GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Cloning, expression analysis and sequence prediction of the CCAAT/enhancer-binding protein alpha gene of Qinchuan cattle

H. Wang[1], L.S. Zan[1,2], H.B. Wang[1,2], C. Gong[1,2] and C.Z. Fu[1]

[1]College of Animal Science and Technology, Northwest A & F University, Yangling, Shaanxi, P.R. China
[2]National Beef Cattle Improvement Center, Yangling, Shaanxi, P.R. China

Corresponding author: L.S. Zan
E-mail: zanls@yahoo.com.cn

Genet. Mol. Res. 11 (2): 1651-1661 (2012)
Received October 19, 2011
Accepted February 16, 2012
Published June 15, 2012
DOI http://dx.doi.org/10.4238/2012.June.15.14

ABSTRACT. CCAAT/enhancer-binding protein alpha (C/EBPα) is an essential transcription factor, regulating the differentiation of adipocytes. We cloned the complete open reading frame of C/EBPα gene of Qinchuan cattle and analyzed its protein structures and expression profile in 15 tissues via DNA cloning, sequencing and RT-PCR. Analysis of the putative protein sequences revealed that C/EBPα consists of alpha helices, random coils and a few extended strands. A significant transmembrane structure was observed in amino acid region 233 to 252. A basic leucine zipper domain was also found in amino acid region 277 to 340, which is characteristic of C/EBPs. Homologous comparison with various species indicated that the C/EBPα gene of Qinchuan cattle shares 97, 95, 94, 94, and 93% similarity in amino acid sequences with *Sus scrofa*, *Homo sapiens*, *Rattus norvegicus*, *Oryctolagus cuniculus*, and *Mus musculus*, respectively, implying strong sequence conservation of C/EBPα during evolution. RT-PCR revealed that the mRNA expression level of bovine C/EBPα gene in subcutaneous fat is much higher than that

in the other 14 tissues, and the relative quantity in fat tissue increases with cattle age.

Key words: C/EBPα gene; Qinchuan cattle; Expression profile; Cloning; Protein structure prediction

INTRODUCTION

It is an undisputable fact that preadipocyte differentiation and fat deposition are regulated by a large number of 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) as well (MacDougald et al., 1995; Storch and Thumser, 2000; Shimano, 2001; Lee et al., 2003; Imai et al., 2004; Chui et al., 2005). C/EBPs, as critical transcriptional regulators of adipocytes, have a highly conserved basic leucine zipper domain (bZIP) and a variable N-terminal region, and to date, six members of C/EBPs have been discovered: C/EBPα, -β, -δ, -ε, -γ, and -ζ (Williams et al., 1991; Lin et al., 1993; Lekstrom et al., 1998). C/EBPs are also known to regulate the transcription of genes that are important in metabolism, differentiation and inflammation (Hanson, 1998; Poli, 1998; Ramji and Foka, 2002; Zuo et al., 2006). C/EBPα is composed of 353 amino acids (Taniguchi and Sasaki, 1996) and expressed just before the transcription of most adipocyte-specific genes that possess C/EBPα binding sites. C/EBPα is also expressed in basal keratinocytes, and is coordinately upregulated as keratinocytes exit in the basal layer and undergo terminal differentiation (Lopez et al., 2009). Moreover, C/EBPα, as the most important member among C/EBPs, works very closely with other fat transcriptional factors. For example, C/EBPα and PPARγ factors cooperatively orchestrate adipocyte biology by adjacent binding on an unanticipated scale (Lefterova et al., 2008).

All in all, these findings indicate that C/EBPα is a crucial regulator in adipocyte differentiation process. Here, we cloned the complete CDS region of the C/EBPα gene, determined its putative protein sequences, and examined its mRNA expression in different tissues in Qinchuan cattle, which lay a foundation for further functional studies.

MATERIAL AND METHODS

Samples collecting

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

C/EBPα gene cloning

Total RNA from mix tissue samples was extracted using the Trizol reagent (Invitrogen). The RNA samples were treated with DNase I for 30 min to remove the genomic DNA

before reverse transcribing to cDNA via a reverse transcription kit (Fermentas). According to NCBI sequences of the bovine C/EBPα gene (GenBank: NM_176784.2), a pair of polymerase chain reaction (PCR) primers named P1 (P1f: 5'-GGACAGATCTGCCACCATGCAACGCC TGGTGGTCTGGG-3' and P1r: 5'-GCGTGGATCCCTAGCAGTGGCCGGAGGAGGCG-3') was designed to amplify the whole open reading frame. The 20-μL PCR mixture contained 50 ng cDNA, 15 pM of each primer, 1X 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 12 s, annealing at 68°C for 30 s, and extension at 68°C for 35 s, and a final extension for 10 min at 68°C. 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 sequence of the cloned gene was obtained using the ABI 3730 sequencer.

Sequence analysis

Sequence homology analysis was obtained from the BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the deduced amino acid sequence was analyzed by the Protparam program of ExPASy (http://www.expasy.org/cgi-bin/protparam). Protein domains were predicted by the Prosite program of ExPASy (http://www.expasy.org/prosite/). Transmembrane regions, hydrophobic nature and signal peptide prediction were obtained by the TMpred program from Swiss EMBnet node server (http://www.ch.embnet.org/software/TMPRED_form.html), the ProtScal program of ExPASy (http://www.expasy.org/cgi-bin/protscale.pl), and the SignalP program from CBS Prediction Servers (http://www.cbs.dtu.dk/services/SignalP/), respectively. Secondary and tertiary structures were predicted via the Prosite database from ExPASy (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_consensus.pl), and the CPHmodels-3.0 program from CBS Prediction Servers (http://www.cbs.dtu.dk/services/CPHmodels/). Phylogenetic and molecular evolutionary analysis was conducted by the ClustalX software, and the results were exported by the Tree View software.

Tissue expression profile analysis

C/EBPα gene expression profile in Qinchuan cattle was analyzed by the ABI 7500 RT-PCR system (Applied Biosystems). mRNA from 15 tissue samples was extracted using the Trizol reagent (Invitrogen) and reverse transcribed with the Fermentas kit (Fermentas). One pair of RT-PCR primers (P2f: 5'-ATCTGCGAACACGAGACG-3' and P2r: 5'-CCAGG AACTCGTCGTTGAA-3') for the Qinchuan cattle C/EBPα gene was designed to amplify 73-bp products. Another pair of primers (P3f: 5'-CCAACGTGTCTGTTGTGGAT-3' and P3r: 5'-CTGCTTCACCACCTTCTTGA-3') was designed to obtain 80-bp products of the bovine GAPDH housekeeping gene (GenBank: AV610889), which served as the endogenous control. The PCR system in 20-μL reaction volume consisted of 50 ng cDNA, 0.4 μM of each primer, 1X SYBR® Premix Ex TaqTMα, and 1X 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 73-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 melting curve. All quantitative RT-PCRs were performed in triplicate, based on a standard curve method.

Statistical analysis

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

$$\triangle CT = CTmean\ (C/EBP\alpha) - CTmean\ (GAPDH) \quad \text{(Equation 1)}$$

$$\triangle\triangle CT = CTmean\ (Sample) - CTmean\ (Max\ sample) \quad \text{(Equation 2)}$$

$$RQ = 2^{-\triangle\triangle CT} \quad \text{(Equation 3)}$$

Once the efficiency of both reactions reached 100%, the expression ratio between samples equal relative quantification (RQ; Julie et al., 2009).

RESULTS AND DISCUSSION

C/EBPα gene cloning and putative protein sequence BLAST analysis

DNA sequencing results showed that the nucleotide sequences obtained shared 99% similarity with the bovine C/EBPα sequence (GenBank: NM_176784.2), implying that the Qinchuan cattle C/EBPα gene coding region was successfully cloned, and that the length of the whole CDS region was 1062 bp.

The putative protein sequence consisted of 353 amino acids and was consistent with the finding of Taniguchi and Sasaki (1996). Further cross-species BLAST analysis showed that the Qinchuan cattle C/EBPα shared a variable level of similarities in amino acid sequence with different animals (Table 1). As can be seen in Table 1, the highest similarity to Qinchuan cattle was 97%, obtained with *Sus scrofa*, followed by *Homo sapiens*, *Rattus norvegicus*, *Oryctolagus*, and *Mus musculus* with 95, 94, 94, and 93%, respectively. A relatively high protein homology among mammals was observed, suggesting a good sequence conservation of C/EBPα. To better understand the bovine C/EBPα relationship and potential evolutionary process, we obtained the phylogenetic tree via the ClustalX software (Figure 1). The results showed that the Qinchuan cattle C/EBPα had a close relatedness with mammals when compared to distant species such as *Danio rerio* and *Salmo salar*. However, the mammalian closeness was discriminatory between specific different species. Overall, the relatively high degree of similarity of the bovine C/EBPα protein sequence with that of other mammals implied a similar potential function that C/EBPα may have among those animals.

Putative C/EBPα protein structure analysis

Primary, secondary and tertiary structures of virtually translated bovine C/EBPα amino acid sequences were analyzed in our study. Prediction analysis revealed that C/EBPα consisted of 98 alpha helices, 243 random coils and 12 extended strands (Figure 2A). A substantial

number of outside to inside transmembrane helices made of random coils existed in the amino acid region from 233 to 252, scored as 551 points (>500 is considered to be significant; Figure 2B). Protein transmembrane areas are generally considered less conserved regions (Siepel et al., 2005). In our study, we only found one membrane-spanning region, concurring with the good conservation of C/EBPα. One bZIP, consisting of alpha helices and ranging from 277 to 340 amino acid residues, was observed, consistent with the characteristics of C/EBPs (Figure 2C) (Croniger et al., 2001; Gomez-Santos et al., 2005). The discovery of bZIP, C/EBPs' predominant characteristic, is generally considered to be closely related to the transcriptional functions in both human and mouse adiposysis (Yeh et al., 1995; Rosen et al., 2002), suggesting the potentially similar effects that C/EBPα may have among mammals.

Table 1. Comparison of bovine C/EBPα amino acid sequences with other GenBank recorded animals.

Species	Protein	
	GenBank accession No.	Similarity (%)
Sus scrofa	XP_003127063	97
Homo sapiens	NP_004355	95
Rattus norvegicus	NP_036656	94
Oryctolagus	XP_002711561	94
Mus musculus	NP_031704	93
Macaca mulatta	XP_001108401	83
Taeniopygia	XP_002188412	68
Ornithorhynchus	XP_001509536	67
Gallus gallus	NP_001026630	63
Salmo salar	NP_001133403	54
Danio rerio	NP_571960	54
Xenopus laevis	NP_001085156	53

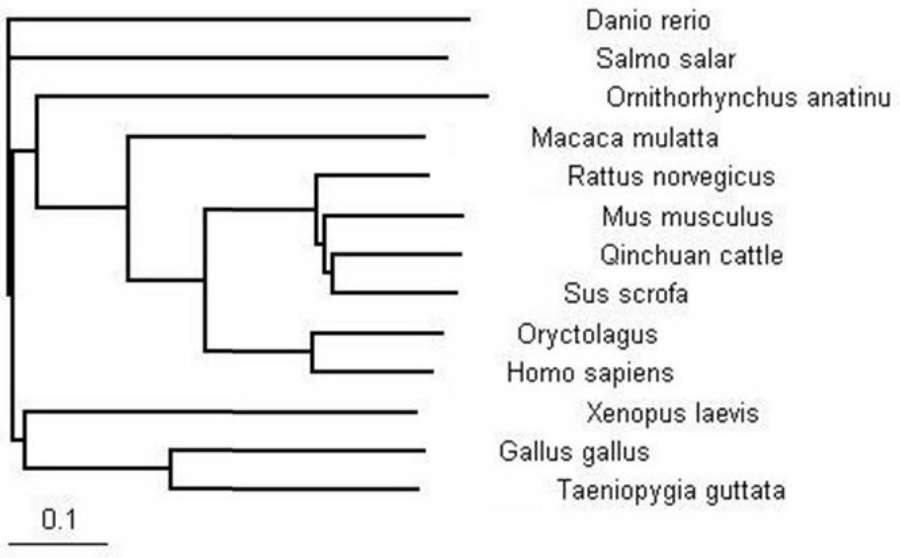

Figure 1. Phylogenetic dendrogram obtained by distance matrix analysis of the Qinchuan cattle C/EBPα had a close relatedness with mammals such as *Sus scrofa* and *Homo sapiens*.

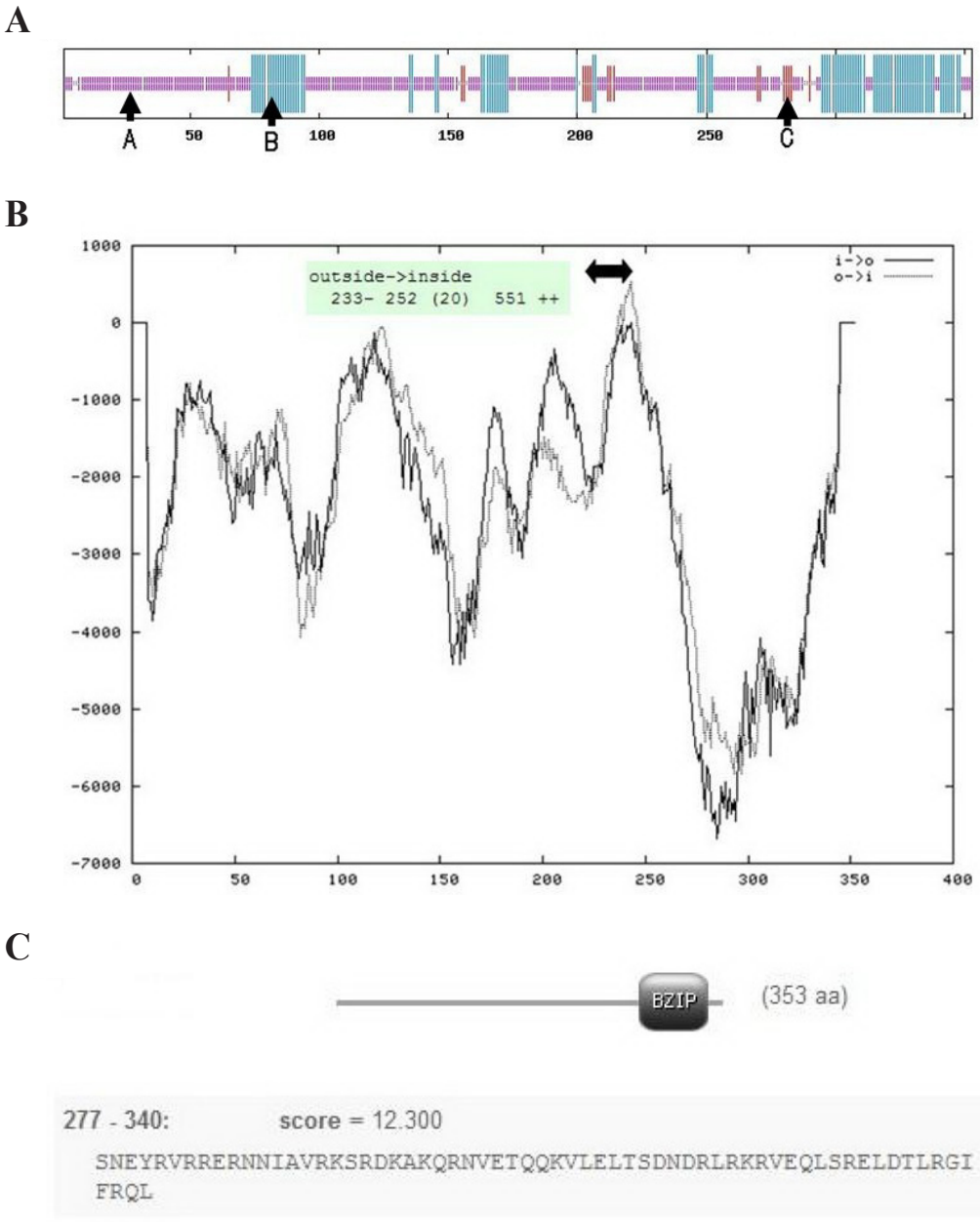

Figure 2. A. Prediction of the bovine C/EBPα primary structure. Area A represents random coils, area B represents alpha helices, and area C represents extended strands. **B.** Prediction of the bovine C/EBPα transmembrane area using the TMPRED program. Y-axis stands for the scores for transmembrane area, while x-axis is protein sequences of bovine, "o>i-" represents transmembrane structure from inside to outside orientation, "i->o" represents transmembrane structure outside to inside orientation, and symbol ++ indicates strong preference of this orientation. **C.** Prediction of functional domain of the bovine C/EBPα. BZIP = Basic leucine zipper domains.

Based on the ProtScal results, a less critical hydrophobic area from 168 to 180 amino acids (Figure 3) was observed. There were no significant signal peptides according to the SignalP program results (Figure 4).

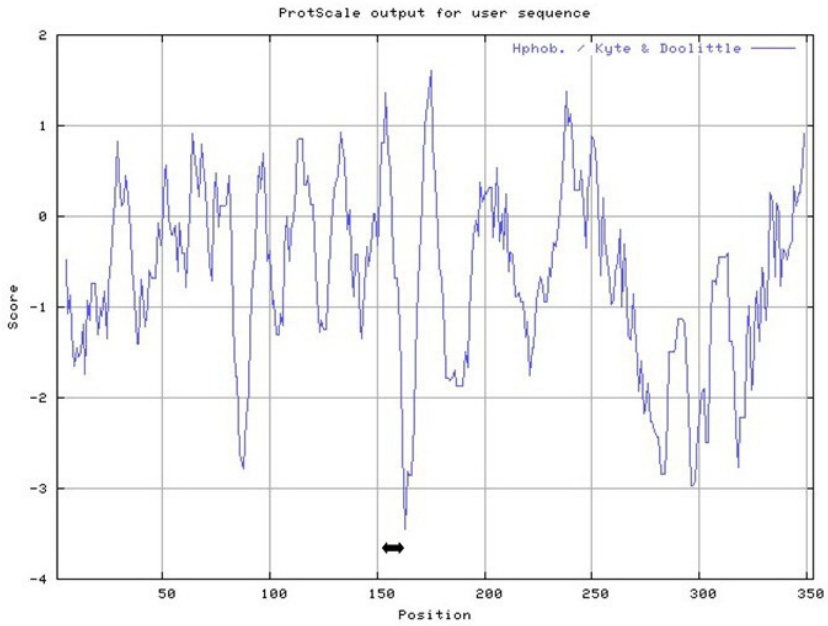

Figure 3. Prediction of one less critical hydrophobic area in the bovine C/EBPα.

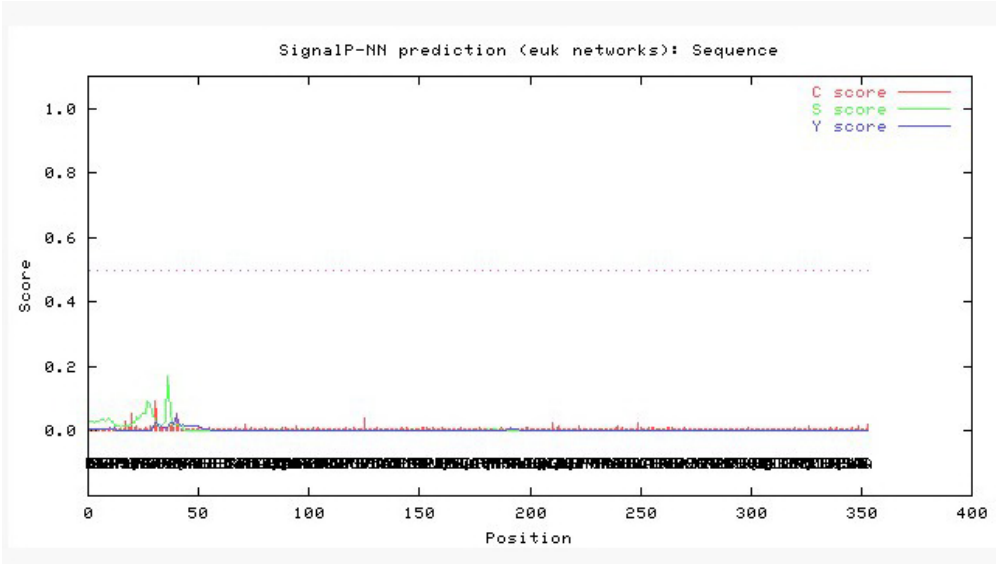

Figure 4. Signal peptide prediction of the bovine C/EBPα. No significant signal peptides were observed.

Additionally, since C/EBPα had good evolutionary conservation, we looked into the tertiary structure via the CPHmodels-3.0 software and compared Qinchuan cattle with *S. scrofa* as well as *H. sapiens*. As can be seen from Figure 5, even homologous comparison showed that Qinchuan cattle had a closer relationship with *S. scrofa* than with *H. sapiens*; still, their three-dimensional structures were in accordance with one another.

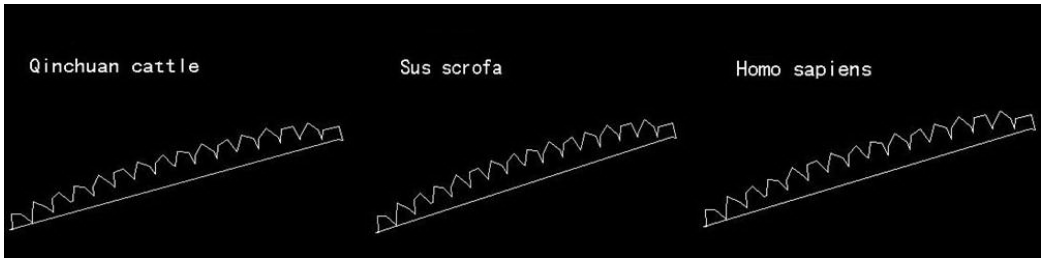

Figure 5. Three-dimensional structure of C/EBPα gene coded proteins.

C/EBPα gene expression profiles

C/EBPα has been detected in adipose tissue, placenta, liver, and a variety of other organs, such as reproductive tissues, and cells of the inflammatory system (Birkenmeier et al., 1989; Chumakov et al., 1997). In order to enhance the understanding of the gene products' role in different tissues of Qinchuan cattle, it was necessary to determine the C/EBPα tissue expression profiles via RT-PCR technology. Figure 6A shows that the C/EBPα gene was found to express in all 15 tissue samples analyzed, suggesting that C/EBPα may have multiple functions in body metabolism. However, even though C/EBPα was observed in all the tissues studied, the quantities were different. Specifically speaking, the greatest RQ was observed in subcutaneous fat (RQ = 9.76) and was significantly higher than that in omasum (RQ = 0.08), brain (RQ = 0.05), heart (RQ = 0.12), etc., implying that C/EBPα may be involved more in fat metabolism. Bennett et al. (2003) reported that C/EBPα expressed at reduced levels in cells with low adipogenic potential, and expressed at high levels in preadipocytes that spontaneously differentiate, concurring with our present study.

Since the C/EBPα mRNA expression level in subcutaneous fat was much higher than that in the 14 other tissues, providing additional evidence of intrinsic expression patterns of the C/EBPα gene in cattle, different breeding age periods seemed essential to better understand its role during fat deposition. Therefore, fat tissues of Qinchuan cattle from three age periods, including 0, 12 and 24 months with 3 duplicates, representing various fattening periods, were collected. Results of RT-PCR indicated that the RQ of C/EBPα gene expression at 0, 12 and 24 months were 1.00, 2.28, 3.37, respectively, showing a gradual rising trend from 0 month to 24 months (Figure 6B). The quantity of subcutaneous fat in newborn calves was much lower than that in 24-month adult individuals, and the activities of fat metabolism and deposition were also weaker. Cattle need fat to resist the cold weather, and therefore, it is natural for their fat metabolism activities to become stronger and stronger as they grow. In China, generally speaking, farmers start fattening calves when they are 12 to 18 months old, then slaughter them when they are 24 months old, because the effects of fattening are conspicuous at that

time (Hu and Zan, 2001). As can be seen from Figure 6B, when cattle were 24 months old, C/EBPα gene expression reached its peak. Taking 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 manipulated by the C/EBPα gene.

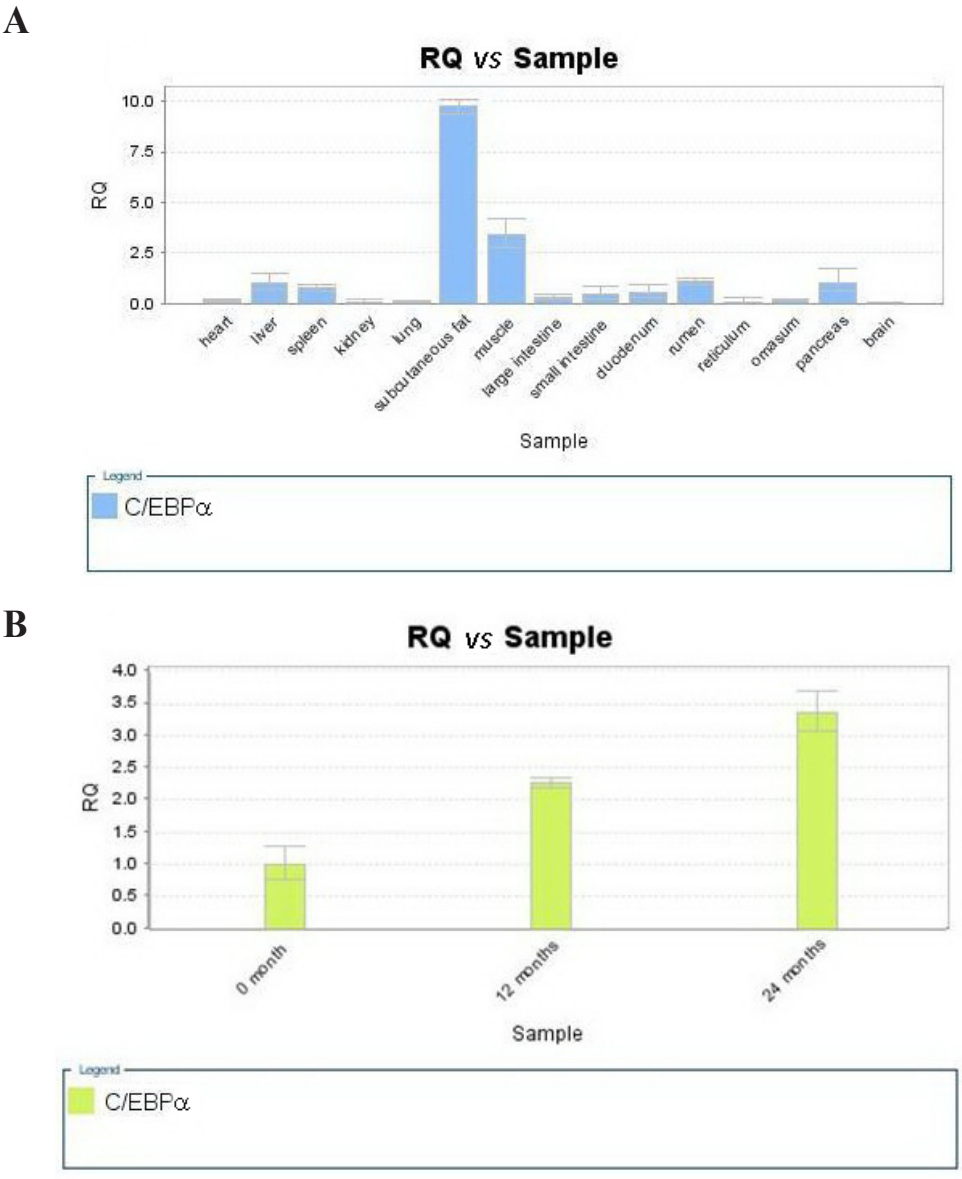

Figure 6. A. mRNA expression profile of C/EBPα in 15 tissues of Qinchuan cattle. **B.** mRNA expression patterns of bovine C/EBPα in the fat tissue of Qinchuan cattle during three different fattening periods. RQ is relative quantity, and the horizontal bars indicate the RQ mean of each group; the expression levels of the C/EBPα gene in fat tissue were much higher than those of other tissues (A), and grew with cattle age (B).

In conclusion, we successfully cloned the complete CDS sequences of the Qinchuan C/EBPα gene for the first time and provided reasonable determination of its putative protein. The analysis of the putative protein structures suggested one significant transmembrane area and one bZIP, corroborating the general characteristics of C/EBPs. The homologous comparison of C/EBPα gene sequences showed a relatively high degree of similarity among mammals, implying a good sequence conservation. C/EBPα was observed to express in all the 15 tissues analyzed of Qinchuan cattle at the mRNA level, and the highest RQ was found in fat tissue. Additional RT-PCR results indicated that C/EBPα gene expression level in fat tissue increased as cattle grew. Although the structures and functions of C/EBPα are well known in humans and mice, information about cattle is rare, further studies of bovine C/EBPα *in vivo* and *in vitro* from DNA or RNA level to protein level should be performed. The present results could offer useful information for specific research on Qinchuan cattle in the future.

ACKNOWLEDGMENTS

Research supported by the National Twelfth ‘‘Five Year’’ Science and Technology Support Project (#2011BAD28B04-03), the China National 863 Program (#2011AA100307), the GMO New Varieties Major Project (#2011ZX08007-002), the National Beef and Yak Industrial Technology System (#CARS-38), the Program for Changjiang Scholars and Innovative Research Team (#IRT0940) and the “13115” Scientific and Technological Innovation Program of Shaanxi Province (#S2010ZDGC109).

REFERENCES

Bennett CN, Hodge CL, MacDougald OA and Schwartz J (2003). Role of Wnt10b and C/EBPα in spontaneous adipogenesis of 243 cells. *Biochem. Biophs. Res.* 302: 12-16.

Birkenmeier EH, Gwynn B, Howard S and Jerry J (1989). Is CCAAT/enhancer-binding protein a central regulator of energy metabolism. *Genes Dev.* 3: 1146-1156.

Chui PC, Guan HP, Lehrke M and Lazar MA (2005). PPARgamma regulates adipocyte cholesterol metabolism via oxidized LDL receptor 1. *J. Clin. Invest.* 115: 2244-2256.

Chumakov AM, Grillier I, Chumakova E, Chih D, et al. (1997). Cloning of the novel human myeloid-cell-specific C/EBP-epsilon transcription factor. *Mol. Cell Biol.* 17: 1375-1386.

Croniger CM, Millward C, Yang J, Kawai Y, et al. (2001). Mice with a deletion in the gene for CCAAT/enhancer-binding protein beta have an attenuated response to cAMP and impaired carbohydrate metabolism. *J. Biol. Chem.* 276: 629-638.

Gomez-Santos C, Barrachina M, Gimenez-Xavier P, Dalfo E, et al. (2005). Induction of C/EBP beta and GADD153 expression by dopamine in human neuroblastoma cells. Relationship with alpha-synuclein increase and cell damage. *Brain Res. Bull.* 65: 87-95.

Hanson RW (1998). Biological role of the isoforms of C/EBP minireview series. *J. Biol. Chem.* 273: 28543.

Hu BL and Zan LS (2001). Association Analysis Between Bovine Carcass Traits and Meat Quality Traits. Master’s thesis. Northwest A&F University, Yangling.

Imai T, Takakuwa R, Marchand S, Dentz E, et al. (2004). Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. *Proc. Natl. Acad. Sci. U. S. A.* 101: 4543-4547.

Julie L, Kirstin E and Nick AS (2009). Real-Time PCR: Current Technology and Applications. Caister Academic Press, Norfolk.

Lee CH, Olson P and Evans RM (2003). Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. *Endocrinology* 144: 2201-2207.

Lefterova MI, Zhang Y, Steger DJ, Schupp M, et al. (2008). PPARgamma and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes Dev.* 22: 2941-2952.

Lekstrom HJ and Xanthopoulos KG (1998). Biological role of CCAAT/enhancer-binding protein family of transcription

factors. *J. Biol. Chem.* 273: 28545-28548.
Lin FT, MacDougald OA, Diehl MA and Lane MD (1993). A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc. Nat. Acad. Sci. USA* 90: 9606-9610.
Lopez RG, Garcia-Silva S, Moore SJ, Bereshchenko O, et al. (2009). C/EBPα and beta couple interfollicular keratinocyte proliferation arrest to commitment and terminal differentiation. *Nat. Cell Biol.* 11: 1181-1190.
MacDougald OA and Lane MD (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Annu. Rev. Biochem.* 64: 345-373.
Poli V (1998). The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J. Biol. Chem.* 273: 29279-29282.
Ramji DP and Foka P (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* 365: 561-575.
Rosen ED, Hsu CH, Wang X, Sakai S, et al. (2002). C/EBPα induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev.* 16: 22-26.
Shimano H (2001). Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog. Lipid Res.* 40: 439-452.
Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, et al. (2005). Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Res.* 15: 1034-1050.
Storch J and Thumser AE (2000). The fatty acid transport function of fatty acid-binding proteins. *Biochim. Biophys. Acta* 1486: 28-44.
Taniguchi Y and Sasaki Y (1996). Rapid communication: nucleotide sequence of bovine C/EBP alpha gene. *J. Anim. Sci.* 74: 2554.
Williams SC, Cantwell CA and Johnson PF (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers *in vitro*. *Genes Dev.* 5: 1553-1567.
Yeh WC, Cao Z, Classon M and McKnight SL (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* 9: 168-181.
Zuo Y, Qiang L and Farmer SR (2006). Activation of CCAAT/enhancer-binding protein (C/EBP) alpha expression by C/EBP beta during adipogenesis requires a peroxisome proliferator-activated receptor-gamma-associated repression of HDAC1 at the C/ebp alpha gene promoter. *J. Biol. Chem.* 281: 7960-7967.