peptide may directly generate from PPET2 as does the ET2 peptide. At present the physiological role of ET-like peptide is unknown.

A canine partial cDNA sequence and a putative amino acid sequence for PPET2 have been reported [9]. The characteristics of the domain structure of dog PPET2 we cloned are well matched with those of human, mouse and rat [3, 13, 18]. However the PPET2 sequence reported by Itoh *et al.* is lacking about 100 amino acids of the c-terminus as compared with those of human, mouse and rat. Although we explored the possibility that the previousy reported PPET2 sequence is in fact a splicing variant form of dog PPET2, using RT-PCR with primer pairs designed to span introns, we observed the presence of no splicing variant in any of the various dog organs (Fig. 3A). Together with this finding, and given the observations obtained from the comparative alignment of our sequence and that reported by Itoh *et al.*, which reveal that: 1) our amino acid sequence corresponding to exon 2 completely coincides with Itoh's, whereas the region corresponding to exon 1 reveals little homology, 2) the nucleotide sequences corresponding to exon 1 and exon 3 by Itoh *et al.* are more homologous to mouse intron 1 and intron 2 than to our exon 1 and exon 3 sequences, and 3) the reported nucleotide sequence corresponding to exon 2 is flanked immediately by ag at the 5' end and by gt at 3' the end, conforming to the GT/AG rule for nucleotide sequences immediately flanking exon borders [19], it is strongly suggested that the dog sequence reported by Itoh *et al.* is from genomic DNA.

Through conventional PCR we could not detect PPET2 gene expression in dog spleen. An absence of the transcript for the PPVIC gene, the murine orthologue to the canine PPET2 gene, also has been reported in mouse and rat spleen [4, 24]. Although a physiological role for ET2 or VIC remains to be elucidated, this peptide does appear to have a functional importance distinct from that of ET1, considering that PPET1 gene expression occurs in highly vascularized organs including spleen, in contrast to the PPET2 and PPVIC genes, and that ET1 is closely related to the regulation of vascular tone. Quantitative gene expression analysis by real-time PCR demonstrates that dog intestine expresses PPET2 mRNA most abundantly of all organs examined. It has been shown that the PPVIC gene is highly expressed in mouse and rat intestine [4, 17, 21, 22, 24] and furthermore that the intestinal expression level increases during late fatal stages toward the high level observed in adult intestine, in accordance with the period during which intestinal villi formation occurs [23]. These findings suggest that ET2 and VIC may play a crucial role in intestine with regard to villous functions and maintenance. A physiological explanation for the fact that the PPET2 and PPVIC genes are expressed to a much lesser degree in liver and kidney than in the intestinal tract is difficult to ascertain because fundamental information such as cellular expression of these genes in liver and kidney has not yet been reported. However these results, which show a very low expression of these genes in the vascularized parenchymal organs, support the findings of the previous reports, that neither ET2 nor VIC expresses in vascular endothelial cells, in contrast to ET1 [1, 25].

Through reproducibility analysis of the quantitative assay system by real-time PCR, it was shown that a slightly

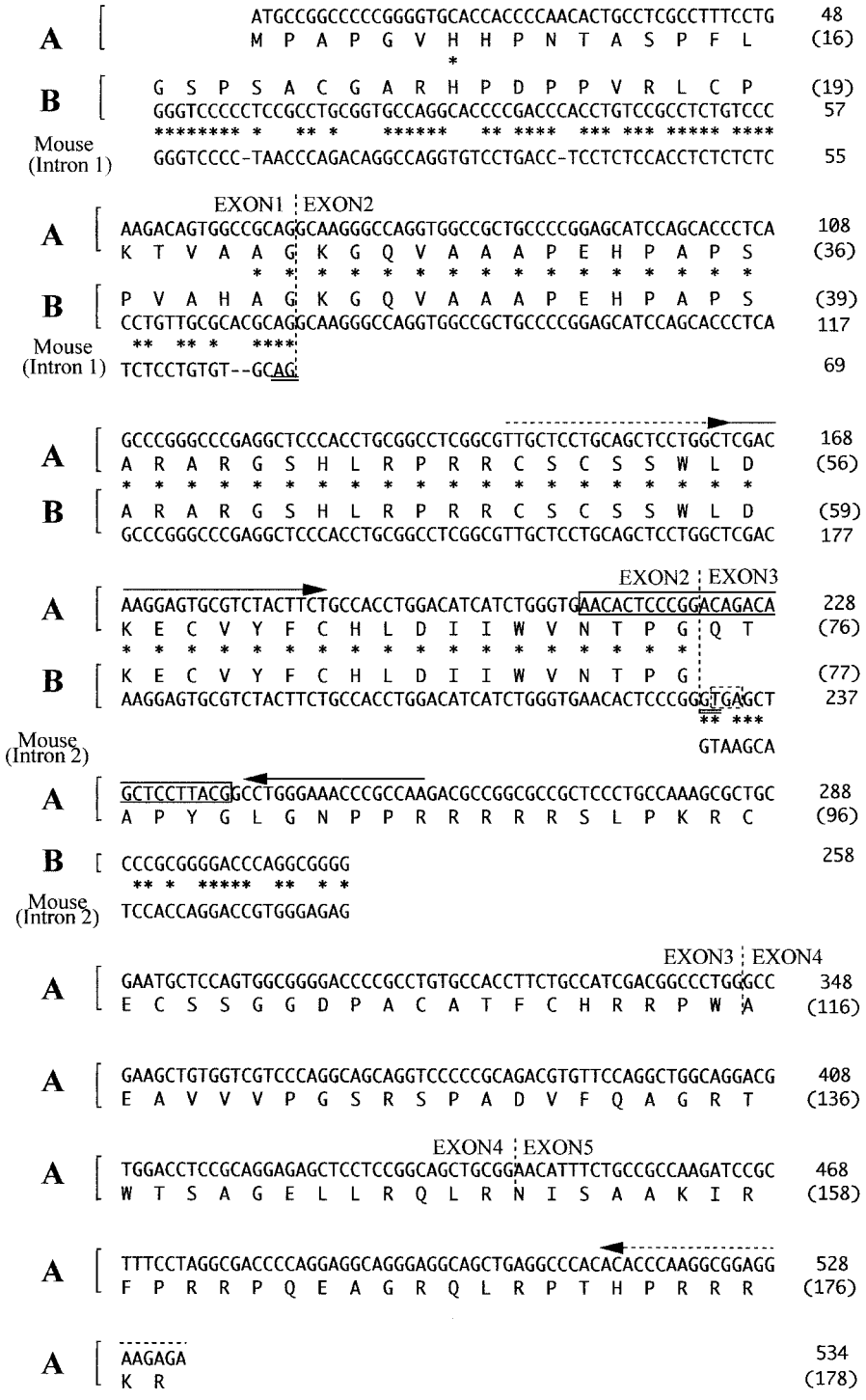

Fig. 2. Alignment of nucleotide and amino acid sequences of dog PPET2. Nucleotide and amino acid sequences of dog PPET2 obtained here (A) along with the sequences reported by Itoh *et al.* [9] (B) are aligned, together with mouse intron 1 and intron 2 sequences [16]. A putative exon organization, predicted based on mouse PPVIC gene structure [16], is also shown on the sequences. Identical nucleotide and amino acid sequences are indicated by asterisks. A stop codon proposed by Itoh *et al.* is enclosed in a dashed box. The ag and gt sequences flanking the nucleotide sequence corresponding to exon 2 are double underlined. The primers used for analysis of the transcript by conventional PCR in Fig. 3A are indicated by dashed arrows. The primers and TaqMan probe used for quantitative analysis of mRNA by real-time PCR are indicated by solid arrows and solid box, respectively. Nucleotide and amino acid sequences are numbered to the right without and with parentheses, respectively.

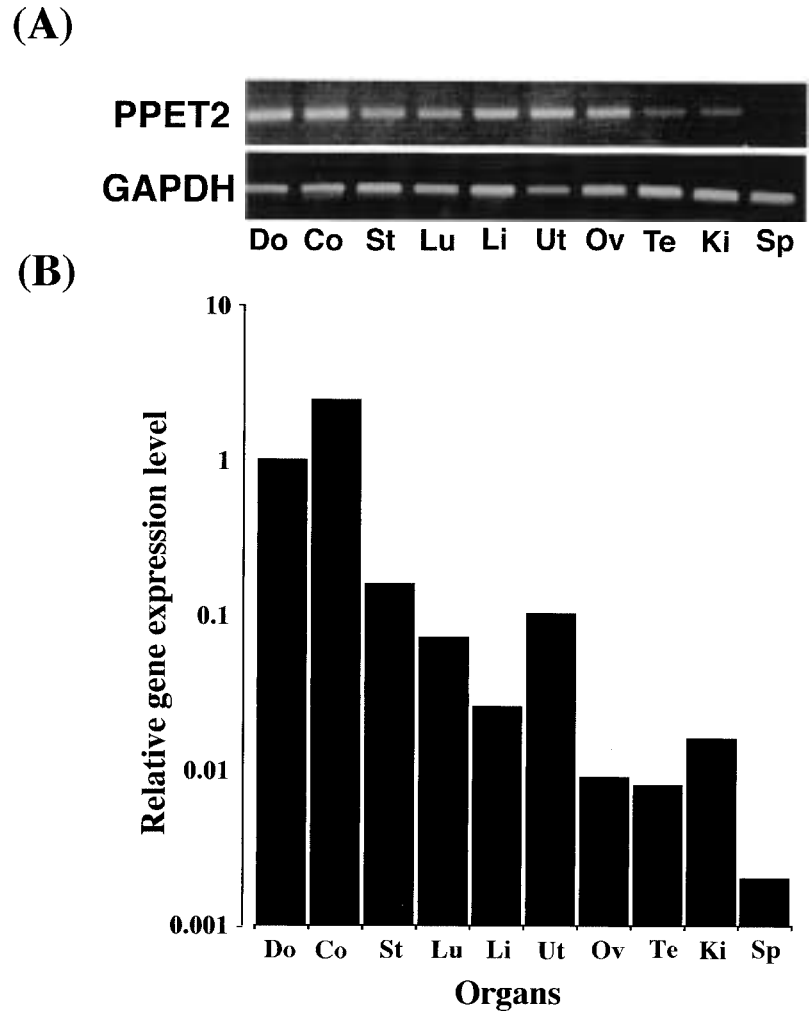

Fig. 3. (A) Organ distribution of dog PPET2 mRNA by conventional PCR. Total RNA was prepared from duodenum (Do), colon (Co), stomach (St), lung (Lu), liver (Li), uterus (Ut), ovary (Ov), testis (Te), kidney (Ki), and spleen (Sp). After RT reaction organ distribution of dog PPET2 mRNA was examined by conventional PCR. GAPDH cDNA was amplified as an internal control. (B) Quantitative analysis of PPET2 mRNA levels by real-time PCR. PPET2 gene expression levels in the organs were quantitatively analyzed by real-time PCR. Each expression level is shown as a value relative to the duodenal level, which is designated as 1.

greater than 1.5-fold increase in gene expression level is detectable as a significant change by the assay system. In addition, based on standard curves created with serially diluted standard templates, it was also shown that this assay system has a wide dynamic range (over 10^7 fold in input cDNA molecules). (Data not shown.)

In conclusion, we showed that dog PPET2 is a polypeptide of 178 amino residues, which is similar in length to human PPET2 and murine PPVIC, contrary to Itoh's report [9]. Additionally, structural characteristics are well conserved among the prepro forms of endothelin family. In other words, regions corresponding to big-form and ET-like peptide within the polypeptides and of the target site for endothelin converting enzyme were found also in our canine PPET2. Together with reliable sequence data, the quantitative gene expression assay system by real-time PCR is useful for studying the physiological and pathophysiological roles of ET2 in dog.

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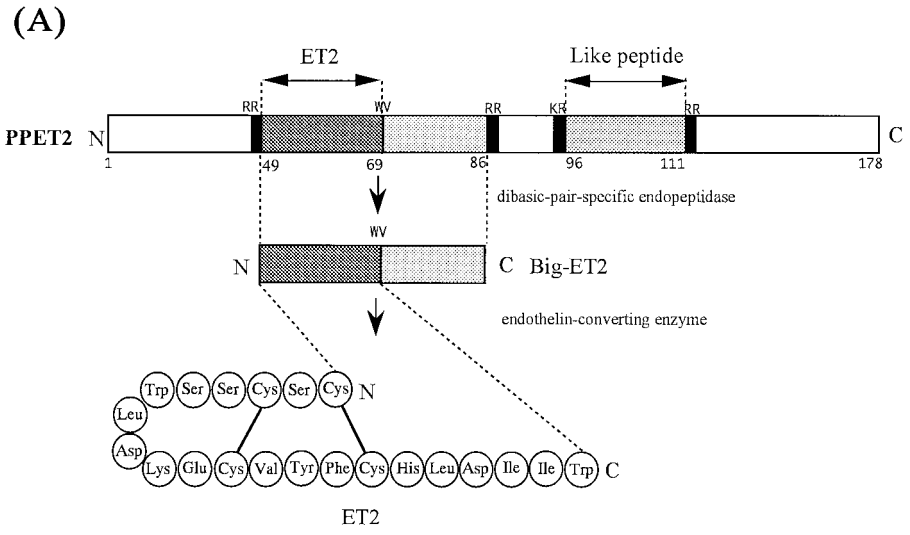

Fig. 4. (A) Putative biosynthesis pathway of dog ET2. Big-ET2 and ET-like peptide regions were present in PPET2, flanking dibasic amino acid pairs (black box). A big-ET2 peptide of 38-amino acids is generated from PPET2 by proteolytic cleavage at the paired basic residues, R^{47}- R^{48} and R^{87}-R^{88}, by dibasic-pair-specific endopeptidase, and mature ET2 peptide is produced from the big-ET2 by cleavage between W^{69} and V^{70} by endothelin converting enzyme. (B) Internal homology within dog PPET2. Mature and ET-like peptide share nine amino acids in common, including four cysteine residues. Identical amino acids are indicated by asterisks. The four common cysteine residues at 1,3,11 and 15 are boxed.

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