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Molecular Cloning, Segmental Distribution and Ontogenetic Regulation of Cationic Amino Acid Transporter 2 in Pigs*

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ABSTRACT : The goal of this study was to elucidate the expression and segmental distribution of the glomerular cationic amino acid metabolism transporter-2 (CAT-2) and thus to improve our understanding of porcine cationic amino acid transporters and amino acid absorption. Porcine CAT-2 was cloned, sequenced and characterized. The predicted amino acid sequence of porcine CAT-2 shared 86.1% and 92.1% identity with human and mouse CAT-2A, respectively. The tissue distribution patterns and ontogenic changes of CAT-2 mRNAs were determined by real-time Q-PCR. The results showed that porcine CAT-2 was highly expressed in the heart and intestinal tract (duodenum, ileum and jejunum). In addition, the mRNA of CAT-2 was found in liver, lung, kidney, brain and muscle. Within the intestinal tract, CAT-2 mRNA was most abundant in the ileum and rarely expressed in the duodenum. In the duodenum, the levels of CAT-2 mRNA reached their peak on day 7 ($p<0.05$) while in the jejunum, levels were low on day 1 and 7 and increased rapidly after day 26 before peaking on days 30 and 60 ($p<0.05$). The levels then dramatically decreased by day 90 ($p<0.05$). In the ileum, levels achieved their maximum on day 30 and then decreased significantly on day 60 ($p<0.05$). (**Key Words** : Amino Acid Transporter, CAT-2, SLC7A2, Ontogenetic Regulation, Molecular Cloning, Pig)

INTRODUCTION

Free amino acids are transported from the lumen of the intestine into the extracellular space by various transport systems that are characterized by differences in structure, substrate specificity, site of expression and regulation. Several distinct transport systems, including system y^+, have been identified based on their ion dependence (i.e. Na^+ and/or Cl^-dependence) as well as by their profile of amino acid acceptance (Palacin et al., 1998). Five transport systems that mediate the uptake of cationic amino acids (CAA) are known: the Na^+ dependent system $B^{0,+}$, Na^+ independent system b^+, system y^+, system y^+L and system $b^{0,+}$ (Van Winkle et al., 1985; Van Winkle et al., 1988; Deves et al., 1992). Only transporters of system b^+, found in preimplantation mouse blastocysts, seem to be specific for CAA, whereas the other systems accept neutral amino acids also.

The y^+ system is Na^+ and pH independent. In recent years, several proteins involved in system y^+ have been identified at the molecular level. There are four known members of a family of cationic amino acid transporters (CAT-1, CAT-2A, CAT-2B, CAT-3) exhibiting system y^+ activity (Deves and Boyd, 1998). The function of a more distantly related isoform CAT-4, which has been identified in humans, remains elusive (Sperandeo et al., 1998; Wolf et al., 2002). Mammalian CAT-1, CAT-2B and CAT-3 mediate high affinity transport (K_m = 70-400 μM), while CAT-2A demonstrates a much lower substrate affinity (K_m = 2-5 μM) and is less dependent on trans-stimulation than system y^+-like CATs (Closs et al., 1993; Kavanaugh et al., 1994; Vekony et al., 2001). Unlike systems y^+L, $b^{0,+}$ and $B^{0,+}$, system y^+ is ubiquitously expressed and functions to accumulate lysine and arginine intracellular amino acid pools for nitrogen metabolism (Verrey et al., 2000).

cDNAs encoding the human homologue of CAT-1

* Sequence data from this article have been deposited with the GenBank Data Library under Accession No. EU155140.

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(hCAT-1) have been reported by two groups of researchers (Yoshimoto et al., 1991; Albritton et al., 1992). The human homologues hCAT-2A and hCAT-2B have also been cloned, and the function of their protein products has been studied in cRNA-injected oocytes and has been compared with hCAT-1 (Closs et al., 1997). In contrast to the detailed information available on the structure and function of the human y^+ system, in pigs only the cDNA of CAT-1 has been cloned (Cui et al., 2005). In this work we cloned and investigated the segmental distribution and the developmental regulation of CAT-2 mRNA to improve our understanding of the relationship between CAT-2 expression and age, y^+ transporter system gene expression and amino acid absorption.

MATERIALS AND METHODS

Animals

A total of 35 littermate purebred Landrace gilts were divided into seven groups at the ages of 1, 7, 26 (5 days post-weaning), 30, 60, 90 and 150 d, respectively. Feed and water were supplied *ad libitum* throughout the experiment. The handing of the animals strictly followed the procedure approved by the Animal Care Committee of South China Agricultural University. Intestinal tissue samples were collected from a total of 70 pigs at different ages: namely suckling (1 and 7 days) and post-weaning (26, 30, 60, 90 and 150 days).

Intestinal tissue sample collection

Pigs were euthanized with an overdose injection of 10% sodium pentobarbital before sampling. The entire small intestine was then removed and dissected free of mesenteric attachments and placed on a smooth, cold surface. The duodenum, jejunum and ileum were separated. The isolated intestinal segments were immediately opened lengthwise following the mesentery line and flushed with ice-cold saline (154 mM NaCl, 0.1 mM PMSF, pH 7.4) and divided into 15-cm segments and deposited in marked tubes. Each tube, which contained approximately 15 g of tissue, was tightly capped and stored at -80°C until further analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg of intestinal tissue samples using TRIZOL reagent (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's instructions. The RNA quality was checked by 1% agarose gel electrophoresis and stained with 10 μg/ml ethidium bromide. The RNA had an OD260:OD280 ratio between 1.8 and 2.0. Synthesis of the first strand cDNA was performed with oligo (dt) 20 and Superscript II reverse transcriptase (Invitrogen).

cDNA cloning strategy

Primers for porcine CAT-2 partial cDNA sequence were designed based on a conserved region of alignment of human and mouse sequence. Then, a 1,887 bp sequence of SLC7A2 was generated by PCR using amplification conditions as follows: 95°C denature 5 min, 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 5 min. On the basis of sequence, porcine SLC7A7 gene-specific primers were synthesized and 3'/5' RACE were carried out according to the manufacturer's instructions (BD Biosciences Clontech). Briefly, the first strand cDNA was generated from 1 μg total RNA using 3' RACE CDS primer A (3' CDS) and 5'-CDS/SMART II A (Clontech) for 3' RACE and 5' RACE, respectively. For 3' RACE, the amplification reaction was performed by 1st touch-down PCR for 40 cycles (94°C for 5 min, 94°C for 30 s, 70°C, 65°C, 61°C for 30 s, respectively, 72°C for 10 min) using GSP2 and the reverse primer UPM. After the first PCR , the second (nest) PCR was performed under similar condition using the nest primer NGSP2 and the reverse primer NUP; for 5' RACE, a similar amplification reaction but a 3-min elongation time was carried out using the forward primer (UPM and NUP) and reverse primer GSP1and NGSP1. The RACE products were gel-purified and cloned into the pGMT vector (Invitrogen). After transformation into *Escherichia coli*, the plasmid purifications from the overnight-grown colonies were done and the cloned cDNA was sequenced. Based on the newly obtained sequence for the full-length cDNA, a pair of PCR primers, forward primer AM1 and reverse primer AM2, were designed to amplify the sequence covering the open reading frame (ORF) of porcine SLC7A2. All primers except those provided by Clontech RACE kit are shown in Table 1.

Sequence and structural analysis

Nucleotide and amino acid sequence alignment were

Table 1. Primers for smart RACE cDNA and ORF amplification

Primer	Application	Sequence
GSP1	1st PCR	5' GACGTAAGTGTACAAATACGCGGACCCAG 3'
NGSP1	2nd PCR	5' ATGGTGGATAAACATCGGCAGAGTTTGG 3'
GSP2	1st PCR	5' GGGGATTCAGCCTGCGAGCCTTCTTC 3'
NGSP2	2nd PCR	5' CTCTGCGTTGCCGTCGTGCTCACC 3'
ZY1	ORF clone	5' AGAATGATTCCCTGCAGAGCAACGC 3'
ZY2	ORF clone	5' GCACTAGCATTCGCTCGTCTTTTCATG 3'

analyzed with DNAMAN and NTI Vector 5.5 software packages. Homology searches were performed using BLAST and FASTA at the National Center for Biotechnological Information (NCBI) and DNA Data Bank of Japan (DDBJ).

Detection of tissue distribution and ontogenetic regulation of porcine SLC7A7 by Real-time RT-PCR analysis

Real-time PCR was performed using one-step SYBR Green PCR Mix (Takara, Dalian, China) containing $MgCl_2$, dNTP, and Hotstar Taq polymerase. 2 μl cDNA template was added to a total volume of 25 μl containing 12.5 μl SYBR Green mix, 0.25 μl RT mix and 1 μM each of forward (CAT-2: 5' ACAACTGGCGAAGAAGTCCG 3', 18S: 5' GGACATCTAAGGGCATCACAG 3') and reverse primers (CAT-2: 5' CTGCCGAGACCCCAAAATAG 3', 18S: 5' AATTCC GATAACGAACGAGACT 3'). Primers for 18S were designed with Primer 5 based on porcine sequence (Accession No. AY390526). We used the following protocol: i) denaturation program (15 min at 95°C); ii) amplification and quantification program, repeated 40 cycles (15 s at 95°C, 15 s at 58°C, 15 s at 72°C); iii) melting curve program (60-99°C with heating rate of 0.1°C s-1 and fluorescence measurement). We used an abundantly expressed gene, 18S, as the internal control to normalize the amount of starting RNA used for RT-PCR for all the samples. Amplification and melting curve analysis was performed in a ABI 7500 (Applied BioSystems). Melting curve analysis was conducted to confirm the specificity of each product, and the size of products was verified on ethidium bromide-stained 2% agarose gels in Tris acetate-EDTA buffer. The identity of each product was confirmed by dideoxy-mediated chain termination sequencing at Takara Biotechnology, Inc. We calculated the relative expression ratio (R) of mRNA by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Real-time PCR efficiencies were acquired by amplification of a dilution series of RNA according to the equation 10 (-1/slope) and were consistent between target mRNA and 18S. Negative controls were performed in which water was substituted for RNA.

Statistical analysis

Developmental data of mRNA abundance were subjected to analysis of variance of mRNA abundance among days 1, 7, 26, 30, 60, 90 and 150 using the Tukey test by SAS (The SAS Institute, Cary, NC). Multiple comparisons of mRNA abundance among duodenum, jejunum, ileum and colon at day 60 were made using the Tukey test by SAS (The SAS Institute, Cary, NC). Data are presented as means±SEM. Significance was determined using the $p<0.05$ level.

RESULTS

Cloning of porcine CAT-2 cDNA sequence

The 3' RACE (~1.7 kb) and 5' RACE (~0.15 kb) products were cloned into the pGMT vector and sequenced. A 3,147 bp segment of cDNA was assembled from the overlapping 3' RACE sequence, known sequence and 5' RACE sequence. The full-length cDNA encoding porcine CAT-2 from porcine was isolated. Sequence analysis of the porcine SLC7A2 cDNA revealed an ORF of 1,974 bp that would encode a protein of 657 residues, 85 bp of 5' untranslated region (UTR) and 1,087 bp of 3' UTR. BLASTn analysis demonstrated that the porcine sequence shared a high degree of sequence identity, especially in coding sequence (CDS) regions (83.7%, 86%, 85.9%, and 83.6%), and homology with human CAT-2A (Accession No. NM_003046), human CAT-2B (Accession No. NM_001008539) and mouse CAT-2A (Accession No. NM_007514) CAT-2, mouse CAT-2B (Accession NM_001044740), as shown in Figure 1. Alignment of amino acid sequence is shown in Figure 2. Hydrophobicity prediction suggested 14 putative membrane-spanning domains within porcine CAT-2 (Krogh et al., 2001), similar to other mammalian CAT-2s. Analysis of the amino acid sequence by ScanProsite (de Castro et al., 2006) revealed several consensus sites for post-translational modification. Three consensus sites for protein kinase C phosphorylation were located at 381-383 and 473-476.

Tissue distribution of porcine CAT-2 mRNA

The tissue distribution of CAT-2 mRNA at day 6 is shown in Figure 3. The porcine CAT-2 mRNA was detected in brain, liver, kidney, heart, lung, small intestine and muscle. The heart had the highest CAT-2 mRNA abundance while brain and kidney had the lowest ($p<0.05$). Compared with kidney, the lung, brain, liver and muscle, and small intestine had higher CAT-2 mRNA abundance ($p<0.05$).

Expression of porcine CAT-2 mRNA along the longitudinal axis

The intestinal distribution of CAT-2 mRNA at day 60 is shown in Figure 3. The ileum had the highest CAT-2 mRNA abundance while the duodenum had the lowest ($p<0.05$) Compared to duodenum and ileum, the CAT-2 mRNA level was medium in the jejunum ($p<0.05$).

Developmental regulation of CAT-2 mRNA relative abundance

Developmental regulation of CAT-2 mRNA relative abundance is shown in Figure 4. The CAT-2 mRNA had the highest level on day 7 in the duodenum ($p<0.05$). It had the

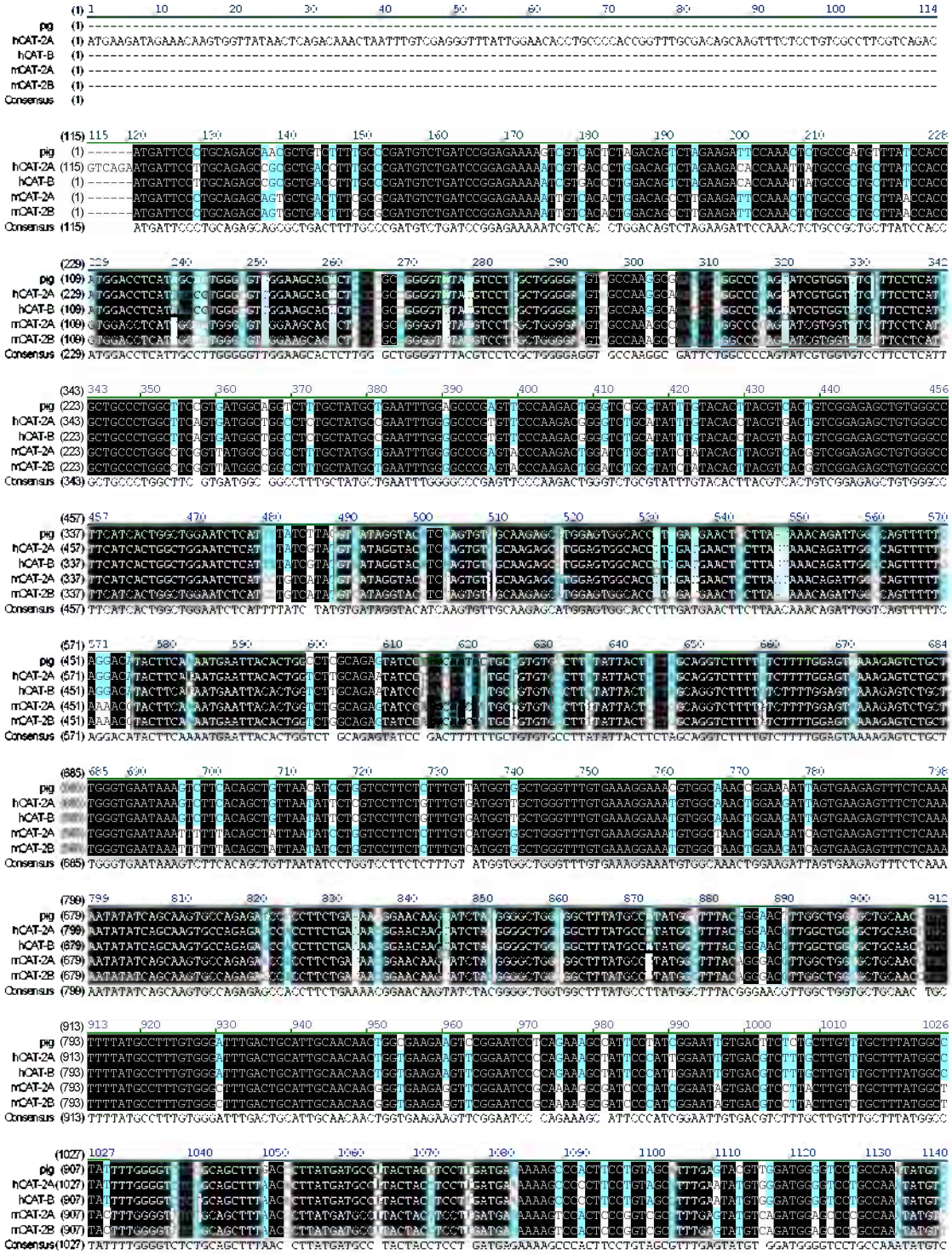

Figure 1. i) Comparison of code sequence of CAT-2 from pig, human and mouse CAT-2A, B. Identical nucleic acids are shown in black background. The porcine code sequence of CAT-2 (Accession No. EU155140) shows 83.7%, 86%, 85.9%, and 83.6% homology with the human CAT-2A (Accession No. NM_003046), human CAT-2B (Accession No. NM_001008539) and mouse CAT-2A (Accession No. NM_007514) CAT-2, mouse CAT-2B (Accession NM_001044740) respectively.

highest level on days 30 and 60 in the jejunum ($p<0.05$) and decreased dramatically on day 90 ($p<0.05$). There was no difference between day 1, 7, 26, 90, and 150 ($p>0.05$). In the ileum, CAT-2 mRNA had the highest abundance on day 30; after day 30 it then decreased dramatically on day 60 ($p<0.05$).

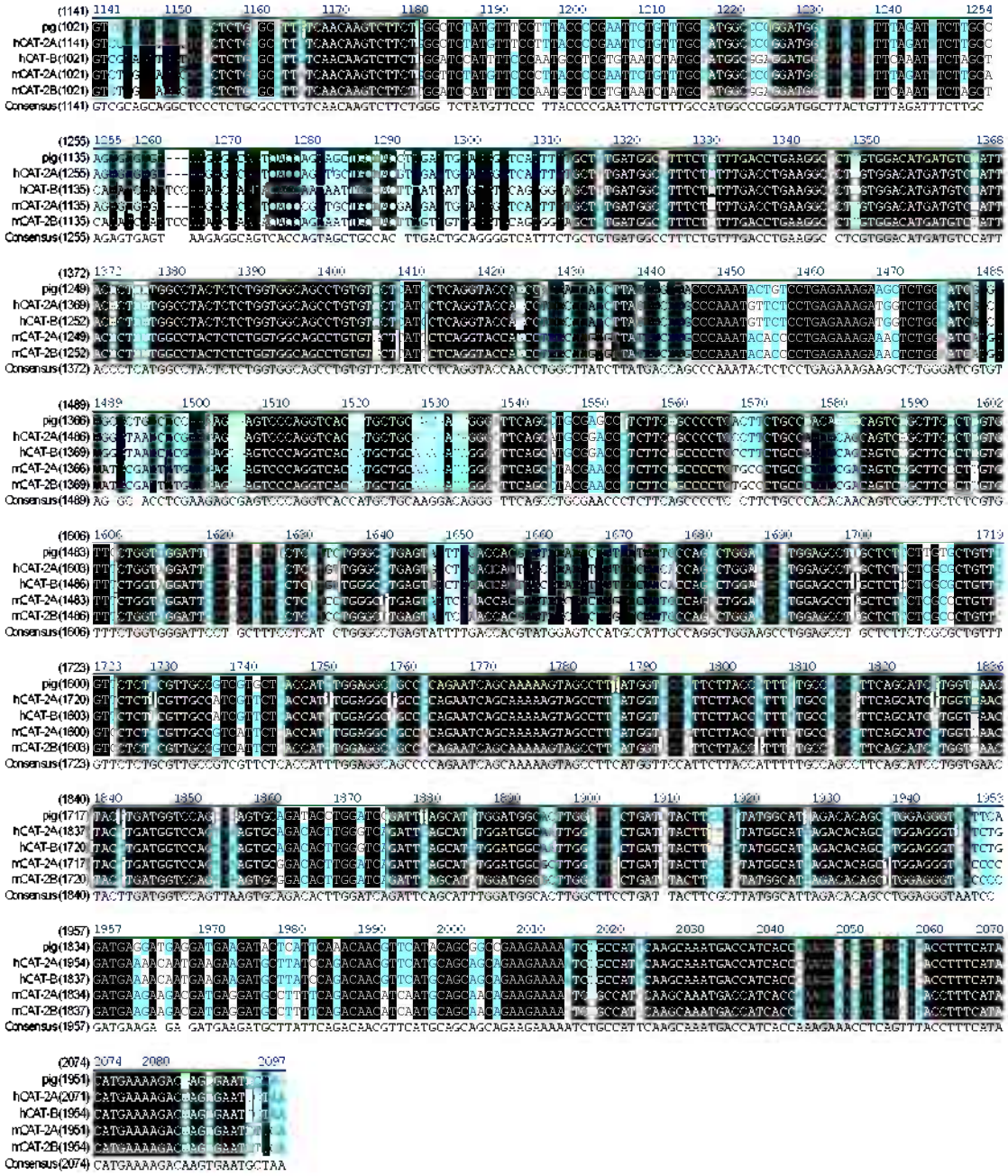

Figure 1. ii) Comparison of code sequence of CAT-2 from pig, human and mouse CAT-2A, B. Identical nucleic acids are shown in black background. The porcine code sequence of CAT-2 (Accession No. EU155140) shows 83.7%, 86%, 85.9%, and 83.6% homology with the human CAT-2A (Accession No. NM_003046), human CAT-2B (Accession No. NM_001008539) and mouse CAT-2A (Accession No. NM_007514) CAT-2, mouse CAT-2B (Accession NM_001044740) respectively.

DISCUSSION

CATs play very important roles in the absorption of cationic amino acid, including lysine, which is an important essential amino acid for pigs. Unlike systems y^+L, $b^{0,+}$ and $B^{0,+}$, system y^+ is ubiquitously expressed and functions to accumulate lysine and arginine into the intracellular amino acid pools for use in nitrogen metabolism (Verrey et al., 2000). Therefore, the system y^+ cationic amino acid transport family is the primary amino acid transport system utilized by most tissues for lysine and arginine uptake (Broer, 2002; Closs, 2002). The two isoforms, CAT-2A and CAT-2B, result from alternative splicing of primary transcripts of the same gene. The two forms only differ in a short stretch of 42 amino acids, shown to be responsible for the differences in the transport properties of the CAT proteins (Closs et al., 1993). The cDNAs encoding the human homologues hCAT-2A and hCAT-2B have been

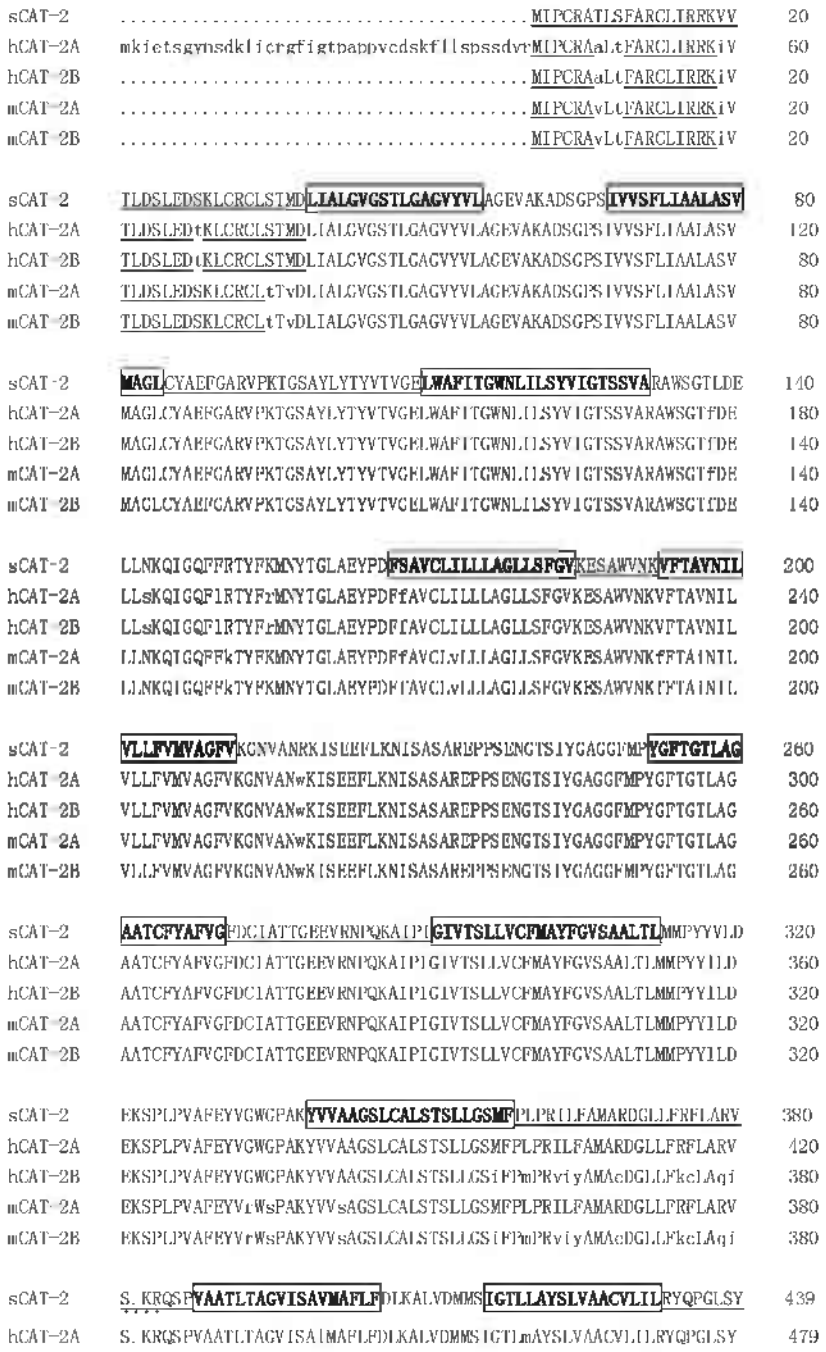

Figure 2. i) Comparison of amino acid sequence of CAT-2 from human and mouse CAT-2A, B. Amino acid sequence determined from the porcine CAT-2 cDNA (Accession No. EU155140) is shown on the top line. Amino acid sequence for human CAT-2A, human CAT-2A, mouse CAT-2A and mouse CAT-2B are shown below. Putative membrane-spanning domains are indicated by open box and in bold. Potential sites for protein kinase C phosphorylation are stressed by black dots and putative intracellular domains are underlined. Dots indicate the absence of amino acid. Lower case indicates difference in amino acid to procine CAT-2.

cloned. The function of their protein products has been studied in cRNA-injected oocytes and has been compared with hCAT-1. In their functional domains (42 amino acids), both hCAT-2A and hCAT-2B differ only by one residue from the respective mouse proteins. Thus, CAT-2 proteins demonstrate a higher interspecies conservation than CAT-1 proteins that are overall 86.5% identical between mouse and human and differ by seven residues in the functional domain. When expressed in Xenopus oocytes, the transport properties of the human CAT-1 and CAT-2B isoforms showed clear differences: hCAT-1 had a 3-fold higher substrate affinity and was more sensitive to trans-stimulation than hCAT-2B (Closs et al., 1997).

In this study, the complete porcine mRNA sequence of

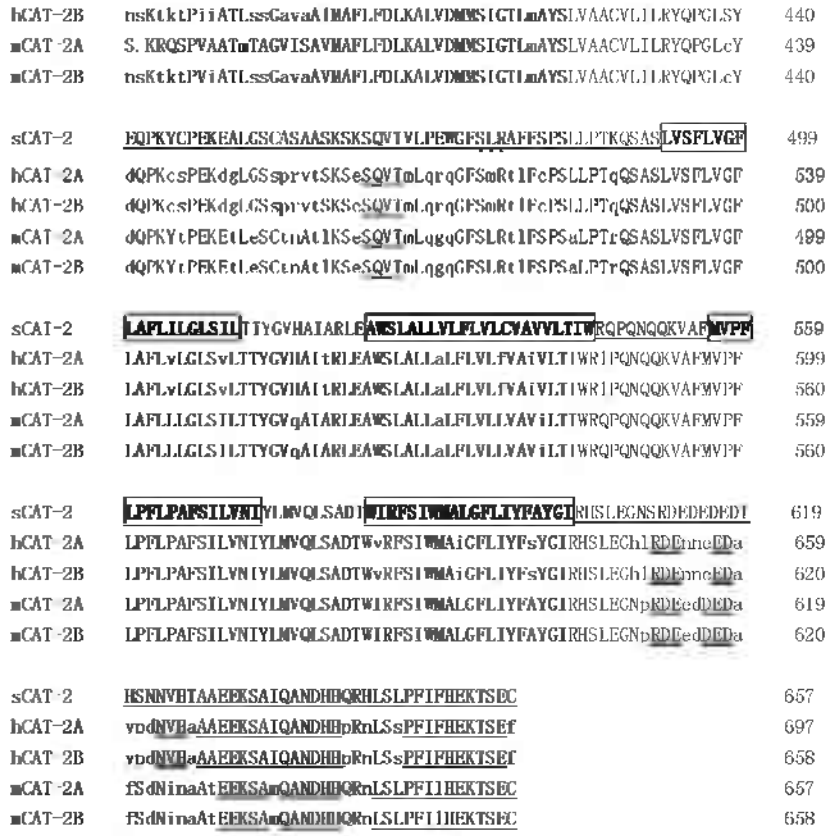

Figure 2. ii) Comparison of amino acid sequence of CAT-2 from human and mouse CAT-2A, B. Amino acid sequence determined from the porcine CAT-2 cDNA (Accession No. EU155140) is shown on the top line. Amino acid sequence for human CAT-2A, human CAT-2A, mouse CAT-2A and mouse CAT-2B are shown below. Putative membrane-spanning domains are indicated by open box and in bold. Potential sites for protein kinase C phosphorylation are stressed by black dots and putative intracellular domains are underlined. Dots indicate the absence of amino acid. Lower case indicates difference in amino acid to procine CAT-2.

the CAT-2 gene was cloned. Sequence analysis of the porcine SLC7A2 cDNA revealed a putative ORF of 1,974 bp that would encode a protein of 657 residues. BLASTn or BLASTp analysis demonstrated that the porcine sequence shared a high degree of sequence identity with the human and mouse CAT-2 gene. Hydrophobicity prediction suggested 14 putative membrane-spanning domains within porcine CAT-2, similar to other mammalian CAT-2s. Porcine CAT-2 cDNA has high homology with human and mouse CAT-2A, and less with human and mouse CAT-2B. Therefore, we presume this represents the porcine CAT-2 homology with human CAT-2A (SLC7A2 transcript variant 1). The clone of this porcine cDNA will facilitate the study of porcine CAT-2A function.

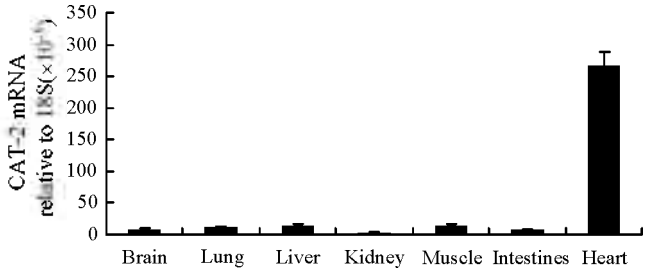

Figure 3. Tissue distribution of porcine CAT-2 in the heart, liver, lung, kidney, brain, muscle and intestine. The heart had the highest CAT-2 mRNA abundance while the brain, kidney had the lowest ($p<0.05$). All samples were normalized using 18S expression as an internal control in each real-time PCR. Relative levels of CAT-2 mRNA were analyzed by the 2 (-Delta Ct) method. Data are presented as the mean±SE (n = 5), in arbitrary units.

The tissue distribution of the mRNA suggests the primary function of this gene (Lee et al., 2008). Unlike the porcine $b^{0,+}$ CAT mRNA, which is most abundant in the small intestine (Zhi et al., 2008), the heart had the highest CAT-2 mRNA abundance and the brain and kidney had the lowest CAT-2 mRNA abundance. In the intestines, the ileum had the highest CAT-2 mRNA abundance and the duodenum had the lowest CAT-2 mRNA abundance. The different distribution of transporters along the intestinal axis from proximal to distal parts and from the crypt to villus may be attributable to the unique morphological characteristics of the intestine and substrates, even though further research is needed to confirm this. However, there are no research data to compare with these results at this

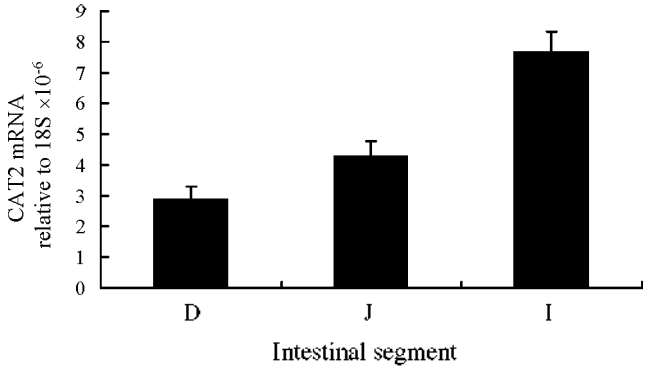

Figure 4. Relative mRNA expression of porcine CAT-2 along longitudinal axis of intestine at day 60. All samples were normalized using 18S expression as an internal control in each real-time PCR. Relative levels of CAT-2 mRNA were analyzed by the 2 (-Delta Ct) method.

point. Another concern of this study is that even though the intestine is physiologically divided into four segments (duodenum, jejunum, ileum and colon), there is still the possibility that the mRNA levels are different within each segment. More knowledge of the function of each gene is needed to further understand the precise pattern of distribution.

The intestine undergoes dramatic structural and functional changes after birth, such as increasing in dry mass and absorptive surface area, and changing of membrane permeability and fluidity (Buddington et al., 2001). In addition to these nonspecific changes, the absorptive capability per cell and the expression of transporters also alters with aging. In this study, the results showed the CAT-2 mRNA abundance generally increased with age in the jejunum and ileum before day 30, although there was some variability among intestinal segments. There are many differences between this result and other research on segmental ontogenetic regulation of the CAT-2 in Lantang pigs conducted by our colleague (unpublished data), and research is ongoing to clarify these issues. The next goal of our research is to investigate the mRNA expression of different amino acid levels (especially lysine) in growing pigs, not only *in vivo* but *in vitro* by primary cultures of swine intestinal epithelia cells.

In conclusion, we have cloned a cationic amino acid transporter CAT-2 from the pig. This cationic amino acid transporter shows significant homology with human and murine CAT-2A. Further studies are needed to identify its function in porcine nutrition and physiology. The mRNA expression of amino acid transporter CAT-2 was not only differentially regulated by age but also differentially distributed along the small intestine of piglets at early and growing stages of life. Further research on ontogenetic regulation of protein expression and total capacity of the whole small intestine is still needed to fully understand

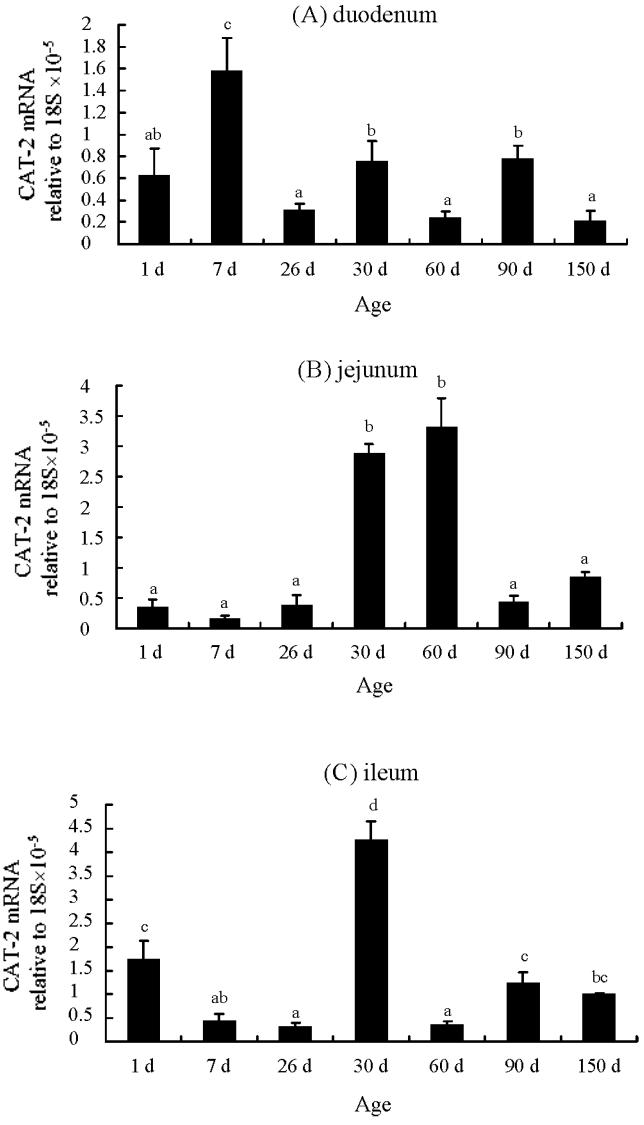

Figure 5. Relative mRNA expression of porcine CAT-2 in pig duodenum, jejunum and ileum during post-natal development. All samples were normalized using 18S expression as an internal control in each real-time PCR. Relative levels of CAT-2 mRNA were analyzed by the 2 (-Delta Ct) method. Bars that share a common superscript do not differ ($p>0.05$). Data are presented at the mean±SE (n = 5), in arbitrary units.

ontogenetic regulation of CAT-2.

ACKNOWLEDGMENT

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