



Cloning and Distribution of Facilitative Glucose Transporter 2 (SLC2A2) in Pigs*

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ABSTRACT : Glucose is the main energy source for mammalian cells and its absorption is co-mediated by two different families of glucose transporters, sodium/glucose co-transporters (SGLTs) and facilitative glucose transporters (GLUTs). Here, we report the cloning and tissue distribution of porcine GLUT2. The GLUT2 was cloned by RACE and its cDNA was 2,051 bp long (GenBank accession no. EF140874). An AAATAA consensus sequence at nucleotide positions 1936-1941 was located upstream of the poly (A)⁺ tail. Open reading frame analysis suggested that porcine GLUT2 contained 524 amino acids, with molecular weight of 57 kDa. The amino acid sequence of porcine GLUT2 was 87% and 79.4% identical with human and mouse GLUT2, respectively. GLUT2 mRNA was detected at highest level in porcine liver, at moderate levels in the small intestine and kidney, and at low levels in the brain, lung, muscle and heart. In the small intestine, the highest level was in the jejunum. In conclusion, the mRNA expression of GLUT2 was not only differentially regulated by age, but also differentially distributed along the small intestine of piglets, which may be related to availability of different intestinal luminal substrate concentrations resulting from different food sources and digestibility. (**Key Words** : GLUT2, Molecular Cloning, Small Intestine, Pig)

INTRODUCTION

Glucose supply is necessary for mammalian cells and is the essential ingredient for protein and lipid synthesis. Blood glucose concentration is maintained in a narrow range, mediated by the coordinated regulation of 3 processes, namely absorption, production and consumption. Glucose absorption from the lumen, the first step, plays a central role in glucose utilization, metabolism and homeostasis. Glucose absorption in the intestine is co-mediated by two families of glucose transporters located in enterocytes (Scheepers et al., 2004). Glucose is firstly transported into the epithelium by Na⁺-bound-SGLTs (Sodium/Glucose Cotransporters) and this transcellular

pathway is powered by a downhill gradient of Na⁺ across the apical membrane. Then, the accumulated glucose in the enterocytes is transported by GLUT2 (Glucose Transporter 2) located in the basolateral membrane into the blood (Wood and Trayhurn, 2003) with the aid of facilitative diffusion down its concentration gradient.

GLUT family comprises of 13 isoforms. Each isoform has its own characteristics including different affinity with glucose and different transport activity to support diversity of glucose utilization in distinct tissues. It has been showed that GLUTs have highly conserved amino acid sequence, putative secondary structure of 12 membrane spanning domains and several motifs (sugar transporter signatures) which are essential for the transport activity (Joost and Thorens, 2001). The GLUTs have been divided into three classes based on sequence homology (Zhao, 2007). GLUT2 is a member of Class I, whose members are distinguished by the presence of a putative glycosylation site on loop 1.

Litter has been known about the characteristics of porcine GLUT2 gene, even though several research groups have studied the characteristics of GLUT2 gene, including molecular cloning of the gene by low-stringency screening of rat (Thorens et al., 1988) and human (Fukumoto et al., 1988) liver cDNA libraries with a GLUT1 cDNA probe as

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well as examination of its tissue distribution in human liver, small intestine and kidney. To study the role of porcine GLUT2 in glucose absorption from the lumen, we cloned the full-length cDNA of porcine GLUT2 and examined its tissue distribution in the small intestine.

MATERIALS AND METHODS

Tissue sample collection

Tissue samples were collected from 60-day old purebred Landrace pigs. The pigs were fed diets *ad libitum* with free access to water, and euthanized with an overdose injection of 10% sodium pentobarbital 12 h after the last meal. The entire small intestine was removed and dissected free of mesenteric attachments and placed on a smooth and cold surface. The duodenum, jejunum and ileum were separated. The isolated intestinal segments were immediately opened lengthwise following the mesentery line, flushed with ice-cold saline (154 mM NaCl, 0.1 mM PMSF, pH 7.4) and divided into 15-cm segments. Each tube, which contained approximately 15 g of tissue, was tightly capped, frozen in liquid nitrogen immediately and stored at -80°C . The brain, lung, liver, kidney, muscle and heart were also collected.

RNA extraction

Total RNA was isolated from 100 mg of tissue using TRIZOL reagent (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's instructions. The RNA quality was examined by 1% agarose gel electrophoresis after staining with 10- $\mu\text{g}/\text{ml}$ ethidium bromide. The RNA had an OD260:OD280 ratio between 1.8 and 2.0.

RACE of GLUT2

In order to obtain full-length GLUT2 cDNA sequence, 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed using SMARTRACE technology (Clontech) following the manufacturer's guidelines. The 5'- and 3'-RACE-ready first-strand cDNA was synthesized using 4 μg total RNA from porcine small intestine. Primers were designed based on the partial porcine GLUT2 sequence in GenBank (Accession no. AF054835). The 5'-RACE reactions were carried out with the gene-specific primer (GSP1), 5'- ACTCGGCCACCATGAACCAGGGGATG -3' followed by the nested gene-specific primer (NGSP1) 5'- TCCAGTAGCACAAGTCCCACCGA-3'. The 3'-RACE reactions were carried out the gene-specific primer (GSP2) 5'- ATGTCGGTGGGACTTGTGCTACTGGAT -3', followed by the nested gene-specific primer (NGSP2) 5'- CTGTGCTTCCAGTACATTGCGGACTTCT-3'. The PCR program was 94°C for 3 min, then touchdown PCR for 15 cycles, 94°C for 30 s, 70°C for 30 s, decreasing 3°C every 5 cycles and 72°C for 3 min followed by 25 cycles of 94°C

for 30 s, 61°C for 30 s, 72°C for 3 min, and a final extension step of 72°C for 10 min. The resulting PCR products were gel-purified, cloned and sequenced. The sequence of 5' and 3' RACE products was verified in three independent clones.

Sequence analysis

Sequence analysis was performed using DNASTAR program, the National Center for Biotechnology Information (NCBI) BLAST site (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the SWISSPROT Scan-Prosit program (<http://us.expasy.org/tools/scanprosite/>). The multiple sequence alignment was performed with ClustalW (<http://genome.cs.mtu.edu/map.html>).

Quantification of mRNA 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. Total RNA was prepared using TRIZOL reagent (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's instructions. Equal amounts of DNase I treated-RNA from all samples were used for synthesis of the first strand cDNA with oligo (dt)20 and Superscript II reverse transcriptase (Invitrogen). 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 and reverse primers. Primers for GLUT2, based on RACE results, were sense 5'- ATCCTGCTTGGTCTATCTGCTGT -3', and Antisense: 5'-GCTTTTCCTTGCTTTGGCTTC -3'. Primers for 18S (sense:5'- AATTCCGATAACGAACGAGACT -3', antisense:5'- GGACATCTAAGGGCATCACAG -3') were designed based on porcine sequence in the GenBank (Accession no. AY390526). We used the following protocol for the reaction: i) denaturation program (15 min at 95°C); ii) amplification and quantification program, repeated 45 cycles (15 s at 95°C , 15 s at 58°C , 15 s at 72°C); iii) melting curve program ($60-99^{\circ}\text{C}$ 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. Amplification and melting curve analysis was performed with 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. The relative expression ratio (R) of mRNA was calculated by $2^{-\Delta\text{Ct}}$ (Livak and Schmittgen, 2001). Real-time PCR efficiencies were acquired by amplification of dilution series of RNA according to the equation $10^{-(-1/\text{slope})}$ and

were consistent between target mRNA and 18S. Negative controls were performed in which water was substituted for RNA.

Statistical analysis

Segmental data of mRNA abundance were subjected to ONE-WAY ANOVA analysis. Multiple comparisons were made using Turkey test by SAS (The SAS Institute, Cary, NC). Data are presented as means \pm SEM. Significance was determined as $p < 0.05$.

RESULTS

Cloning of porcine GLUT2 gene

A RACE strategy was adapted to obtain the full-length cDNA sequence of porcine GLUT2 from small the intestine (Figure 1). The full-length mRNA of porcine GLUT2 is 2,051 bp long (GenBank accession no. EF140874). An AAATAA consensus sequence, presenting in the vast majority of eukaryotic polyadenylation signals (Natalizio et al., 2002), is located at the upstream of the poly(A)⁺ tail (1936-1941). Open reading frame analysis suggests porcine GLUT2 contains 524 amino acids, about 57 kDa MW. The amino acid sequence of porcine GLUT2 is 87% and 79.4% identical with human (accession no. NM_000340) and mouse (NM_031197) GLUT2 (Figure 2), respectively.

Sequence analysis of porcine GLUT2

Porcine GLUT2 keeps the main structural features of the gene as shown in other species previously. The software recorded a hydrophilic maximum of 2.144 with a minimum value of -1.767. The lowest hydrophobicity values correspond to α -helix protein structures, while the maximum values indicate a high likelihood of hydrophobic interactions leading to "core" protein formation. Generally, the hydrophobic sites are adjacent to the hydrophilic ones. Hydropathy plot of porcine GLUT2 (Figure 3) showed that the distribution pattern of its hydrophobic and presumed membrane spanning (TM) segments are similar to the proposed secondary structure of GLUTs, a 12-helix model (Figure 2). The porcine GLUT2 has two large exoplasmic loops between TM1 and 2 (loop 1) and between 6 and 7 (loop 6) in addition to one large exoplasmic C-termini (Figure 2). The loop 6 is the longest. The large loop 1 contains one Asn-glycosylation site (Figure 1), which is a structural characteristic of class I members of GLUT family. Therefore, the hydropathy plot of porcine GLUT2 is more similar to that of another class I member (Bell et al., 1993).

The sequence of porcine GLUT2 retains several sugar transporter family characters. The sequence between amino acids 13 and 488 in porcine GLUT2 matches with major facilitator superfamily (MFS) profile (PS50850). There is a

sugar transport proteins signature 1 (SUGAR_TRANSPORT_1) (accession No. PS00216) between amino acids 356 and 372 (SVFLVEKAGRRslfliG). The sugar transport protein signature 2 (SUGAR_TRANSPORT_2) (accession No. PS00217) is located between amino acids 160 and 185 (IsGLYcGLisglvpmYigEiaptkfR). Besides, porcine GLUT2 conserves other motifs that may be critical for either transport activity or substrate specificity (Zhao, 2007): PESPRY/FLL in loop 6, GRR in loop 8, PMY in TM4, GPGPIP/TW in TM 10, and PETKG in the C-terminal tail.

Scan-Prosit analysis showed that there were three potential N-glycosylation sites (PS00001) located at amino acids 62-65 (NSTE), 297-300(NSSY), 443-446(NWTY) in porcine GLUT2. The first site is located in the putative loop 1 which is a structural characteristic of class I members of GLUT family. However, as to which site could be glycosylated needs further verification. In addition, there are several protein kinase C (PS00005) and casein kinase II (PS00006) phosphorylation sites and N-myristoylation sites (PS00008), one amidation site (PS00009), one cAMP- and cGMP-dependent protein kinase phosphorylation site (PS00004) in porcine GLUT2 sequence. The biological activities of these sites remain to be investigated.

Tissue distribution of GLUT2 in porcine

Expression of GLUT2 mRNA in porcine brain, lung, liver, kidney, muscle, small intestine and heart was analyzed by real-time PCR (Figure 4). GLUT2 mRNA was found in all porcine tissues examined, most abundant in the liver, at intermediate levels in the small intestine, kidney, and at lower levels in the brain, lung, skeletal muscle and heart. Here, we further tested GLUT2 mRNA expression in small intestinal segments (Figure 5), and GLUT2 mRNA expression was found the highest in the jejunum and the lowest in the ileum.

DISCUSSION

GLUT2, a low-affinity and high-capacity glucose transporter, is involved in glucose absorption from the lumen of the small intestine in a cooperative manner with SGLT1. Porcine GLUT2 has not been studied previously. In this study, we reported the full-length porcine GLUT2 cDNA sequence and its deduced amino acid sequence. We found that porcine GLUT2 had high homology with GLUT2 of other mammals and retained the main motifs presumably required for sugar absorption (see results for detail); but however, similar to that in humans, porcine GLUT2 lacked the QLS motif at TM7, which is thought to be an essential part for glucosamine substrate specificity (Uldry et al., 2002).

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1          TGGAAACAAGACCTGGATCTCCACAGAGAGGGAGGCTCAAAT
47      CAGCTTTGCCAGTGTGCTATGTCCAGGTCAGGCTTCTCTGTTTAAAGAGTCCCTCCAC
107     ATGTCAGAAGACAAGATCACCAGGAACTTGGTTTTGCTGTCCTCACTGCCGTGCTGGGT
1      M S E D K I T G T L V F A V L T A V L G
167     TCCTCCAGTTTGGATACGACATTGGTGTGACCAATGCAOCTCAACAGTAATAATAACC
21      S F Q F G Y D I G V T N A P Q Q V I I T
227     CATTATAGACACGTTTTGGGTGTTCCGCTGGATGACCGAAAAGCTATCAACAGCTATGCT
41      H Y R H V L G V P L D D R K A I N S Y A
287     ATCAACAGTACAGAGGAGCTGCCACAGGACCATACCCAGGGGATCCAACACCAACCTCT
61      I N S T E E L P T G P Y P G D P T P T S
347     TGGGCTGAGGAAGAGACTACGGCATCTGCTAGCCTCATCATGCTCTGGTCTCTGCT
81      W A E E E T T A S A S L I I M L W S L S
407     GTGTCCATCTTGAATTTGGTGAATGATTGCATCATTCTTGGTGGGATGCTGGAGAT
101     V S I F A I G G M I A S F F G G M L G D
467     CGGCTTGAAGAATAAAAAGCCATGTTGGTAGCAACATCTCTCATTAGTTGGAGCTCTC
121     R L G R I K A M L V A N I L S L V G A L
527     TTGATGGTTTTGCGAAATGGGACCATCTCATATCTAATAATTTCAGGAAGAGGCATA
141     L M W F S K L G P S H I L I I S G R G I
587     TCAGACTCTACTGTGGCTAATTTTCAGGCTTGGTCCAATGTACATTGGTGAATTGCT
161     S G L Y C G L I S G L V P M Y I G E I A
647     CCAACCAAGTTCAGGGGTGCTATTTGGTGTCTTTCATCAGCTGGCCATTGTCACAGGCATT
181     P T K F R G A I G A L H Q L A I V T G I
707     CTTGTTAGTCAGATCATTAGGCTCGACTTTCTCCTGGCAATCATGAGCTATGGCACATC
201     L V S Q I I G L D F L L G N H E L W H I
767     CTGCTGGTCTATCTGCTGTGCCAGCTGTCTCCAATCTCTGATGCTCTTCTCTGTCCA
221     L L G L S A V P A V L Q S L M L F F C P
827     GAAAGCCCAAGATACTTTACATCAAGCTGGATGGGAAGCCAAAGCAAGGAAAAGCTTG
241     E S P R Y L Y I K L D G E A K A R K S L
887     AAAAACTCAGAGGAAGTATGATGTACCAAAAGACATCACCGAGATGCGAAAAGAAAGG
261     K K L R G S D D V T K D I T E M R K E R
947     GAAGAAGCATCAAGTGAAGAAAGTCTCCATAATTCAGCTCTTCACCAATTCAGCTAC
281     E E A S S E K K V S I I Q L F T N S S Y
1007    CGACAGCCTATCTAGTAGCACTGATGCTGCATATGGCTCAGCAATTTCTGGAATCAAT
301     R Q P I L V A L M L H M A Q Q F S G I N
1067    GGGATCTTTACTACTCAACCAGCATTTTTCAGACAGCTGGAATCAGCCAACTGTTTAC
321     G I F Y Y S T S I F Q T A G I S Q P V Y
1127    GCAACCATGGAGTTGGCGCTATCAACACGATTTTCACTGCTCTCTCTGTTTTCCCTTGT
341     A T I G V G A I N T I F T A L S V F L V
1187    GAGAAGCGAGGGCAGCTCTCTGTTCTAATTGGAATGAGTGGGATGTTTGGTTGTC
361     E K A G R R S L F L I G M S G M F V C A
1247    ATCTTCATGTCGGTGGGACTTGTCTACTGGATAAATGGCTTGGATGAGTTATGTGAGT
381     I F M S V G L V L L D K L P W M S Y V S
1307    ATGACAGCCATATCTCTTTGTGAGTTTCTTTGAAAATGGGCCAGGCCCATCCCTGG
401     M T A I F L F V S F F E I G P G P I P W
1367    TTCATGGTGGCCGAGTTTTTCAGTCAAGGACCAGCCCTGCTGCTTTAGCAATGGCTGCA
421     F M V A E F F S Q G P R P A A L A M A A

1427    TTCAGCAACTGGACCCGAATTTTATTATAGCTCTGTGCTTCCAGTACATTGCGGACTTC
441     F S N W T R N F I I A L C F Q Y I A D F
1487    TGTGGACCTTATGTGTTTTTCTCTTTGCTGGAGTGGCTGGTCTTACTCTGTTTACA
461     C G P Y V F F L F A G V V L V F T L F T
1547    TTTTCAAAGTTCAGAAACCAAGGAAAGTCTTTTGGAAAATGCTGCAGAATCCAA
481     F F K V P E T K G K S F E E I A A E F Q
1607    AAGAAGAGTGGCTCAGCCCAATCACCAGGCTGCTGTAGAAATGGAATTCCTGGAGCT
501     K K S G S A Q S P K A A V E M E F L G A
1667    ACAGAGACTGTAAAGATAAACCCTACTTTTTGAACAGAACTGGAAGGAAGCAGTGT
521     T E T V *
1727    TTAAATAACAATTTTTGAGAATTTATATCTACATTTTGAAGTACTGTTTTATTTTTT
1787    AAAGAGATTTATGGGCTCTGACCAGAAATGTCATTGAACATACAAAAGTATTTTTTTA
1847    ACTTAGGGAATCTATGTTGGGTGTTAAGACTCTGTAATTAAGTAAACCAAAGCTAGCTCA
1907    ACATATTAACAATAACAAAATACACAAAATAAATAAATACATTACCTAACATATAA
1967    GTATAGGAGATAGAAATTTTTATCTGACGCTATAGAGATAGTACCGTAAGGAAAGATG
2027    AAAAAAAAAAAAAAAAAAAAAA
    
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Figure 1. Full-length cDNA and amino acid sequences of porcine GLUT2. The predicted amino acid sequence is shown beneath its coding sequence. The AAATAA consensus sequence is indicated by an open box. The predicted glycosylation sites are highlighted in bold italics.

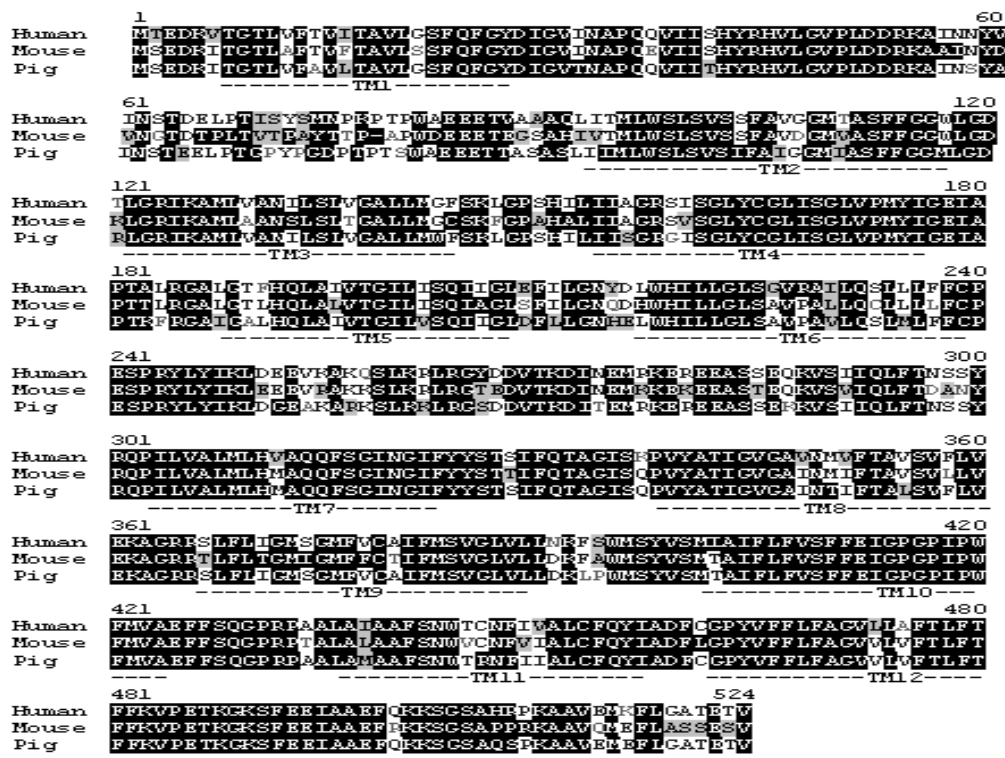


Figure 2. Multiple sequence alignment of the deduced amino acid sequence of porcine GLUT2 (GenBank accession no. EF140874) with the sequences of human (NM_000340) and mouse (NM_031197). Residues highlighted by black shading background represent absolutely conserved amino acids and residuals highlighted by grey shading indicates a single non-conserved residue at that position. Positions of predicted membrane-spanning helices (TM) are indicated by the numbered dashed lines at the bottom of the sequence alignment.

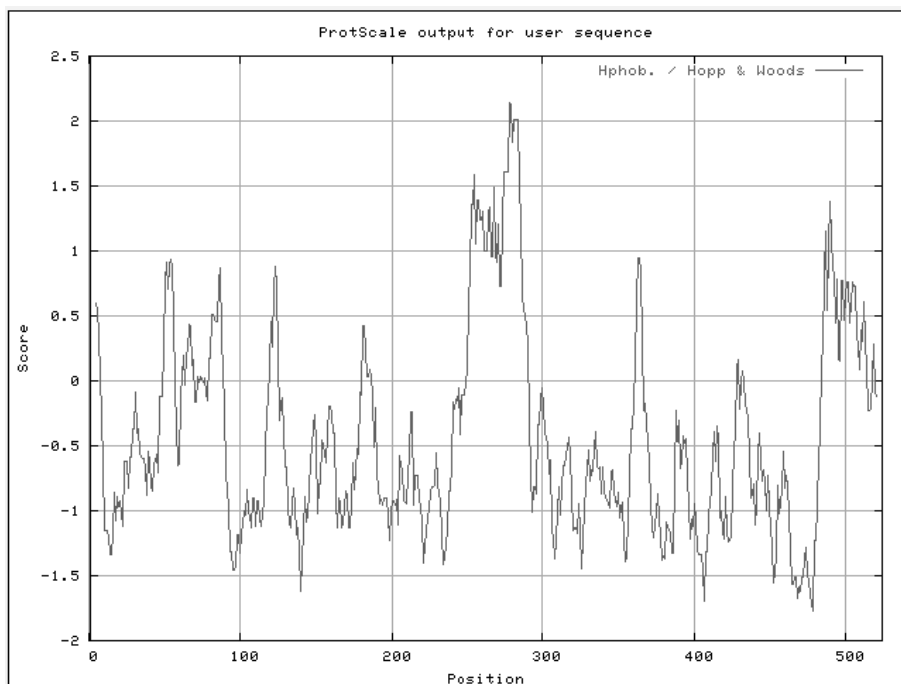


Figure 3. Hydropathy plot analysis of porcine GLUT2 by ProtScale.

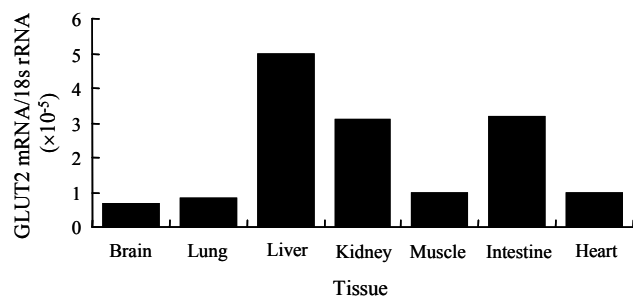


Figure 4. Q-RT-PCR analysis of tissue distribution of porcine GLUT2.

Our data demonstrated that GLUT2 was expressed in the liver, small intestine, brain and kidney, which is consistent with the expression pattern in human (Fukumoto et al., 1988). In previous studies, tissue distribution of GLUT2 has been examined in various mammals, but not in pigs. In liver, GLUT2 contributes to the bidirectional transport of glucose in and out of hepatocytes. In the kidney, GLUT2 is located in the basolateral membrane where it participates in the release of reabsorbed glucose (Wright et al., 2004). In the small intestine, GLUT2, located in the basolateral membrane, is cooperative with SGLT1 for glucose absorption. However, in recent years, GLUT2 expression has been confirmed at the enterocyte brush-border membrane (Affleck et al., 2003; Gouyon et al., 2003). Apical GLUT2 became a major pathway of intestinal sugar absorption and was especially highly expressed after meals. This finding leads to a new perspective for diabetes therapy (Kellett and Brot-Laroche, 2005). GLUT2 is responsible for transporting glucose out of the lumen of airway of the lungs. Its mRNA and protein were detected at the brush-border membrane of human bronchiolar epithelial H441 cells (Medina et al., 2006). With light and electron microscopic immunohistochemistry, rat GLUT2 was found expressed throughout the brain, especially in the limbic areas and related nuclei, and therefore, was hypothesized to be involved in cerebral glucose sensing (Arluison et al., 2004).

In this study, we report for the first time that porcine GLUT2 was expressed in the muscle and heart. Interestingly, SGLT1 mRNA was also detected in heart previously (Zhou et al., 2003). These results suggest that SGLT1 and GLUT2 may contribute to glucose transport in the cardiac muscle. A lot of previous studies have proved that the main glucose transporter in muscle from mammals was GLUT4 (Seki et al., 2006; Owens et al., 2007). However, recent data has illustrated that GLUT2 was also detected in the muscle from adult zebrafish, which was lower than that in testis, brain, skin, kidney, and intestine (Castillo et al., 2009). These results indicate that GLUT2 might also play an essential role in glucose transport in muscle.

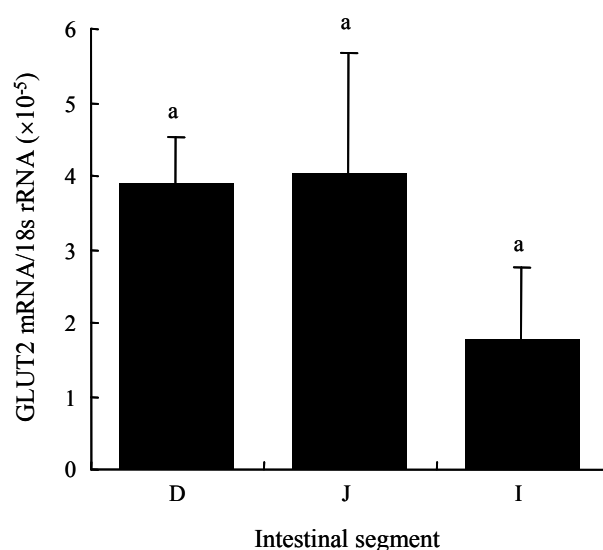


Figure 5. Relative mRNA expression of porcine GLUT2 mRNA along longitudinal axis of the intestine. All samples were normalized to 18S expression. Relative levels of GLUT2 mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method. Bars that share a common superscript do not differ ($p > 0.05$). Data are presented as the means \pm SE ($n = 5$). D: duodenum, j: jejunum, I: ileum.

Our detailed analysis of GLUT2 expression in the small intestine of pigs indicates that the jejunum has the greatest level. This is consistent with the observation made in broilers (Gilbert et al., 2007). In the small intestine of sucking newborn piglet, there was a proximal-to-distal gradient of GLUT2 expression accounting for a site-specific effect of glucose utilization (Cherbuy et al., 1997). In the present work, GLUT2 mRNA expression level in both duodenum and jejunum was relatively higher than that in the ileum, suggesting that glucose absorption mainly occurs in the fore- and mid-gut but not in hindgut of the small intestine.

In conclusion, the present study indicated that mRNA expression levels of GLUT2 were differential in different segments of the small intestine of piglets. It appears that substrate concentrations in the intestinal lumen due to different food sources and digestibility exert certain influences on GLUT2 mRNA expression levels in the small intestine. But this needs to be determined in future studies.

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