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Full Length Research Paper

Molecular cloning, expression analysis and sequence prediction of CCAAT/enhancer-binding protein beta gene of Qinchuan cattle

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CCAAT/enhancer-binding protein beta (C/EBPβ), as an essential transcriptional factor, regulates the differentiation of adipocytes and the deposition of fat. Herein, we cloned the whole open reading frame (ORF) of bovine C/EBPβ gene and analyzed its putative protein structures via DNA cloning and sequence analysis. Then, the expression profile of this C/EBPβ gene in fifteen kinds of tissues of Qinchuan cattle was conducted by real-time polymerase chain reaction (RT-PCR) technology. One significant outside to inside transmembrane structure located in amino acid region from 190 to 208 was observed in the putative protein sequences. Besides, one basic leucine zipper domain (bZIP) in amino acid area from 274 to 337 was found, concurring with the main characteristic of C/EBPs. Homologous comparison of the amino acid sequences from C/EBPβ cloned in this study and those from different species indicated C/EBPβ gene of Qinchuan cattle shared 97, 95 and 91% similarity with *Homo sapiens*, *Sus scrofa* and *Oryctolagus cuniculus* respectively, indicating a good sequence evolutional conservation of C/EBPβ. RT-PCR results revealed bovine C/EBPβ gene mRNA expression level of subcutaneous fat was the highest among all the analyzed tissues, and the relative quantity (RQ) in fat tissue increased as cattle grew. All in all, the present results could be used as basic but important genetic resource and information to inspire additional and specific studies on Qinchuan cattle.

Key words: CCAAT/enhancer-binding protein beta, molecular cloning, expression analysis.

INTRODUCTION

According to previous researches, both preadipocytes differentiation and fat deposition are regulated by many transcriptional factors such as peroxisome proliferator-activated receptor gamma (PPARγ, sterol regulatory element-binding proteins (SREBPs), fatty acid binding proteins (FABPs) and CCAAT/enhancer-binding proteins (C/EBPs) (Ormond and Lane, 1995; Shimano, 2001; Lee et al., 2003; Takeshi et al., 2004; Chui et al., 2005; Zhao et al., 2011). C/EBPs, as critical transcriptional regulators of adipocytes, share highly conserved basic leucine zipper domain (bZIP) and C-terminal basic amino acid-rich DNA binding protein (Jia et al., 2009). C/EBPs are known to regulate the transcription of genes important for proliferation, differentiation and inflammation (Ramji and Foka, 2002; Ying et al., 2006; Najla and Gregory, 2009; Jia et al., 2009; Zhao et al., 2011).

The transcriptional factor CCAAT enhancer binding protein beta (C/EBPβ) as one important member of C/EBPs C/EBPα, –β, –δ, –ε, –γ and –ζ is involved in the differentiation and function of adipocytes (Lekstrom and Xanthopoulos, 1998; Evan and Ormond, 2006; Zhao et al., 2011). C/EBPβ composed of 348 amino acids dimerizes not only with the other members of C/EBPs but also with other kinds of bZIP proteins (Yamaoka et al., 1997; Newman and Keating, 2003; Cai et al., 2008).

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Table 1. Primers used for Qinchuan cattle C/EBPβ gene cloning and its expression profiles analysis.

Primer pair	Genbank accession no.	Primer sequences (5' > 3') (Forward / Reversed)	Fragment size (bp)
C/EBPβ-P1	NM_176788	F: GGACAGATCTGCCACCATGCAACGCCTGGTGGTCTGGG R: GCGTCTCGAGCTAGCAGTGGCCGGAGGAGGCG	1063
C/EBPβ-P2	NM_176788	F: TTCCTCTCCGACCTCTTCTC R: CCAGACTCACGTAGCCGTACT	79
GAPDH-P3	AV_610889	F: CCAACGTGTCTGTTGTGGAT R: CTGCTTCACCACCTTCTTGA	80

"C/EBPβ-P1" refers to primers used for gene cloning; "C/EBPβ-P2" and "GAPDH-P3" refer to primers used for expression profiles analysis; fragments "AGATCT" and "CTCGAG" are restriction sites for Bgl Ⅱ and Xho Ⅰ respectively; fragment "GCCACC" represents Kozak sequence.

Inhibition of C/EBPβ activity blocks not only C/EBPα and PPARγ2 expression, but also renders the preadipocytes dependent on exogenous PPARγ2 ligand for their differentiation into adipocytes (Jonathan et al., 2001). It is no doubt that the differentiations of preadipocytes into mature fat cells is regulated by a cascade of transcription factors that interact in a complex fashion to control expression of several adipogenic genes. High-quality beef has better marblings which are directly or indirectly influenced by the fat quantity among muscles (Saymore et al., 2011). C/EBPβ has taken an active part during adipogenesis in 3T3-L1 preadipocytes and may have important effects in fat deposition (Croniger et al., 2001). C/EBPs are found in liver, adipose tissue, intestine, lung, reproductive tissues and cells of the inflammatory system (Sirois and Richards, 1993; Chumakov et al., 1997; Pall et al., 1997; Zhao et al., 2011). The action of C/EBPβ in human and mice has been addressed in several studies; however, specific expression pattern of C/EBPβ in the different tissues of Qinchuan cattle has not been studied so far. Herein, we cloned the complete ORF region of Qinchuan cattle C/EBPβ gene, analyzed its putative protein sequences, and examined its mRNA expression in different tissues, which would lay a foundation for further functional studies for Chinese indigenous cattle in the future.

MATERIALS AND METHODS

Samples collection

Fifteen tissue samples from three two-year old pure-breed Qinchuan cattle (Experiment farm of Naional Beef Cattle Improvement Center, Yangling, Shaanxi, China) were obtained, including heart, liver, spleen, lung, muscle, subcutaneous fat, large intestine, small intestine, rumen, reticulum, omasum, duodenum, pancreas, testis and brain. All samples were promptly frozen in liquid nitrogen and stored at -80°C.

C/EBPβ gene cloning

Total RNA from mix tissue samples was extracted by trizol reagent kit (INVITROGEN). The RNA samples were treated with DNase 1 for 30 min to remove the genomic DNA before reverse transcribing to cDNA via reverse transcription kit (FERMENTAS). According to NCBI sequences of the bovine C/EBPβ gene, a pair of polymerase chain reaction (PCR) primers named C/EBPβ P1 (Table 1) was designed to amplify the whole open reading frame. The 20-μl PCR reaction mixture contained 50 ng cDNA, 15 pM each primer, 1 × buffer, 1 mM $MgSO_4$, 0.2 mM dNTPs and 0.4 U KOD - Plus - Ver.2 (TOYOBO). PCR conditions were as follows: Initial denaturation step at 95°C for 10 min, 35 cycles of denaturation at 98°C for 10 s, extension at 68°C for 35 s, and a final extension for 10 min at 68°C (two-steps method). The PCR products were analyzed on a 0.8% agarose gel, recovered from the gel and then cloned into PMD - 19T - simple vector (TAKARA). After verification via bacterial colony PCR, the detailed sequencer of cloned gene was obtained through ABI 3730 sequencer.

Sequence analysis

Sequence analysis was performed by several kinds of software (Que et al., 2011). Sequence homology analysis was obtained from Blastp suite programme of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the deduced amino acid sequence was analyzed by the Protparam programme of ExPASy (http://www.expasy.org/cgi-bin/protparam). Protein domains were predicted by Prosite programme of ExPASy (http://prosite.expasy.org/cgi-bin/prosite/ScanView.cgi?scanfile= 294556012219.scan.gz).

Transmembrane regions, hydrophobic nature and signal peptide prediction were obtained by TMpred programme from Swiss EMBnet node server (http://www.ch.embnet.org/software/TMPRED_form.html), ProtScal programme of ExPASy (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1), and SignalP programme from CBS Prediction Servers (http://www.cbs.dtu.dk/services/SignalP/), respectively. Primary, secondary and tertiary structures were predicted via NPSA database (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_dsc.pl), prosite database from ExPASy (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_consensus.pl), and CPHmodels-3.0 programme from CBS prediction servers (http://www.cbs.dtu.dk/cgi-bin/nph-webface?jobid=cphmodels,4D81F778029AC618&opt=none).

Phylogenetic and molecular evolutionary analysis was conducted by Clustalx software, and the results were exported by Tree View software. Currently, there was no high-resolution structural data available for predictive sequences; therefore, computer modelling is one of the necessary tools to elucidate the position of domains within the polypeptide chain.

Table 2. Comparison of bovine C/EBPβ amino acid sequences with other Genbank recorded animals.

Species	Genbank accession no.	Similarity (%)
Bos taurus	NM_176788	99
Homo sapiens	NP_005185	97
Sus scrofa	NP_004355	95
Oryctolagus cuniculus	XP_002723973	91
Paralichthys olivaceus	BAB40971	73
Monodelphis domestica	XP_001369325	72
Rattus sp	AAB21102	69
Mus musculus	NP_034013	69
Gallus gallus	NP_990584	68
Anolis carolinensis	XP_003220672	66
Taeniopygia guttata	XP_002187599	66
Danio rerio	NP_571959	50
Xenopus laevis	NP_001165638	47

Tissue expression profile analysis

C/EBPβ gene expression profile in Qinchuan cattle was analyzed by ABI 7500 RT-PCR system (Applied Biosystems). mRNA from fifteen tissue samples were extracted via trizol reagent (Invitrogen) and reverse transcribed via fermentas kit (Fermentas). One pair of RT-PCR primers C/EBPβ P2 was designed to amplify 79-bp products from Qinchuan cattle C/EBPβ gene (Table 1). Another pair of primers GAPDH-P3 was designed to obtain 80-bp products of bovine GAPDH housekeeping gene, which played as the endogenous control (Table 1). The PCR system in 20-μl reaction volume consisted of 50 ng cDNA, 0.4 μM each primer, 1 × SYBR® Premix Ex TaqTMII, 1 × ROX reference dye. PCR conditions were as follows: Initial denaturation step at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, and extension at 60°C for 34 s to amplify 79-bp products and another 40 cycles of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s to obtain the melt curve. All the quantitative RT-PCR reactions were performed in triplicate, based on a standard curve method.

Statistical analysis

The expression levels of C/EBPβ gene were analyzed via $2^{-\triangle\triangle CT}$, where CT value represented the cycle number at which the fluorescence intensity trace of each reaction intersected the threshold line (Livak and Schmittgen, 2001). The specific formulas were as follows:

$$\triangle CT = CT_{mean\ (C/EBP\beta)} - CT_{mean\ (GAPDH)}$$

$$\triangle\triangle CT = CT_{mean\ (Sample)} - CT_{mean\ (Max\ Sample)}$$

$$RQ = 2^{-\triangle\triangle CT}$$

Once the efficiency of both reactions reached 100%, the expression ratio between samples equals RQ.

RESULTS AND DISCUSSION

C/EBPβ gene molecular cloning

DNA sequencing results showed the obtained nucleotide sequences shared 99% similarity with bovine C/EBPβ gene sequences (GenBank: NM_176788), implying Qinchuan cattle C/EBPβ gene coding region was cloned successfully for the first time, and the length of the whole ORF region was 1047-bp with no introns.

Putative Qinchuan cattle C/EBPβ protein structure analysis

Protein sequence blast analysis

The putative protein sequence consisted of 348 amino acids and further cross-species blast analysis showed Qinchuan cattle C/EBPβ shared a variable level of similarities on amino acid sequence with different animals (Table 2). Except for bovine sequences, the highest similarity to Qinchuan cattle was 97%, obtained from *Homo sapiens* followed by *Sus scrofa* and *Oryctolagus cuniculus* with 95 and 91%, orderly. To better understand bovine C/EBPβ relationship and potential evolutional process, we obtained the phylogenetic tree via Clustalx software (Figure 1). Results showed Qinchuan cattle C/EBPβ had a close relatedness with mammalians when compared to distant species such as *Gallus gallus* and *Danio rerio* etc. However, the mammalian closeness was discriminatory between specific different species. Overall, the relatively high degree similarity of bovine C/EBPβ protein sequence with other mammals implied good evolutional sequence conservation.

Transmembrane regions and bZIP domains

Since *H. sapiens*, *S. scrofa* and *O. cuniculus* showed relatively high protein similarities with Qinchuan cattle; we analyzed the basic structures of the four animals to better understand C/EBPβ compositions in different species.

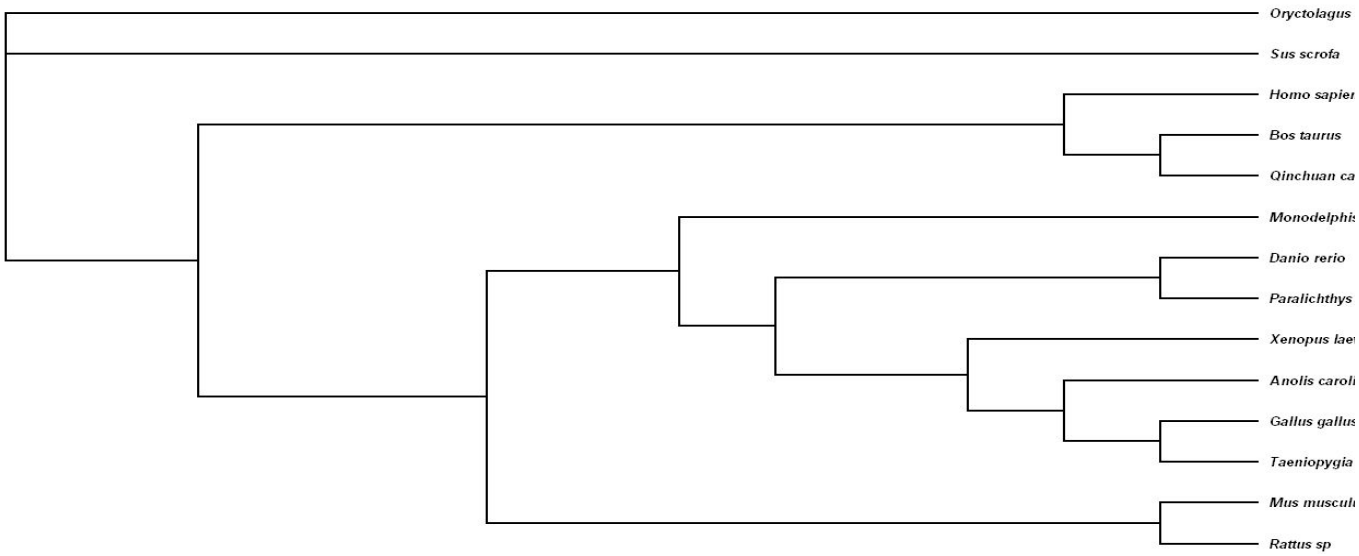

Figure 1. Phylogenetic dendrogram obtained by distance matrix analysis of C/EBPβ from 14 species. Qinchuan cattle C/EBPβ had closer relatedness with *H. sapiens*.

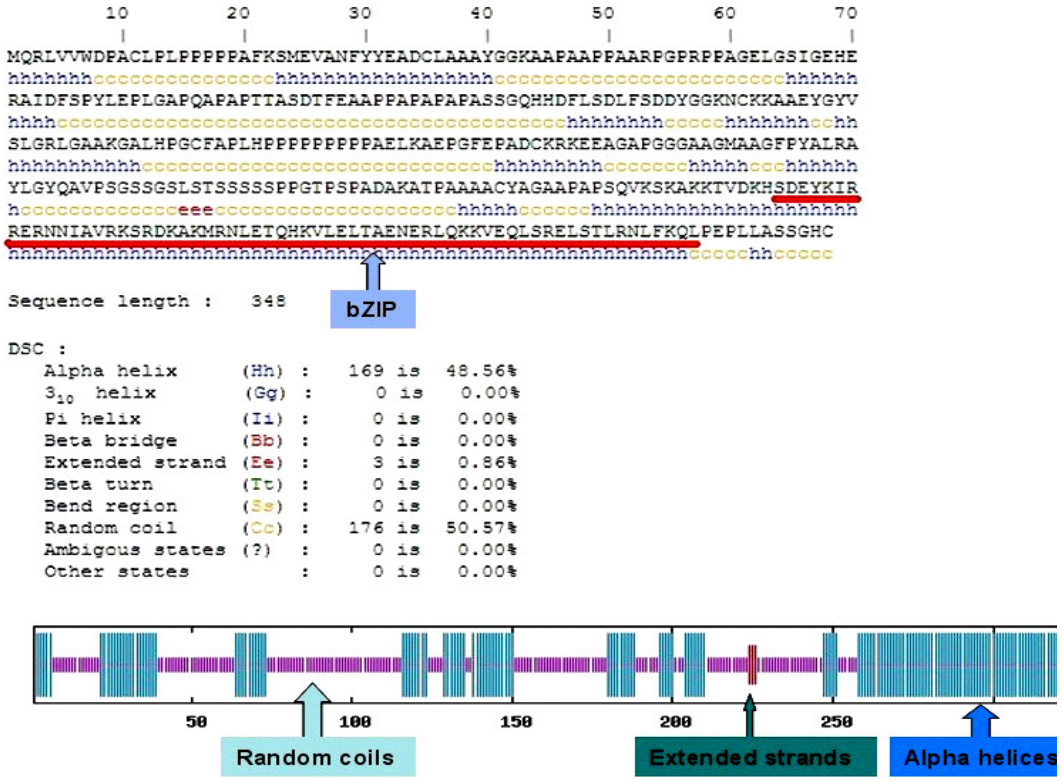

Figure 2. Prediction of Qinchuan cattle C/EBPβ elementary components. bZIP, The basic leucine zipper domains.

A less critical hydrophobic area from 168 to 180 amino acid residues was observed and no significant signal peptides was found according to the ProtScal results and SignalP programme results of Qinchuan cattle C/EBPβ respectively. Analysis on the other three animals exhibited similar results.

Prediction analysis uncovered that bovine C/EBPβ consisted of 169 alpha helices, 176 random coils and 3

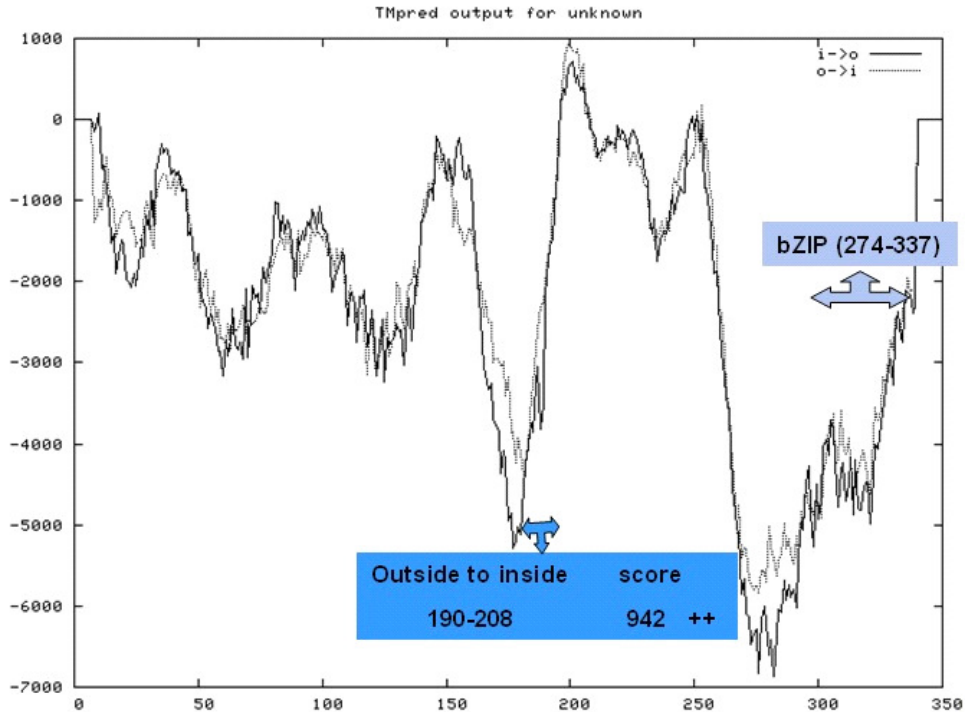

Figure 3. Prediction of Qinchuan cattle C/EBPβ transmembrane areas using TMpred programme. Y axis stands for the scores for transmembrane area (> 500 was considered to be significant) while X axis is protein sequences of Qinchuan cattle C/EBPβ; "i-> o" represents transmembrane structure from inside to outside orientation; "o->i" represents transmembrane structure outside to inside orientation; ++ indicates strong preference of this orientation; bZIP, the basic leucine zipper domains.

extended strands (Figure 2), and the first two structures took up over 98% in amino acid residues. Similar situation was also observed when we probed into the basic structures of *H. sapiens*, *S. scrofa* and *O. cuniculus* C/EBPβ proteins.

A significant outside to inside (o-i) transmembrane helices in amino acid region from 190 to 208 with N-terminus outside was found via TMpred Programme, and it scored 942 points (> 500 was considered to be significant (Figure 3); Hotmann and Stoffel (1993). Protein transmembrane areas were generally considered as less conserved regions yet played important roles. In our study, only one membrane-spanning region was found, concurring with the good conservation of C/EBPβ Fundamentally, membrane regions anchor the protein to the cell membrane and form a pore though which the transport of a wide variety of substrates occurs (Siepel et al., 2005). The predictive results indicated C/EBPβ might be membrane protein or secretory protein. Remarkable outside to inside transmembrane helices were also observed in nearly overlapped location of the other three species, concurred with cross-species comparative results.

One bZIP which consisted of 64 alpha helices amino acid residues from 274 to 337 was observed in Prosite programme, consistent with the main characteristics of C/EBPs (Croniger et al., 2001). During animal embryogenesis, bZIP factors are necessary for the proper development of organs and tissues such as the liver, bone, heart and fat (Darlington et al., 1998; Eferl et al., 1999). The bZIP structures were found in *H. sapiens*, *S. scrofa* and *O. cuniculus* too. Interesting fact that we have found was that all the bZIP structures from those four animals were made of the exact of the same 64 amino acid residues (Figure 4). The discovery of bZIP, C/EBPs' predominant characteristic, was generally considered to be closely related to the transcriptional functions of humans and mice's adiposysis (Yeh et al., 1995; Evan et al., 2002), suggesting the putative amino acids sequence did belong to C/EBPs.

Additionally, we probed into the tertiary structure via CPHmodels-3.0 software and compared Qinchuan cattle with the other three mammalians. Even the homologous comparison showed Qinchuan cattle had a closer relationship with *H. sapiens* than with the other two, still their three dimensional structures were in accord with each other (Figure 5).

C/EBPβ gene expression profiles

In order to enhance the understanding of the gene

Figure 4. The prediction of functional domain of Qinchuan cattle C/EBPβ. bZIP, The basic leucine zipper domains; bZIP, structures from those four animals were made of the exact same 64 amino acid residues.

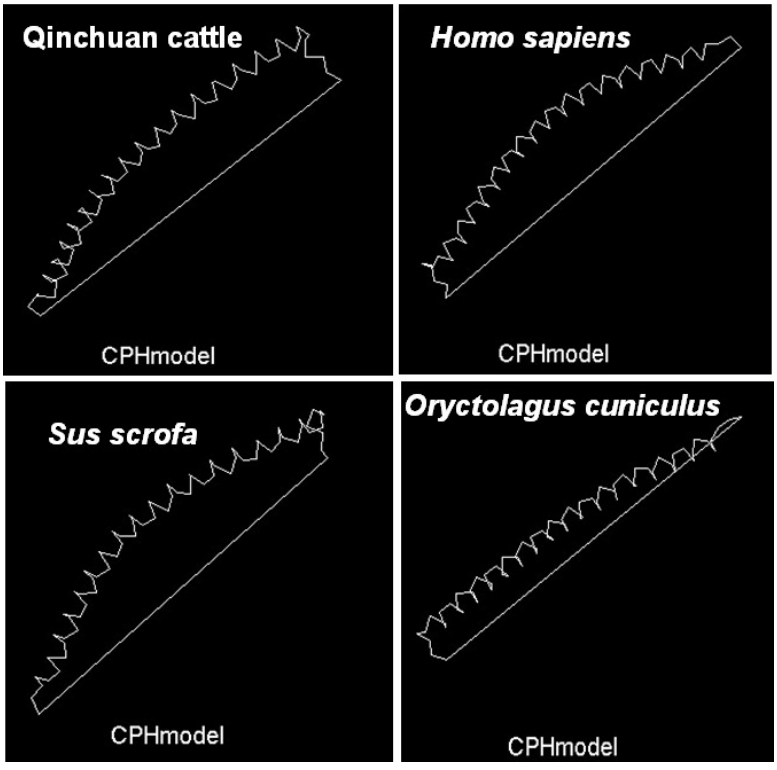

Figure 5. Three dimensional structures of C/EBPβ gene coding proteins.

products' role in various tissues of Qinchuan cattle, it was necessary to provide the C/EBPβ tissue mRNA expression profiles via RT-PCR technology. Figure 6a illustrated C/EBPβ gene was found to express in all the analyzed 15 tissue samples, suggesting C/EBPβ may have multiple functions during body metabolising (Claudia et al., 2007; Jeske et al., 2009; Jennifer et al., 2009; Zhao et al., 2011). However, even C/EBPβ was observed in all these tissues, the quantities varied from one to the other. Specifically speaking, the highest RQ

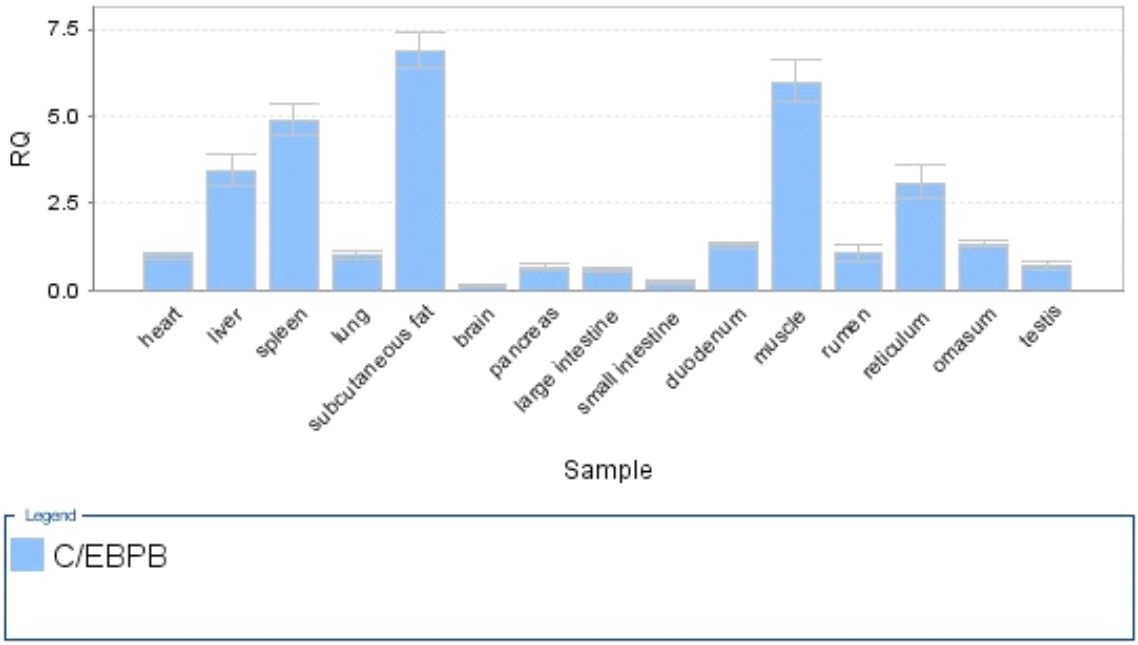

Figure 6a. The mRNA expression profile of C/EBPβ gene in 15 kinds of tissues in Qinchuan cattle. RQ is relative quantity, X axis delineates 15 tissues; the expression levels of C/EBPβ gene in fat tissue was higher than that in the other tissues.

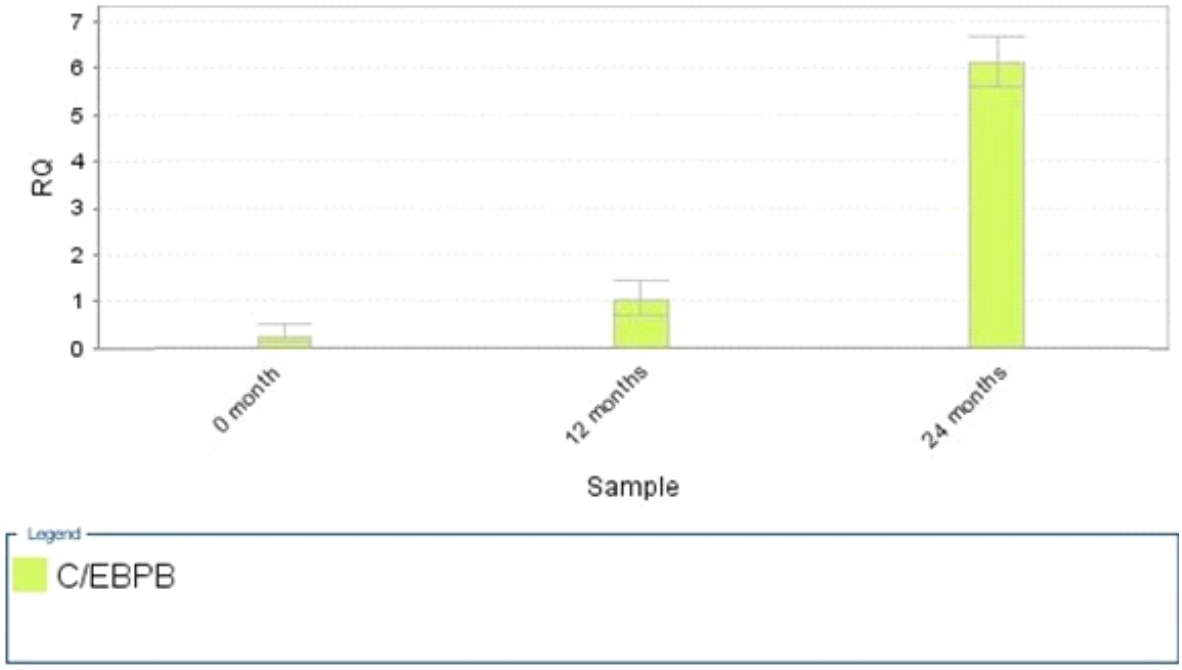

Figure 6b. The mRNA expression patterns of C/EBPβ gene of fat tissue of Qinchuan cattle during three different fattening periods. RQ is relative quantity; X axis delineates three fattening periods (0, 12 and 24 months).

was observed in subcutaneous fat (RQ = 6.88), followed by muscle (RQ = 5.58), spleen (RQ = 4.89), liver (RQ = 3.45), reticulum (RQ = 3.08), omasum (RQ = 1.33), duodenum (RQ = 1.30), rumen (RQ = 1.05), lung (RQ = 1.00), heart (RQ = 1.00), testis (RQ = 0.70), pancreas (0.67), large intestine (RQ = 0.58), small intestine (RQ = 0.23) and the lowest RQ was obtained from brain (RQ = 0.13), implying C/EBPβ may be involved more in fat

metabolism than in any other tissues.

Since the C/EBPβ mRNA expression level in subcutaneous fat was much higher than that in all the other 14 tissues, providing additional evidence of C/EBPβ gene intrinsic expression patterns in cattle, different breeding age periods seemed essential to better understand its role during fat depositing. Therefore, fat tissues of Qinchuan cattle from three age periods including 0, 12 and 24 months with 3 duplicates were collected, and represented various fattening periods. Results of RT-PCR indicated the RQ of C/EBPβ gene expression in 0, 12 and 24 months were 0.24, 1.00 and 6.10, respectively, showing a rising trend from 0 to 24 months (Figure 6b). The quantity of subcutaneous fat in new born calves was much lower than that in 24-month adult individuals, indicating the activities of fat metabolism or deposition was weaker then. Animals need fat to maintain their body temperature to live, and it is a common sense that fat depositing ability was better when animals grow up. In China, generally speaking, farmers started to fatten the calves when they were 12 to 18 months old, then slaughtered them when they were 24 months old, because the effects of fattening were conspicuous at that time (Hu and Zan, 2001). When cattle were 24 months old, the C/EBPβ gene expression reached its peak, showed their fat metabolism activities became stronger when compared with new-borns. Taken these two facts together, it is not hard for us to discover the internal connections that the formative process of mature adipocytes may be directly or indirectly mediated by C/EBPβ gene. Though we know preadipocytes differentiation and fat deposition are regulated by a large amount of factors, we can not ignore the importance of C/EBPβ.

In conclusion, we have successfully cloned the complete ORF sequences of Qinchuan cattle C/EBPβ gene for the first time, provided reasonable analysis of its putative protein, and compared it with those from other animals via several softwares. Although the structures and functions of C/EBPβ have been well studied in humans and mice, the corresponding information for cattle was rare. The present results could offer basic but useful information to the specific researches of C/EBPβ gene in Qinchuan cattle in the future. However further analysis on bovine C/EBPβ *in vivo* and *in vitro* from DNA or RNA level to protein level should be performed to reveal the role of C/EBPβ in cattle, which should be helpful for the beef quality improvement of Qinchuan cattle.

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