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Full Length Research Paper

Tissue-specific alternative splicing and expression of ATP1B2 gene

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The Na^+-K^+-ATPase is an essential transport enzyme expressed in all animal tissues, where it generates ion gradients to maintain membrane potential and drive the transport of other solutes. It also balances metabolism and body temperature. In this study, the characterization of three novel bovine ATP1B2 splice variants, designated as ATP1B2-AS1, ATP1B2-AS2, and ATP1B2-AS3, is discussed. All three novel splice isoforms were derived from a complete transcript (ATP1B2-complete) by alternative splicing. The pattern of splicing to produce the ATP1B2-AS1 and ATP1B2-AS2 isoforms was intron retention; these isoforms were found in liver, kidney, muscle and breast tissues. For the ATP1B2-AS3 isoform, splicing was by exon inclusion and this isoform was only found in muscle tissue. As demonstrated by real-time polymerase chain reaction, the isoforms were all expressed at significantly lower levels than the complete ATP1B2 gene transcript in all the tissues studied. After heat-stress, the expression levels of the different transcripts were lower in different tissues; however, the expression of the ATP1B2-complete transcript increased in heart and lung tissues. The results of this research provide some useful information for further studies into the function of the bovine ATP1B2 gene. Alternative splicing (AS) is recognized as the major contributor to protein diversity from limited gene pool. ATP1B2-AS2 was the splice of intron retention found from ATP1B2 in liver, kidney, muscle and breast tissues. In the study, ATP1B2-AS2 showed that many of the amino acid residues were in an unfavorable energy environment. It is interesting to speculate that this may be the perfect transcript to respond to heat-stress. So, AS may become the appropriate pathway to tackle heat-stress and reduce the economic losses in cows.

Key words: ATP1B2 gene, alternative splicing, alternative splicing mechanism.

INTRODUCTION

The impact of heat stress on dairy

Genetic progress in milk production is closely related to an increased feed intake. However, overfeeding results in raised metabolic heat increment which requires effective thermoregulatory mechanisms to maintain body temperature in the thermoneutral zone and in physiological homeostasis. Heat stress is a major contributing factor to the low fertility of dairy cows (Ray et al., 1992; Thompson et al., 1996; al-Katanani et al., 1999; Ingraham et al., 1974).The accurate measurement of when cows enter heat stress is complicated because the responses to heat stress affect not only the energy balance, but also water, sodium, potassium and chlorine metabolism. The metabolism of an animal is always in a state of dynamic equilibrium in which the influx of nutrients is balanced by the production of energy in catabolic and anabolic processes. Cows require nutrients for among other things, maintenance of biological processes, reproduction, and lactation. The separation of metabolism into maintenance and production is somewhat artificial because energy metabolism is affected by complex interrelationships among all physiological processes (Hayssen and Lacy, 1985). How to help the cow avoid physiological dysfunction and for it to better fit its

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environment are very important, not only for the comfort of the cow, but also for the benefit of the dairy industry.

Concept and structure of Na^+-K^+-ATPase

The Na^+-K^+-ATPase is a transmembrane carrier protein widespread in all eukaryotic cell membranes where it generates ion gradients to maintain membrane potential and drive transport of other solutes. Na^+-K^+-ATPase also keeps metabolism and body temperature balanced (Barcroft et al., 2002). Vague et al. (2004) reported that Na^+-K^+-ATPase regulates the balance of Na^+ and K^+, and that low Na^+-K^+-ATPase activity impaired hydronium transport leading to disturbances in energy and metabolism. In addition to transport of ions, the Na^+-K^+-ATPase is important in signal transduction (Xie, 2003; Tian and Xie, 2008), intercellular adhesion and cell migration (Gloor et al., 1990; Rajasekaran et al., 2007; Vagin et al., 2006, 2008; Geering, 2008). The minimal functional unit of the Na^+-K^+-ATPase consists of a catalytic α subunit and an N-glycosylated β subunit that is required for maturation and membrane targeting of the enzyme. There are four isoforms of the Na^+-K^+-ATPase α subunit (α_1, α_2, α_3 and α_4) and three isoforms of the Na^+-K^+-ATPase β subunit (β_1, β_2, and β_3) (Blanco and Mercer, 1998; Crambert et al., 2000).

The β_2 subunit gene [ATP1B2; has six introns and seven exons, the messenger ribonucleic acid (mRNA) total length is 933 bp and is located in 19 chromosome in bovine) was explored in Chinese Holstein cows. It has eight potential N-glycosylation sides; the removal of one or more of the N-glycosylation sites or the prevention of glycan-calnexin interactions prevents α-assembly and export of the β_2 subunit (Tokhtaeva et al., 2010). However, which of the N-glycosylation sites could undergo change and still maintain the functional structure of the Na^+-K^+-ATPase (NKA) so that it could play a role in the stress response has not yet clearly been established. In the process of heat stress, the ATP1B2 can maintain the stability of NKA (Melissa et al., 2007) and inhibit decrease of NKA activity (Wang et al., 2009), and so can maintain constant body salt, water balance and body temperature.

Mechanisms and the role of splice

Splicing is the process by which the introns are excised and the exons ligated to form a translatable message (Sammeth et al., 2008). Alternative splicing (AS) is now recognized as the major contributor to protein diversity because of the important role it plays in generating diversity and complexity from the limited gene pool (Cáceres and Kornblihtt, 2002). Current estimates suggest that at least 60% of genes can produce two or more mRNAs by AS. The potential for generating diversity is demonstrated by the more than 500 protein isoforms. It is now estimated that 95% of human genes are split genes, with coding exons split by noncoding introns (Chacko and Ranganathan, 2009), and that 21% of 21,755 bovine genes are alternatively spliced (Ray et al., 1992). There are numerous examples that splicing and its regulation play key roles in the control of cell growth, differentiation, and disease (Zhou et al., 2002; Will and Luhrmann, 2011; Tazi et al., 2009). AS can alter the encoded protein and disrupt its structure and function as a result. Such disruptions in the protein structure are frequently associated with diseases (Arnold, 2006). Thus, the regulation of AS provides an essential control step in post-transcriptional gene expression. The endocrine system is likely to be one of the important systems that direct AS in a tissue and cell-specific manner. Here, we describe the current understanding of the AS and the split gene mechanisms in the ATP1B gene in different tissues with particular reference to heat-stress in Chinese Holstein cattle.

MATERIALS AND METHODS

Tissues

Heart, liver, spleen, lung, kidney, muscle and breast tissues were collected from ten Chinese Holstein cattles that formed two groups, normal (n=5) and heat-stressed (T=33.5°C, THI=85.7) (n=5) (Wang et al., 2009). The tissues were snap-frozen in liquid nitrogen in the slaughterhouse and transported to our laboratory.

Identification of the splice variants of the bovine ATP1B2 gene

A pair of specific primers was designed using the Primer 5.0 software to amplify the bovine ATP1B2 gene (GenBank: NM-174677.2) spanning most of the length of the mRNA sequence (46 to 933 bp). The primers and respective product sizes are shown in Table1. Total ribonucleic acid (RNA) was extracted from the seven tissues using TRIzol reagent (Bioteke, Beijing, China) according to the manufacturer's instructions. Samples were treated with RNase-free DNase (Promega) to remove contaminating genomic deoxyribonucleic acid (DNA) from the RNA preparations. RNA concentrations were measured with a Biophotometer (Eppendorf), and RNA quality was monitored by visualization of ethidium bromide-stained bands in agarose gels after electrophoresis. Samples were stored at -70°C. Next, complementary deoxyribonucleic acid (cDNA) was synthesized with the PrimeScript RT Master Mix first-strand cDNA synthesis kit (TaKaRa, Dalian, China).

The PCR was performed as follows: 95°C for 5 min; 95°C for 45 s, 60°C for 30s , 72°C for 45 s (30 cycles), and 72°C for 8 min. DNA bands were separated by 1% agarose gel electrophoresis and eluted using gel extraction kit (Biomiga, USA). Immediately after purification, the PCR products were subcloned into the pEASY-T3 cloning vector (TaKaRa, Dalian, China), which was transformed into DH5α competent cells. The cells were subsequently propagated in Luria Bertani (LB) medium overnight at 37°C. The plasmids were then purified using a Plasmid Miniprep kit (Biomiga, USA) and the cDNA inserts were sequenced by the ABI PRISMTM 3730 DNA Sequencer (Applied Biosystems) and BigDye terminator v3.1 sequencing kit (Shanghai Sangon, China). Multiple sequence alignments were performed using DNAStar software for the identification of possible splice variants.

Table 1. List of primer sets used in this work.

Primer	Primer sequence (5′-3′)		Target	Fragment size (bp)
ATPIB2-cDNA	F:GGTGGTTGAGGAGTGGAAGGAG	R:GACATTCCAGGAGCATCCACAG	Clone	888
B-actin gene	F:GCACAATGAAGATCAAGATCATC	R:CTAAVAGTCCGCCTAGAAGCA	qRT-PCR	173
ATPIB2-complete	F:CCGTCAAGATGGTCATTC	R:ATCAAGCCTGGTGTAGCC	qRT-PCR	256
ATPIB2-ASI	F:GAGCAGGGTGCGGGTTGGA	R:AGAGTTGGGGCGGAGGAAGG	qRT-PCR	258
ATPIB2-AS2	F:CAACTTCGTTATGTTCCCTG	R:ACTTGTCTCGTCATCGTC	qRT-PCR	226
ATPIB2-AS3	F:GGCATCCAACTTCTTTCC	R:TGTAGCCAATCGGTCCTG	qRT-PCR	235

Bioinformatics and sequence analysis

The prediction of AS and transcript diversity and mRNA expression of ATP1B2 (GenBank: NM-174677.2) was carried out using ASTD (http://www.ebi.ac.uk/astd/main.html). Sequence analysis was performed with DNAStar software and National Center for Biotechnology Information -Basic Local Alignment Search Tool (NCBI BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.html) .The mRNA was translated to the amino acid sequence using the translate tools on the ExPASY Proteomics Server (Serverhttp://www.expasy.ch/tools/dna.html). The tertiary structure of ATP1B2 protein was predicted with SWISS-MODEL/Template Identification (http://swissmodel.expasy.org/workspace/index.html) (Kiefer et al., 2009; Larionov et al., 2007).

Quantification of ATP1B2 transcript variants

Quantitative real-time PCR analysis was performed on a 20 µl mixture containing 50 ng cDNA, 0.4 µM of sense and antisense primers, 6.8 µl dH2O, 10.0 µl SYBR® Premix Ex Taq™ (2×), and 0.4 µl ROX Reference Dye (50×) (TaKaRa, Dalian, China). The reaction mixture was denatured for 30 s at 95°C and incubated for 40 cycles (denaturing for 5 s at 95°C, annealing for 31 s at 61°C). The primers used in the experiment are shown in Table 1. The PCR was monitored using an ABI PRISM 7000HT Fast Real-Time PCR system. For each alternatively spliced variant studied, a specific standard curve was created using a series of dilutions from the corresponding plasmids. All samples were analyzed within the same real-time PCR run, thereby eliminating the need for a calibrator sample. In addition to melting curve reading, product identity was confirmed by 1% agarose gel electrophoresis. Alternatively spliced transcription values were normalized against the content of the housekeeping gene β-actin (GenBank: NM_173979.3) in each sample (Table 1). Relative quantification of ATP1B2 gene expression was done using the standard curve method for relative real-time PCR (Crick, 1979).

RESULTS AND DISCUSSION

Identification of the bovine ATP1B2 transcript variants

The 888 bp ATP1B2 cDNA was amplified from all the seven tissues. The cDNAs were purified, cloned, and sequenced. ASTD showed only one type of AS for the cattle ATP1B2 gene (GenBank.NM-174677.2) (Figure 1A). However, for the Chinese Holstein cattle ATP1B2 gene cDNA, the DNAStar predicted possible four transcripts. Presence of the full length transcript designated ATP1B2-complete (888 bp) (Figure 2A), was discovered and shown in all the seven tissues. In addition, three novel transcripts of the ATP1B2 gene were also identified. Two of the splice transcripts, ATP1B2-AS1 (995 bp, insert 107 bp at 719 bp, intron retention) (Figure 2B) and ATP1B2-AS2 (920 bp, insert 32 bp at 719 bp, intron retention) (Figure 2C), discovered in liver, kidney, muscle and breast, were the products of intron retention AS. The third splice transcript ATP1B2-AS3 (937 bp, insert 49 bp at 45 bp, exon inclusion) (Figure 2D), is the product of exon inclusion and was discovered only in muscle tissue. The most primitive form of AS is intron retention; however, the most commonly described form of AS is exon skipping/inclusion (Tokhtaeva et al., 2010). Several other mechanisms exist, including the use of alternative 3' and 5' splice sites, mutually exclusive exons, alternative promoter usage, and alternative poly-adenylation (Graveley, 2011). We found that two AS mechanisms occurred in the bovine ATP1B2 gene. This is commonly observed because many genes in physiological systems have been reported to undergo a combination of different alternative splicing events, employing various mechanisms.

Protein structure predictions of the ATP1B2 splice transcripts

The cloned sequences of the four transcript variants were translated to give the amino acid sequence in Figure 3, such as ATP1B2-complete (281aa), ATP1B2-AS1 (326aa), ATP1B2-AS2 (302aa) and ATP1B2-AS3 (310aa). The InterPro database at EMBL-EBI showed that ATP1B2-complete, ATP1B2-AS1 and ATP1B2-AS2 proteins belonged to the Na^+-K^+-ATPase family; the ATP1B2-AS3 protein is not in the database indicating that it is an unknown protein. The ANOLEA program was used to calculate the energy environment of each of the amino acid residues in the protein chains of three of the proteins; ATP1B2-complete (Figure 4A), ATP1B2-AS1 (Figure 4B) and ATP1B2-AS2 (Figure 4C). The three proteins were found to have perfect non-local environments (NLEs) for most of amino acids in the protein chain; however, some of the residues in ATP1B2-

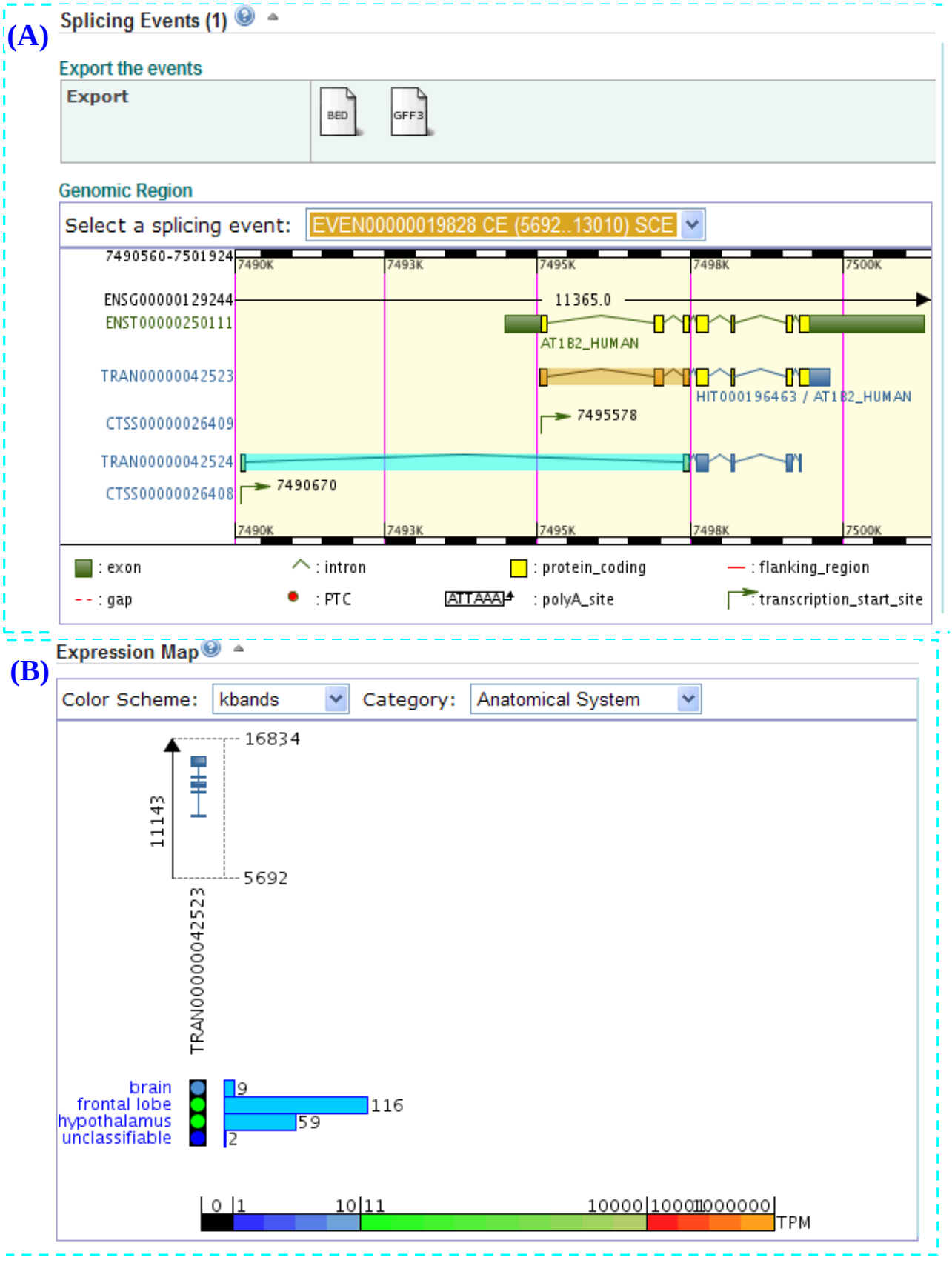

Figure 1. Splice variants and expression of the ATP1B2 gene in the ASTD database; A**,** splice variants of the human ATP1B2 gene; B, expression of the human ATP1B2 gene in various tissues.

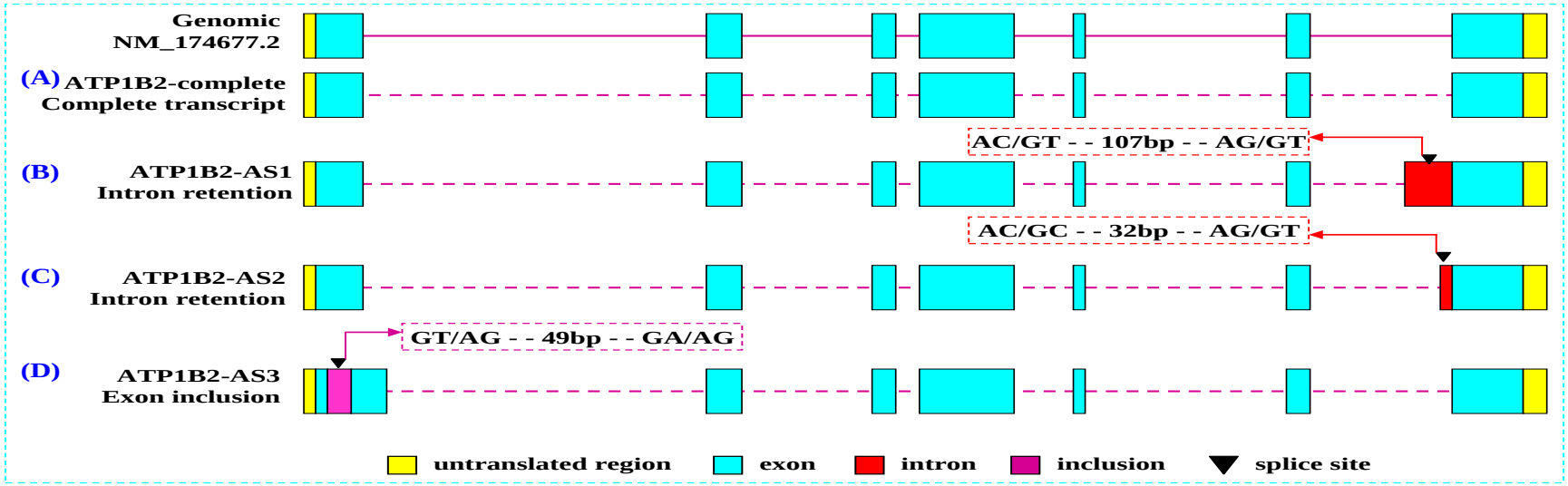

Figure 2. Genomic structure and alternative splicing patterns of the bovine ATP1B2 gene; A, the full length transcript (888 bp); B, splice transcript ATP1B2-AS1 (995 bp); C, splice transcript ATP1B2-AS2 (920bp); D, splice transcript ATP1B2-AS3 (937 bp).

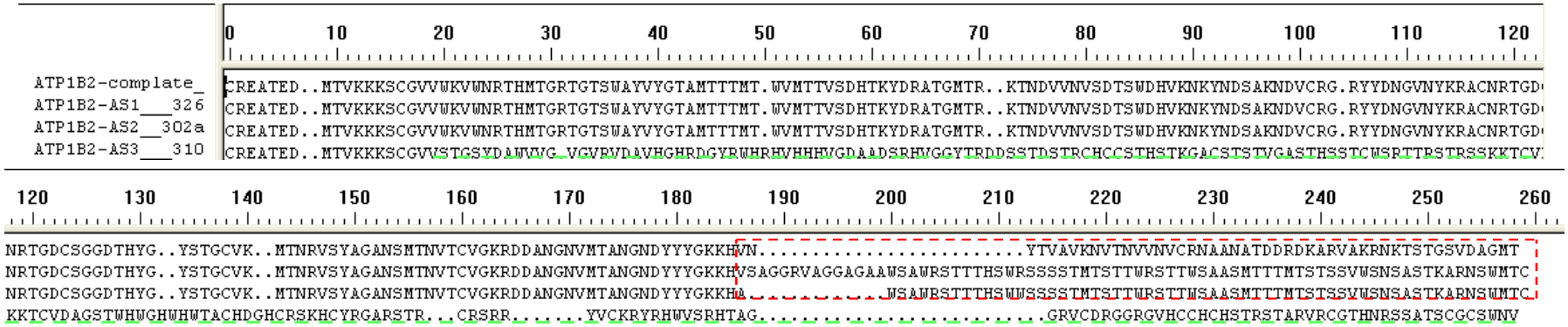

Figure 3. The different splice transcript amino acid sequence of bovine ATP1B2 gene.

AS2 had low negative energy indicating an unfavorable energy environment for those amino acids.

The 3D structure of ATP1B2-complete (Figure 5A), ATP1B2-AS1 (Figure 5B) and ATP1B2-AS2 (Figure 5C) were predicted using SWISS-MODEL (Figure 5). Although, the ATP1B2-AS1 and ATP1B2-AS2 proteins are very similar to ATP1B2-complete, differences exist in the secondary

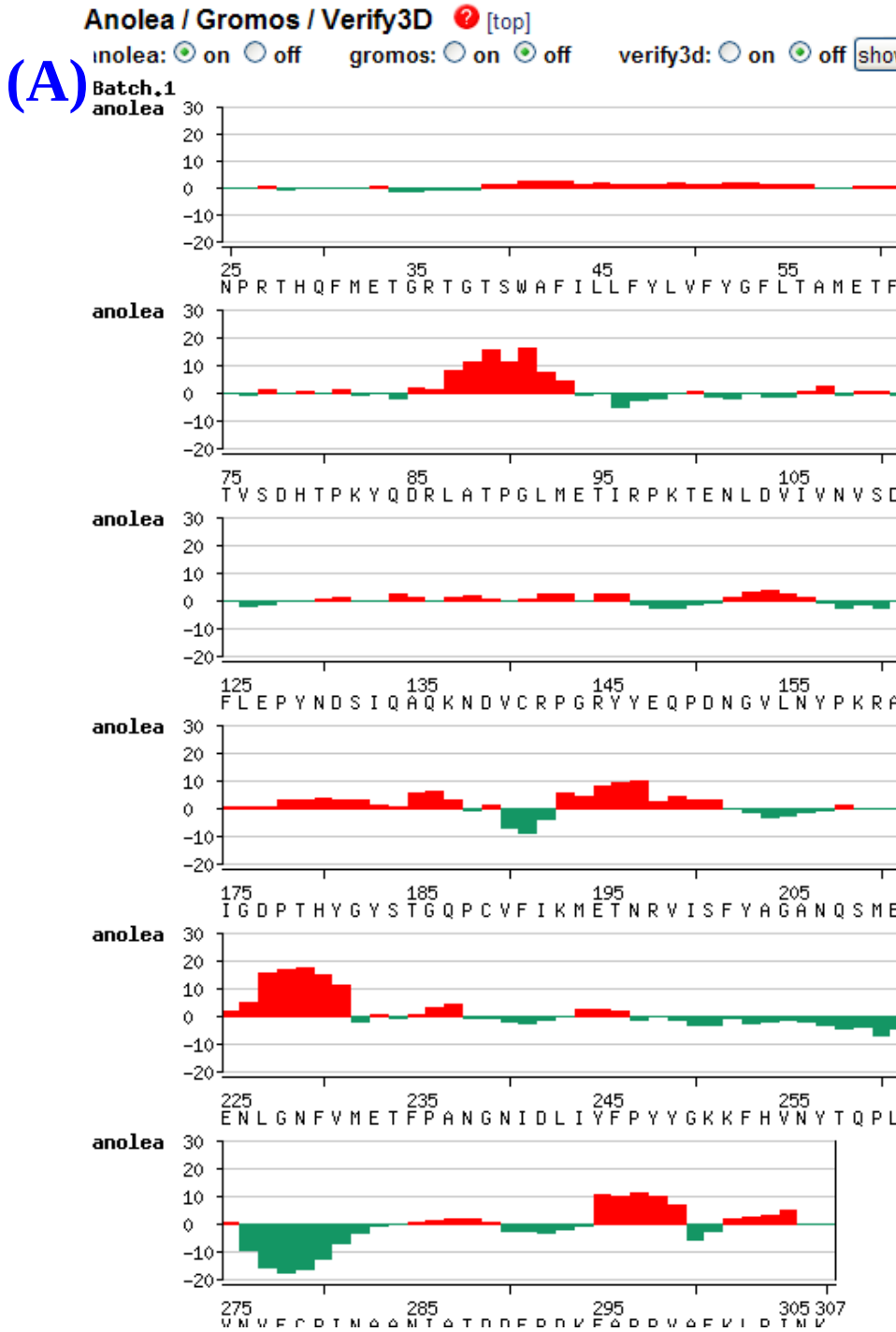

Figure 4. Energy values for the amino acid residues in the ATP1B2 protein chains calculated using the ANOLEA program; A, ATP1B2-complete; B, ATP1B2-AS1; **C** ATP1B2-AS2. Negative energy values (green) indicate a favorable environment; positive energy values (red) indicate an unfavorable environment.

structure of the folds and coiled coil, and in the combine power of amino acids. AS is now recognized as the major contributor to protein diversity (Tarn and Steitz, 1997) in the process of generating diversity from a limited number of genes, by which the introns are excised and the exons ligated to form a translatable message (Hastings and Krainer, 2001; Hertel and Graveley, 2005; Staley and Guthrie, 1998; Keren et al., 2010). Like in this study, the four transcript variants were translated from the same gene, but the different genes conding for hormones, their

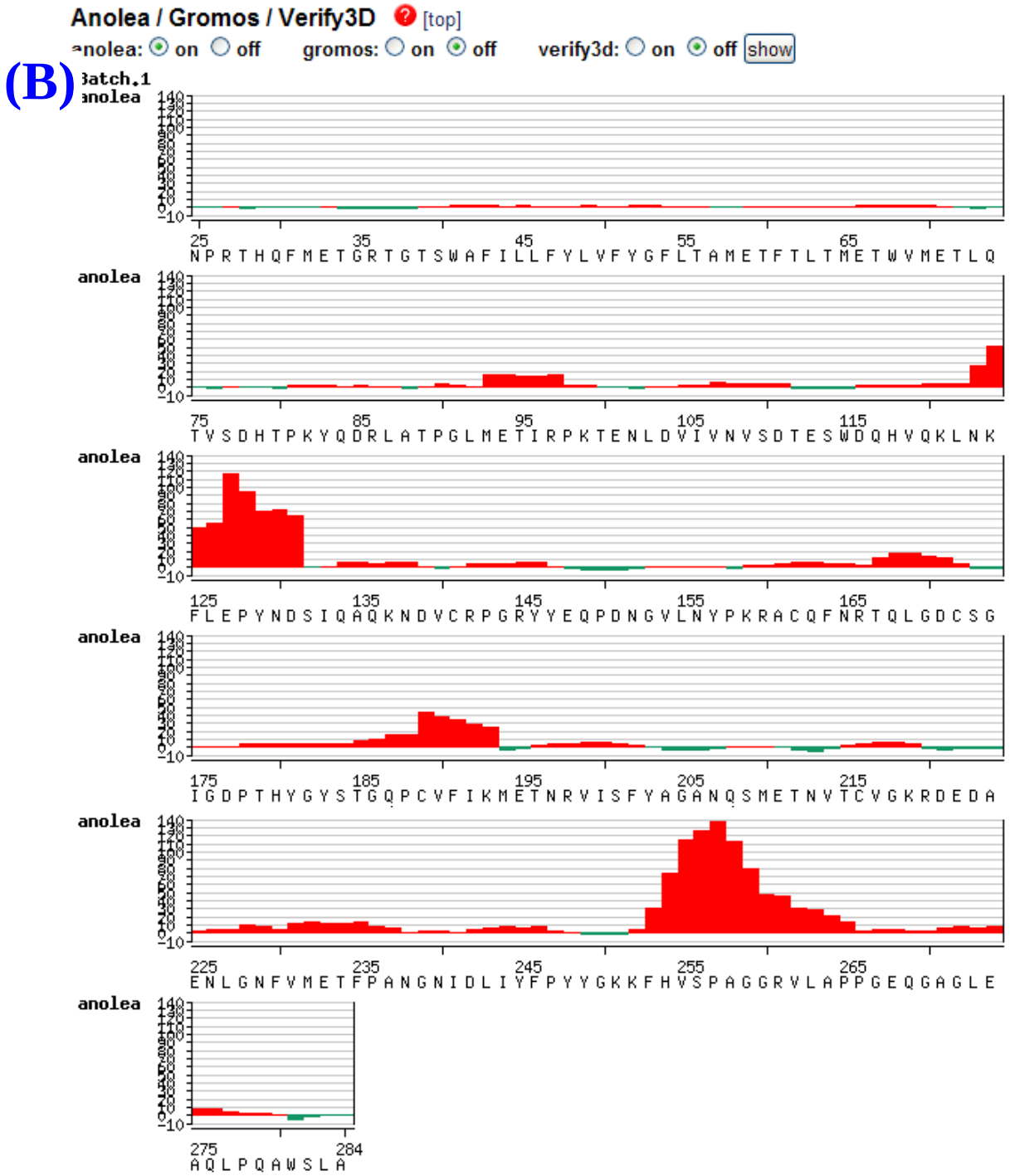

Figure 4. Contd.

binding proteins, and their receptors are particularly well represented (Tange et al., 2004; Cartegni et al., 2006).

Expression of ATP1B2 splice transcript in the normal and heat-stress tissues

The ASTD analysis showed that the ATP1B2 gene was expressed in the frontal lobe, hypothalamus and unclassifiable areas of the brain (Figure 1B). In this study, the four bovine transcripts were effectively amplified by specific primers that spanned the region of AS. The relative quantification of the ATP1B2-complete, ATP1B2-AS1, ATP1B2-AS2 and ATP1B2-AS3 transcripts in different tissues from the normal and heat-stressed cows, was carried out by real-time PCR amplification. ATP1B2-

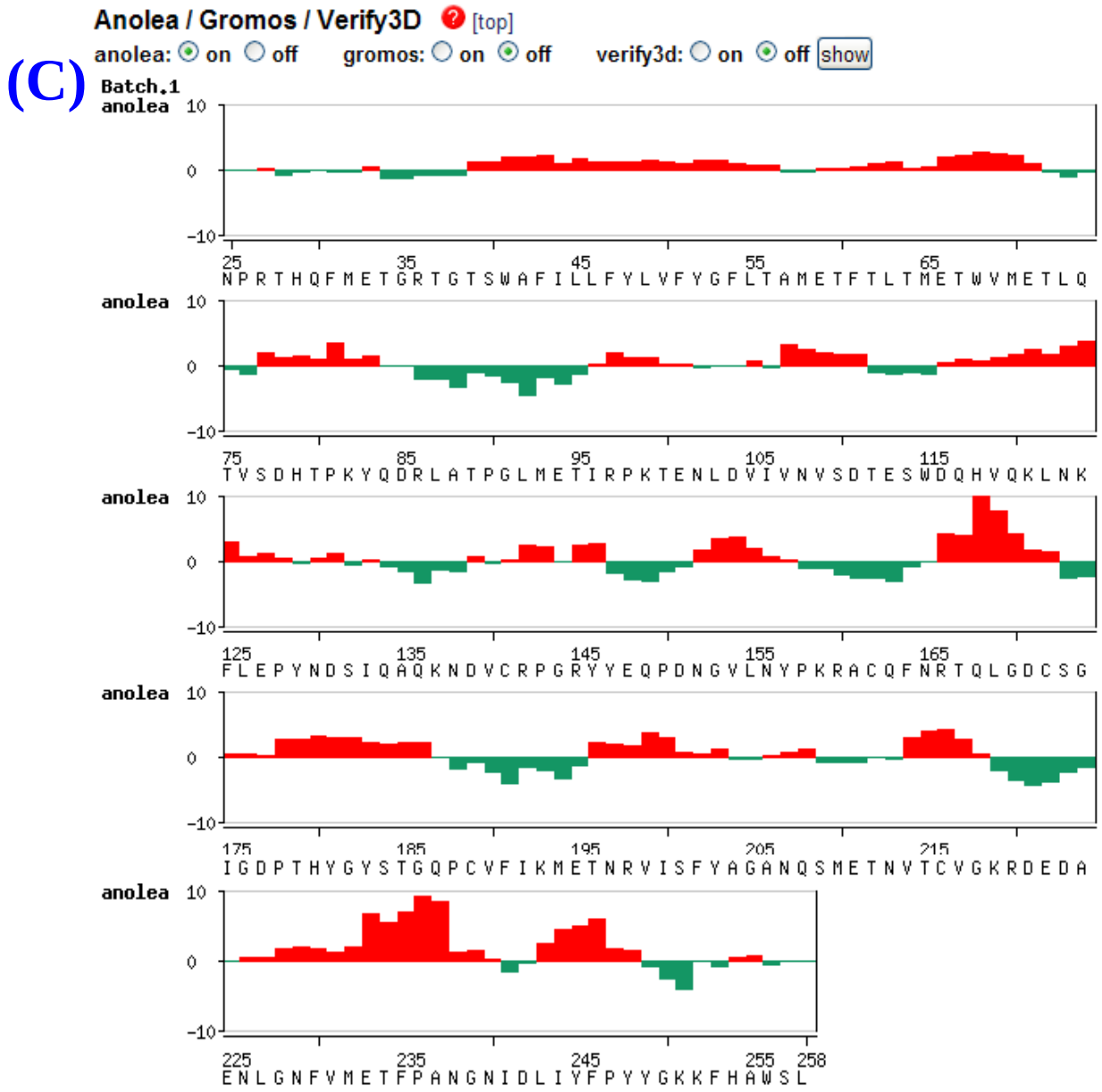

Figure 4. Contd.

complete was expressed in all the seven tissue types; the ATP1B2-AS1 and ATP1B2-AS2 were expressed mainly in liver, kidney and breast tissues, and the ATP1B2-AS3 was expressed only in the muscle tissue (Figure 6A). In the heat-stress, the expression of all the transcripts was reduced in all tissue types; an exception was for ATP1B2-complete expression which was increased in the heart and lung tissues (Figure 6B).

A great deal of ATP is expended in the process of AS. In this study, four kinds of splice transcripts were found to be present together in the seven tissues that we studied whether they were under heat stress or not. Heat stress reduces feed intake in lactating dairy cows. It has been shown that increased dietary sulfur can raise dry matter digestibility and provide sufficient sulfur for the microbial synthesis of the otherwise milk production-limiting sulfur-containing amino acids (Maniatis and Tasic, 2002; Bouchard and Conrad, 1973).

However, when there is increased loss of Na+ in urine and K+ in sweat (Schneider et al., 1988; Shalit et al., 1991; Maltz and Silanikove, 1996; Silanikove, 1998) too much sulfur can change the acid-base balance (Maltz et al., 1994) and blood acid-base balance (Clark et al., 1978; Coppock et al., 1982), and even cause metabolic acidosis (Tucker and Harrison, 1988) and metabolic alkalosis (Clark et al., 1978). The role of the splice

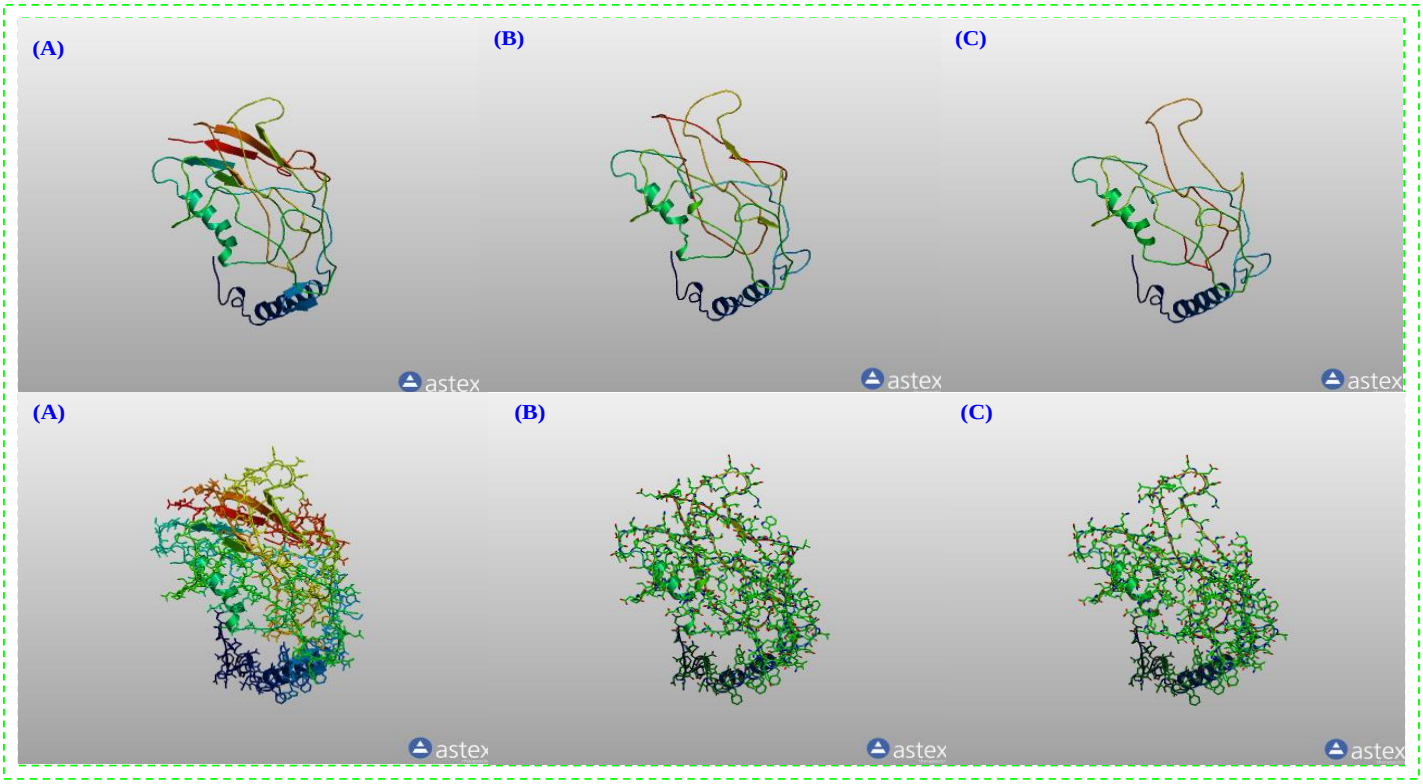

Figure 5. The protein structures predicted by SWISS-MODEL for the ATP1B2 proteins; A, ATP1B2-complete; B, ATP1B2-AS1; C, ATP1B2-AS2.

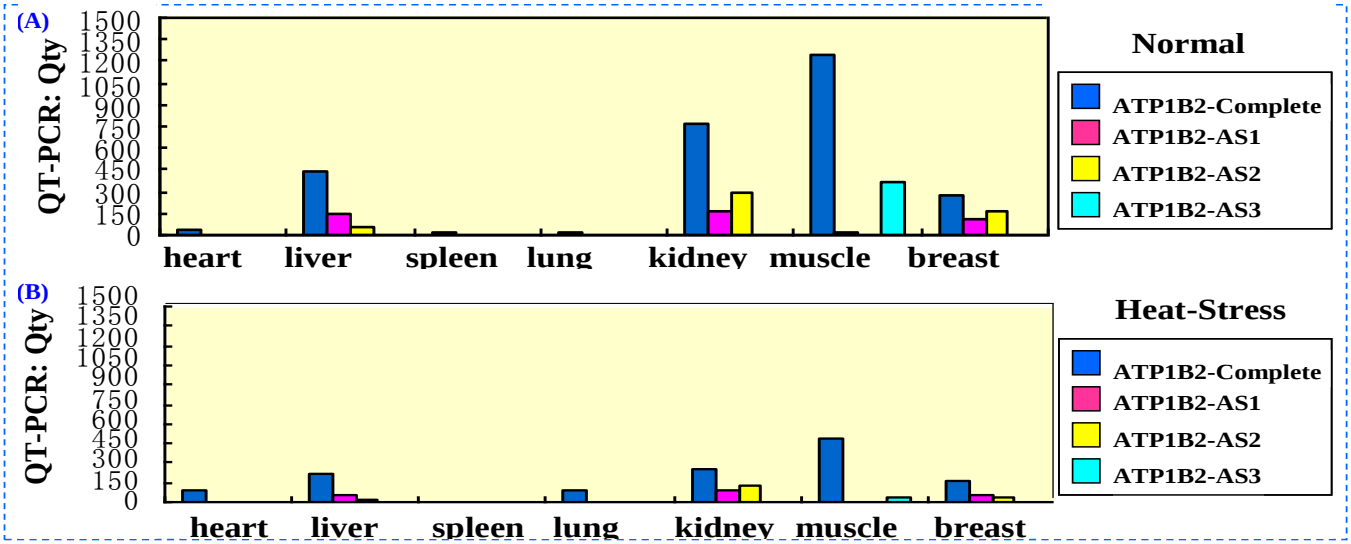

Figure 6. mRNA expression of different ATP1B2 gene transcripts in seven bovine tissues; A, expression in normal tissues; B, expression in heat stress tissues.

transcripts of the ATP1B2 gene in response to heat stress is yet to be elucidated. Many studies have shown that AS changes the expression of the gene mRNA (Maniatis and Reed, 2002) and this is reflected in the occurrence of diseases (Akker et al., 2001; Caceres and Kornblihtt, 2002; Faustinoand, 2003). The increased expression of ATP1B2-complete that was found in the heart and lung tissue also requires further confirmation. But the

regulation of AS provides an essential control step in post-transcriptional gene expression, and the endocrine system is likely to be one of the key systems that directs this in a tissue (Wang et al., 2008).

Link of alternative splicing of ATP1B2 with NKA activity and cattle production in heat stress

From the experimental results, the protein structure underwent significant changes of different ATP1B2 splice transcripts, which have the unique protein conformation, may affect the stability and vitality of the NKA, but they all require experimental verification. NKA activity is less affected by the physiological state and nutritional factors; it is mainly affected by the impact of genetic and environmental factors (temperature, humidity, etc.). For heat stress, there was a sharp decline in production performance of low NKA activity dairy cows (Wang et al., 2009), the search for a splice transcript to stabilize NKA activity in order to maintain good milk production performance during heat stress is recommended; all these needs further in-depth study.

Conclusion

AS is an essential means of generating diversity from a limited number of genes. Different transcript genes for coding and binding their proteins, and also their receptors are particularly well represented. In this study, three novel splice variants of ATP1B2 gene were identified in addition to the main full length expression transcript. The expression of the full length transcript, ATP1B2-complete, increased in heart and lung tissues while one of the splice transcripts, ATP1B2-AS3, was only found in the muscle tissue. The predicted three dimensional structure of one of the splice transcripts, ATP1B2-AS2, showed that many of the amino acid residues were in an unfavorable energy environment. It is interesting to speculate that this may be the perfect transcript to respond to heat-stress. As further knowledge of the AS process and its relation to disease processes is obtained, it may become possible to design novel therapies that direct AS to the appropriate pathway to tackle heat-stress in cows.

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