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Novel splice variants of the bovine *PCK1* gene

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ABSTRACT. *Phosphoenolpyruvate carboxykinase 1* (*PCK1*), also named *PEPCK-C*, is a multiple-function gene that is involved in gluconeogenesis, glyceroneogenesis, reproduction, female fertility, and development of obesity and diabetes. How its many functions are regulated was largely unknown. Therefore, we investigated mRNA expression and possible splice variants of *PCK1* by screening cDNA in nine tissues from Holstein bulls and cows. *PCK1* mRNA was highly expressed in the liver, kidney, ovary and testis; expression levels were low in the heart, spleen, and lung tissues. Expression of this gene was not detected in skeletal muscle. This led to the discovery of five novel bovine splice variants, named *PCK1-AS1*-*PCK1-AS5*. In *PCK1-AS1*, 51 nucleotides in the interior of exon 2 were spliced out. In *PCK1-AS2*, exons 2 and 3 were altered by the alternative 3' and 5' splice sites, respectively. *PCK1-AS3* was truncated from the 3' end of exon 2 to the 5' end of exon 4. In *PCK1-AS4*, exon 5 was completely spliced out. In *PCK1-AS5*, exons 5 and 6 and the 5' end of exon 7 were spliced out. These splice variants (*PCK1-AS1*-*PCK1-AS5*) potentially encoded

shorter proteins (605, 546, 373, 246 and 274 amino acids, respectively), when compared to the complete protein (622 amino acids). Considering the functional domains of the PCK1 protein, it is likely that these splice variants considerably affect the function of this protein; alternative splicing could be one of the mechanisms by which the diverse functions of *PCK1* are regulated.

Key words: Alternative splicing; *PEPCK* gene; Liver; Testis; Bull; Cow

INTRODUCTION

Phosphoenolpyruvate carboxykinase (PEPCK) is a rate-limiting enzyme of gluconeogenesis that catalyzes the formation of phosphoenolpyruvate from oxaloacetate in the liver and renal cortex. Therefore, the enzyme has been thought to be essential in glucose homeostasis. *PEPCK* is involved in glyceroneogenesis in liver and white and brown adipose tissue (Hanson and Patel, 1994). PEPCK is synthesized as two isoforms, cytosolic (PEPCK-C, also called PCK1) and mitochondrial (PEPCK-M, also called PCK2). The two isozymes are encoded by separate nuclear genes. All vertebrate species have both PEPCK isoforms but express them at different levels. For example, most mammals have similar activities of both *PCK1* and *PCK2* in their liver, while the rat and mouse have 90-95% *PCK1* and birds have 100% *PCK2* (Chakravarty and Hanson, 2007). There is accumulating evidence that *PCK1* is a multi-function gene that can play important roles in physiological processes and diseases, such as, glucose and triglyceride/fatty acid metabolism in the liver, kidney and adipose tissues, and obesity and diabetes (Chakravarty et al., 2005). Moreover, the *PEPCK* gene is also involved in aggressive behavior, feeding, extended longevity and reproductive capacity, which was evidenced by over-expressing *PCK1* in skeletal muscle in mice (Hakimi et al., 2007; Hanson and Hakimi, 2008). *PEPCK* of mycobacterium tuberculosis has been shown to trigger cell-mediated immune response by increasing the activity of cytokines, and *PEPCK* may be a promising new subunit vaccine candidate for tuberculosis (Liu et al., 2006). Interestingly, another study suggests that the role that *PEPCK* plays in gluconeogenesis may be mediated by the citric acid cycle, the activity of which was found to be directly related to *PEPCK* abundance. Mouse *PEPCK* levels alone were not found to be highly correlated with gluconeogenesis in the liver (Burgess et al., 2007). However, *PEPCK* is expressed not only in the liver, kidney and adipose tissues but also in other mammalian tissues, including the small intestine, colon, mammary gland, adrenal gland, lung and muscle, which is not associated with gluconeogenesis or glyceroneogenesis, a finding that is not explained by this understanding (Croniger et al., 2002). Therefore, the role of *PEPCK* may be more complex and may involve more factors than previously reported.

Alternative splicing (AS) is an important mechanism for regulating gene expression in cells (Maniatis and Tasic, 2002). The protein and mRNA isoforms produced by alternative processing of primary RNA transcripts may differ in function, structure, localization or other properties (Matlin et al., 2005). Alternative splicing has been proposed as a primary driver of the evolution of phenotypic complexity in mammals for it is known to affect more than half of all human genes (Johnson et al., 2003). About 21% of all bovine genes (21,755) are alternatively spliced (Chacko and Ranganathan, 2009).

Bovine *PCK1* gene is located on chromosome 13, contains 10 exons and 9 introns and encodes 622 amino acids (Agca et al., 2002). We predicted that the *PCK1* gene may exist some variants because multiexon genes have high probability of expression via alternative splicing. Although the mechanisms responsible for the tissue-specific and hormonal regulation of transcription of the gene for *PCK1* have been extensively studied previously, a better understanding of the regulation and function of bovine *PCK1* was sought by using specific primers to determine a detailed transcription profile in 9 different tissues. The potential splice variants and *PCK1* mRNA expression were investigated in various tissues of Chinese bulls and cows, to provide an explanation for the regulation of the tissue-dependent functions of the *PCK1* gene.

MATERIAL AND METHODS

Tissue collection and RNA isolation

Tissue samples (heart, liver, spleen, lung, kidney, skeletal muscle, ovary, testis and sperm) were collected from 10 Chinese Holstein cattle (8 bulls and 2 cows) at the slaughter house at Jinan, Shandong Province, China. Tissues were immediately frozen in liquid nitrogen and kept at -80°C. Total RNA was isolated from tissues using an RNAsimple Total RNA kit (Tiangen), following the manufacturer instructions. RNA concentrations were measured with a Biophotometer (Eppendorf), and RNA quality was monitored by visualization of ethidium bromide-stained bands in 1% agarose gels after electrophoresis. Samples were stored at -80°C before use.

RT-PCR analysis

First-strand cDNA was synthesized from 3-5 μg total RNA using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. A pair of specific primers (Table 1) were designed using PRIMER PREMIER 5.0 software and used to amplify the coding region spanning the 5'-untranslated and 3'-untranslated regions (UTR) of the bovine *PCK1* gene (GenBank accession No. NM_174737). For subsequent PCR amplification, 2 μL reverse-transcribed cDNA were combined with 10 μL Hi-Fidelity Taq buffer, 0.1 μL Hi-Fidelity Taq (Invitrogen, USA), 2.5 μM oligonucleotide mixture, 0.5 μM $MgSO_4$ and 2.5 μM dNTPs in a final volume of 25 μL. PCR was performed as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1.5 min; and 72°C for 10 min. DNA bands were separated by 1% agarose gel electrophoresis and eluted using the Gel Extraction kit (Biomiga, USA).

Table 1. Bovine *PCK1* and *β-actin* gene primers.

Name	Sequence (5'-3')	Length (bp)
PCK1 cDNA	F: CTGACCTGATCGTCCAAAGAG R: CTGGTGCGTTGTATGGATTG	1980
β-actin	F: GCACAATGAAGATCAAGATCATC R: CTAACAGTCCGCCTAGAAGCA	173

Clone sequencing and AS identification

The PCR products were subcloned into the pEASY-T3 cloning vector (TaKaRa, Dalian, China) immediately after purification, which was transformed into DH5α competent cells. Subsequently, the cells were propagated in LB medium overnight at 37°C. The plasmids were then purified using the Endo-free Plasmid Mini kit II (Omega, USA), and the cDNA inserts were sequenced with the ABI PRISMTM 3730 DNA Sequencer (Applied Biosystems) and Big Dye terminator v3.1 Sequencing kit (BGI, China). The splice variants were confirmed by the method described in a previous report (Huang et al., 2011).

Multiple sequence alignments were performed using DNAMAN v5.2.2 software (Lynon Biosoft) for the identification of possible splice variants. The protein secondary structure of identified splice variants was predicted using SWISS-MODEL (http://swissmodel.expasy.org/).

RESULTS AND DISCUSSION

Expression of *PCK1* mRNA in various tissues in cattle

RT-PCR results showed that bovine *PCK1* mRNA expression was tissue-specific (Figure 1). The *PCK1* gene was highly expressed in liver, kidney, ovary and testis tissues, while expression abundance was low in the heart, spleen, sperm and lung tissues. No PCK1 mRNA was detected in skeletal muscle. The high expression of *PCK1* in liver and kidney is consistent with the fact that *PCK1* is involved in gluconeogenesis and glyceroneogensis in liver and the control of acid/base balance in kidney in many mammalian species. There is recent evidence that *PCK1* is closely related to reproduction and female fertility in the mouse (Hanson and Hakimi, 2008). However, the role of *PCK1* in the ovary and testis is unclear, but it most likely participates in either gluconeogenesis or glyceroneogenesis or reproduction and fertility in the cow.

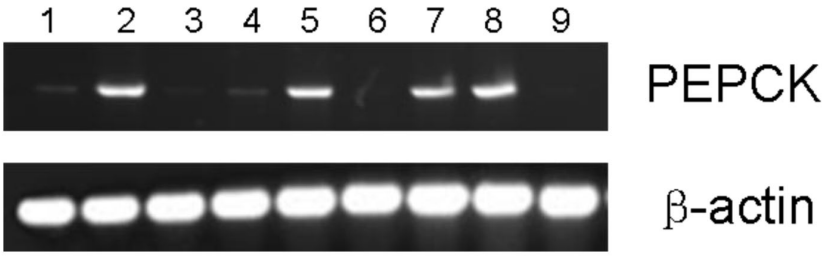

Figure 1. Expression pattern of PCK1 mRNA in various tissues in cattle. *Lane 1* = heart; *lane 2* = liver; *lane 3* = spleen; *lane 4* = lung; *lane 5* = kidney; *lane 6* = skeletal muscle; *lane 7* = ovary; *lane 8* = testis; *lane 9* = sperm.

Identification of the bovine *PEPCK* transcript variants

After the amplification of the full-length *PCK1* transcript with specific primers for *PCK1* mRNA reference sequence (Table 1) using cDNA from mammary tissues as templates, the PCR product of approximate 1.9 kbp in size was found in 7 tissues. It was purified, subcloned and sequenced. Comparing the sequence obtained from each sample with the bovine

PCK1 genomic reference sequence (GenBank accession No. AC_000170) and the *PCK1* mRNA reference sequence (GenBank accession No. NM_174737.2), five novel splice variants of the bovine *PCK1* gene, named *PCK1-AS1*-*PCK1-AS5* (sequences submitted to NCBI, GenBank accessions: JQ733516-JQ733520), were detected (Figure 2). The result showed that the expression of *PCK1* AS was tissue dependent (Table 2). The full-length transcript, designated as *PCK1*-complete, was found in 6 tissues (Figure 1 and Table 2).

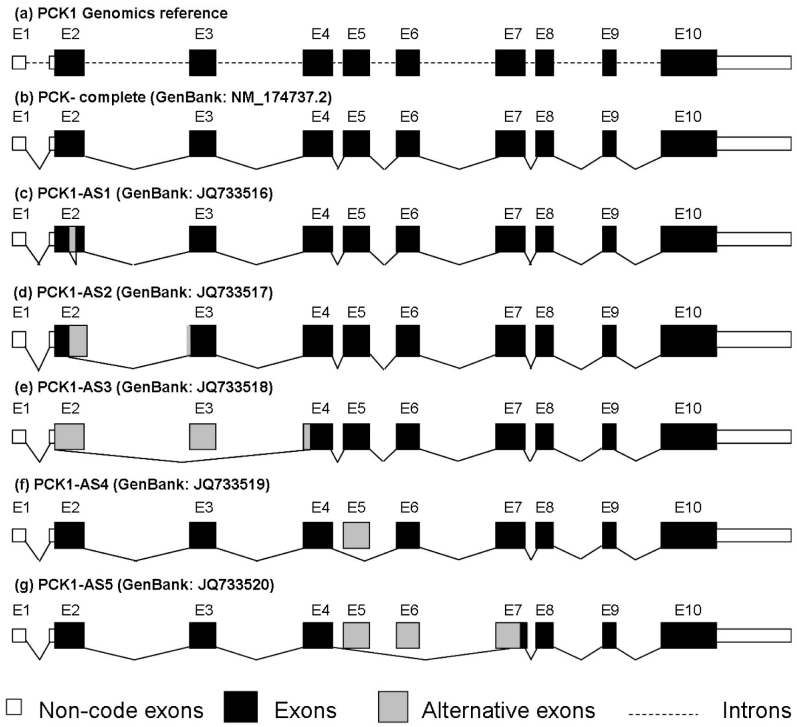

Figure 2. Genomic structure and alternative splicing patterns of the bovine *PCK1* gene.

Table 2. Splice variants of the *PCK1* gene in the tissues of bull and cow.

PCK1 splice variants	Length (bp)	Tissue	Putative protein (aa)
PCK1-complete	1980	Liver, kidney, testis, ovary, heart, lung	622
PCK1-AS1	1929	Liver	605
PCK1-AS2	1891	Liver	546
PCK1-AS3	1725	Liver	373
PCK1-AS4	1792	Testis	246
PCK1-AS5	1725	Liver	274

In comparing with *PCK1-complete*, *PCK1-AS1* had 51 bp deleted in exon 2 and encoded a putative protein with 605 amino acid (aa) residues. In *PCK1-AS2*, exon 2 and exon 3 of the PCK1 gene were altered by the alternative 3' splice site and alternative 5' splice site, respectively. In the *PCK1-AS2* transcript, a 90-bp sequence was spliced out, and a new initia-

tor codon (ATG) was generated, which was predicted to encode a protein with 546 aa (Figure 3). The *PCK1*-AS3 was truncated from the 3' end of exon 2 to the 5' end of exon 4 of the *PCK1-complete* gene. The *PCK1-AS2* had a total of 546 bp, where the normal initiator codon was spliced out. Therefore, this variant was predicted to encode a protein with 373 aa. Comparison of the predicted proteins encoded by the *PCK1-AS3* and *PCK1-complete* splice variants revealed that the putative PCK1-AS3 isoform lost the domain of the PEP carboxykinase N-terminal domain superfamily (Figures 4 and 5). The splicing pattern of *PCK1-AS4* belongs to exon skipping. The *PCK1-AS4* was spliced out in exon 5 of *PCK1-complete*, and the consequent amino acid sequence was changed with the early occurrence of the stop codon. The variant transcript was translated into a 246-aa protein which is apart of the PCK1-complete protein. The variant transcript of *PCK1-AS5* was only translated into a 274-amino acid attributed to the splicing of exon 5, exon 6 and 5' end of exon 7.

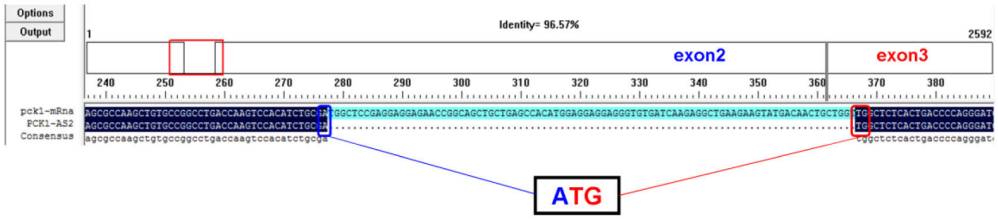

Figure 3. Alternative splicing pattern of the bovine *PCK1-AS2*. In the *PCK1-AS2*, the exon2 and exon3 of the bovine *PCK1-AS2* were altered by the alternative 3' splice site (A3SS) and alternative 5' splice site (A5SS), respectively. The last oligonucleotide of exon2 C-terminal was A and the N-terminal of exon3, just altered 5 oligonucleotides, starting with TG. Therefore, a new initiator codon (ATG) was generated.

Many genes encode a far greater number of proteins in all multicellular organisms by alternative splicing (Black, 2003). They can be expressed in a tissue-specific and temporally regulated manner under the control of pre-mRNA-binding proteins (Jelen et al., 2007). The mRNA and protein isoforms produced by alternative processing of primary RNA transcripts may differ in structure, function, localization or other properties (Matlin et al., 2005).

As shown in Figure 4, the protein three-dimensional structures and domains of identified splice variants were predicted using SWISS-MODEL (http://swissmodel.expasy.org/). Alternative splicing likely has a large impact on the function of the *PCK1* gene. The expression of *PCK1-AS4* mRNA is testis-specific, and it encodes a 246-aa protein, which is different from the other isoform structures of the bovine *PCK1* gene through the alteration of the reading frame. Another four *PCK1* splice variants and *PCK-complete* were expressed in the liver. Alternative splicing is very frequent in the testis, which is the site of an extensive adult developmental program (Elliott and Grellscheid, 2006). Why is alternative splicing relatively abundant in the testis and liver? A relatively unique feature of the testis is that it is the site of an extensive adult developmental process. However, other factors may also be important in driving the extent of alternative splicing in the testis, since alternative splicing is also particularly high in the adult liver, which is terminally differentiated and not particularly complex in cell type content (Yeo et al., 2004). The present study demonstrates various bovine *PCK1* splice variants existing in liver and testis tissues, which can explain in part the multiple functions of the *PCK1* gene.

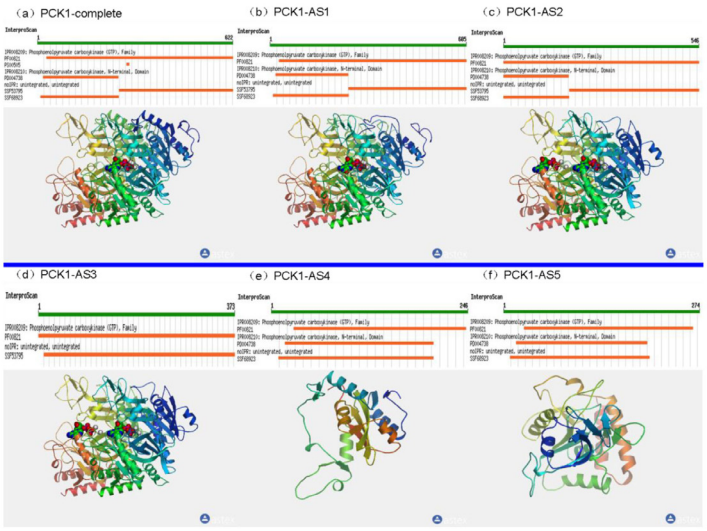

Figure 4. Putative protein structures and functional domains of the bovine *PCK1* splice variants identified. The transcript of PCK1-complete contains 5 domains: PR008209, phosphoenolpyruvate carboxykinase (GTP; PF00821: 29-622 PEPCK); IPR008209, phosphoenolpyruvate carboxykinase (GTP; PS00505: 284-292 PEPCK_GTP); IPR008210, phosphoenolpyruvate carboxykinase, N-terminal (PD004738: 18-258 PEPCK_N); noIPR: unintegrated, unintegrated (SSF53795: 260-622 SSF53795); noIPR: unintegrated, unintegrated (SSF68923: 10-259 SSF68923). The domain of PCPCK_N and SSF68923 in the PCK1-AS3 transcript was completely spliced out and the domain of PEPCK was partly spliced out. In PCK1-AS4 and PCK1-AS5, the domain of SSF53795 was completely lost and the domain of PEPCK was partly spliced out.

```
#MEGA
!Title PEPCK;
!Format
   DataType=Protein
   NSeqs=6 NSites=622
   Identical=. Missing=? Indel=-;

!Domain=Data;
#PEPCK-complete MPPQLSDGLN YSAKIVRGSL DSLPQAVRSF VESSAKLCRP DQVHICDGSE EENRQLLSHM EEEGVIKRLK KYDMCWLALT  80
#PEPCK-AS1      .......... .......... .......... .......... ....                    ........ ..........  63
#PEPCK-AS2                                                                           ........ M...       4
#PEPCK-AS3
#PEPCK-AS4      .......... .......... .......... .......... .......... .......... .......... ..........  80
#PEPCK-AS5      .......... .......... .......... .......... .......... .......... .......... ..........  80

#PEPCK-complete DPRDVARIES KTVIITREQR DTVPIPKNGL SQLGRWMSEE DFEKAFNIRF PGCMKGRTMY VIPFSMGPLG SPLSKIGIEL 160
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... .......... ..........  143
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... .......... ..........  84
#PEPCK-AS3
#PEPCK-AS4      .......... .......... .......... .......... .......... .......... .......... ..........  160
#PEPCK-AS5      .......... .......... .......... .......... .......... .......... .......... ..........  160

#PEPCK-complete TDSPTYVTSH KIMTRMGTSV LEALGDGEFV KCLHSVGCPL PLKKPLVNNW ACNPELTLIA HLPDRKEIIS PGSGYGGNSL 240
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... .......... ..........  223
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... .......... ..........  164
#PEPCK-AS3
#PEPCK-AS4      .......... .......... .......... .......... ...NSGHHQP QGPE.VLRGC VSQRLW.DQP GHDEPYSPGM 240
#PEPCK-AS5      .......... .......... .......... .......... .RARSGTPRM GSLVPTPTHG SALQPASAP. LTLTGSLQRA 240

#PEPCK-complete LGKKCFALRM ASRLAKEBGW LAEHMLILGI TNPKGQKKYF AAAFPSACGK TNLAMMNPTL PGWKVECVGD DIAWMKFDQQ 320
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... .......... ..........  303
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... .......... ..........  244
#PEPCK-AS3      .......... .......... .......... .......... .......... .......... .......... ..........  71
#PEPCK-AS4      ESKV.G---- ---------- ---------- ---------- ---------- ---------- ---------- ----------  246
#PEPCK-AS5      CPLRASSSBG .GL.VSLWST RLSAGSTVCL WGRP------ ---------- ---------- ---------- ----------  274

#PEPCK-complete GNLRAINPEN GFPGVAPGTS VRTNPNAIKT IQKNTIFTNV AETSDGGVYW EGIDQPLAAR VKLISWKGKE WDPKDGEPCA 400
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... .......... ..........  383
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... .......... ..........  324
#PEPCK-AS3      .......... .......... .......... .......... .......... .......... .......... ..........  151
#PEPCK-AS4      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  246
#PEPCK-AS5      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  274

#PEPCK-complete HPNSRPCTPA SQCPIIDPDW ESPEGVPIEG IIPGGRRPVG VPLVYEALSW QHGVFVGAAM RSEATAAAEY KGKVIMHDPF 480
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... .......... ..........  463
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... .......... ..........  404
#PEPCK-AS3      .......... .......... .......... .......... .......... .......... .......... ..........  231
#PEPCK-AS4      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  246
#PEPCK-AS5      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  274

#PEPCK-complete AMGPFPGYNF GQYLAHWLSM AQRPAAKLPK IFHVNWFRKD KAGKFLWPGF GENSKVLEWM FNRVVGEGGA SVTPIGYIPD 560
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... .......... ..........  543
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... .......... ..........  484
#PEPCK-AS3      .......... .......... .......... .......... .......... .......... .......... ..........  311
#PEPCK-AS4      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  246
#PEPCK-AS5      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  274

#PEPCK-complete EDALDLRGLG DVDVKELPHI SKEFWEEEVE EIQKYLEEQV NVDLPPEIKN QVLALKQRIS QM                    622
#PEPCK-AS1      .......... .......... .......... .......... .......... .......... ..                    605
#PEPCK-AS2      .......... .......... .......... .......... .......... .......... ..                    546
#PEPCK-AS3      .......... .......... .......... .......... .......... .......... ..                    373
#PEPCK-AS4      ---------- ---------- ---------- ---------- ---------- ---------- --                    246
#PEPCK-AS5      ---------- ---------- ---------- ---------- ---------- ---------- --                    274
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Figure 5. Multiple alignments of the predicted amino acid sequences of the bovine PCK1 transcript variants. (.) = same amino acid when PCK1 transcript as compared to the PCK1-complete; (-) = end of translation.

In conclusion, our findings contribute to a better understanding of the complex bovine *PCK1* gene. Considering the functional domains of the PCK1 protein, it is very likely that these splice variants affect the function of the protein considerably and that alternative splicing is one of the mechanisms by which the diverse functions of the bovine *PCK1* gene are regulated.

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