Cloning, Characterization of Landes Goose C/EBPβ gene and its mRNA Expression response to Dietary Betaine Supplementation in Fatty Liver

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Abstract—The protein synthesised from the C/EBPβ gene interacts specifically with a C/EBP-recognition sequence and acts as a transcriptional activator. It plays a key role in lipometabolism and therefore is considered as a candidate gene for goose fat liver forming. In order to get the sequence, four pairs specificity primers were designed for amplification of C/EBPβ gene in Landes geese. The fragments of C/EBPβ gene in geese were obtained by PCR amplification and splicing. The length of the complete DNA sequence was 2130 bp (Genebank accession number GU068582), including the promoter regions and a 984 bp open reading frame coding 327 amino acid(aa). High percentages of G and C nucleotides were found in the coding region and 5'flanking region comprising a CpG island. The corresponding mRNA is present at high levels in the liver and adipose tissue compared to others. This indicated that it was closely related with lipid metabolism. In liver, the expression of C/EBPβ mRNA was higher in geese overfed than that of control group (P<0.01). Compared with the overfed groups, the geese that were fed the betaine supplemented diet showed the decreased expression on the transcriptive level in the livers.

Keywords- Overfeedings; Landes geese; C/EBPβ gene; Betaine

I. INTRODUCTION

Geese fat livers (also known as “foie gras”), regarded as one of 3 dainties in the world, have dedicate texture, beautiful flavor and contain plenty of unsaturated fatty acid. Now, the study of genes of goose fat liver forming was mostly focused on lipoprotein lipase (LPL) genes (Davail et al., 2000), the thyroid hormone responsive Spot 14 genes(Su et al., 2009), Adipocyte fatty acid-binding protein(ap2) genes(Su et al., 2009), but these are not enough to illustrate the mechanism of goose fat liver forming. Therefore, more genes that regulate the fatness trait need to be studied.

Betaine was presumed to act both as a methyl donor and as an osmoprotectant, it was initially introduced to the feed industry as a replacement for methionine and choline in poultry and fish diets (Kidd et al.,1997). In addition, betaine enhances the synthesis of methylated compounds including carnitine and phospholipids (Carter et al., 1995; Chiang et al., 1996). Moreover, betaine has been accepted as a hepatoprotective agent against alcoholic (Barak et al.,1997) and non-alcoholic steatosis (Neuschwander-Tetri, 2001). Therefore, betaine can be used to enhance the resistance to imbalance between lipid synthesis (increased) and secretion (reduced) due to its hepatoprotective effect.

The C/EBP subfamily includes structurally similar but genetically and functionally distinct proteins - C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ. The gene of them contain a highly conserved carboxyl-terminal basic-leucine zipper (bZIP) domain that consists of a basic region, involved in DNA recognition, and an adjacent helical structure, the leucine zipper, that mediates subunit dimerisation (Wandel and Ziegler-Heitbrock et al.,1995). The pleiotropic transcriptional effects of C/EBP result from different mechanisms, such as their tissue and embryonic developmental stage-specific expression, leaky ribosomal reading, posttranscriptional modifications, and variable DNA binding specificities (Lekstrom-Himes et al., 1998). Deletion of the C/EBPβ gene in mice causes death in utero, largely due to defective gluconeogenesis and adipogenesis (Croniger et al., 1997). Loss of C/EBPβ in mice also causes defective development and differentiation of hepatocytes (Diehl et al., 1998), myelomonocytes (Natsuka et al., 1992), adipocytes (Darlington et al., 1998), and neurons (Taubenfeld et al., 2001). Given the diverse effects of C/EBPβ, it is conceivable that its association with different cellular factors in a gene context and signal-specific manner may regulate its activity. A number of in vitro studies demonstrate that C/EBPs are induced during adipocyte differentiation and play key roles in adipocyte differentiation (Z. Cao et al., 1991; C. Manchado et al., 1994; W.C. Yeh et al., 1995; E. J Schwarz et al., 1997).

In this study, we cloned the DNA of landes goose C/EBPβ, analyze its sequence characteristics, tissue distribution, and determined the effects of dietary betaine supplementation on the mRNA expression level of C/EBPβ in landes goose fatty liver.

II. MATERIALS AND METHODS

A. Animals

A total of 18 healthy male Landes geese (Anser anser; BW=4.0±0.01 kg), obtained from Xingyun Jiangsu group, were

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fed a commercial diet to the age of 10 weeks. From 10 to 12 wk, the feed restriction was released progressively to increase the volume of the digestive tract and to initiate the metabolic adaptation to overfeeding. At 13 weeks (85 d), the geese were divided into three groups. Geese in the first group (n=6) continued the control diet and were allowed to feed ad libitum (150 g/d) on a diet containing 2600 kcal and 138 g/kg protein and up to 500 g/kg grass. The remaining geese (n=12) were switched to a overfeeding diet (420 g/d), which consisted of two thirds salted and boiled maize (3370 kcal/kg, 90 g protein/kg and 4.5g fat/kg) to which 0.4% waterfowl fat was added and one-third (by volume) of water. Geese fed the overfeeding diet were fed six meals per day for three weeks (with the overfeeding diet without betaine). Of the geese that were fed the overfeeding diet, six were also fed betaine (Genetime Biotech Co., Nanjing, China) as a dietary supplement (1g/d/goose).At week 15, all the geese were slaughtered for the collection of liver of All individuals and the tissues of first group: pituitary gland, skeletal muscle, heart, liver, spleen, spinalcord, lung, and kidney. The protocol for the treatment of geese was approved by the Nanjing Agricultural University Animal Care and Use Committee. The tissues were collected, snap frozen in liquid nitrogen, and stored at -80 °C.

B. DNA : RNA isolation and RT-PCR

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C. Real-time Quantitative PCR

Primer sequences were designed using Primer 5.0 and synthesized by TaKaRa (table 1). The specificity of the amplification product was further verified by electrophoresis on a 0.8% agarose gel and cDNA sequencing. Results for each gene are presented as ratios relative to β-actin to correct for differences in the amounts of template cDNA used. Real-time quantitative PCR was carried out in a final volum of 20 μl containing 1μl RT, 1μl EX Taq HS DNA polymerase (TAKARA, Japan), 4 μl 5×PCR Buffer (100mM Tris-HCl pH8.3, 500 mM KCl) , 0.3mM dNTP, 3.75mM MgCl2, 0.5 Mm each primer. The PCR cycling program was as follows: Initial denaturation of 1min at 95°C, 40 cycles of 95°C for 10 s, annealing for 15s, 72°C for 15s, plate-reading every other 0.2 °C from 65°C to 94°C for drawing melting curves; final extension step of 72 °C for 5min.

D. Cloning and sequencing of PCR product

With on-line resource of NCBI, we designed 4 pairs of primers (table1) for geese according to the C/EBPβ gene sequence of chicken , Zebra Finch, Quail, human, and hamster in the Genebank. PCR products were separated by electrophoresis on a 1.0% agarose gel and purified with a gel extraction kit (Watson Biomedical Inc., Shanghai, China). The purified PCR product was ligated into pGEM-T vector (Invitrogen). The positive clone were sequenced on an automatic sequencer (ABI 3730, Invitrogen).

E. Sequencing and phylogenetic analysis

The nucleotide sequences of the clones were assembled and identified using the NCBI BLAST search program. Complete cDNA sequences were aligned using the DNAMAN software package (Lynnon Biosoft, Quebec, Canada). The GC content of the sequences was predicted with the MethPrimer design software (http://www.urogene.org/methprimer/index1.html). Amino acid similarities, including identities and positives, were analyzed with the BLASTP program in conjunction with the BLOSUM 62 scoring matrix. Multiple peptides were aligned with the ClustalW multiple sequence alignment program. Following ClustalW alignment using the minimum evolution method (Kumar, 2004), the p-distances (proportion of differences) with a high resolution of branching pattern were calculated. The reliability of the constructed tree was tested by bootstrap analysis using the MEGA 4.1 program.

III. RESULTS

A. Cloning and characterisation of the gC/EBPβ gene

After sequencing and assembling, the total coding region and 1.1 kb of 5’ flanking DNA of lande goose C/EBP β gene were obtained and submitted to GenBank (accession number: GU068582). Like the C/EBPβs from other species, the goose C/EBPβ gene is intronless and contains an uninterrupted reading frame of 984 bp. In the flanking sequences, a TATA- box and a CAP signal are present at 296 bp and 262 bp 5’ up to the gC/EBP β ORF. The sequence is shown as figure 1. The peptide strand of gC/EBPβ consists of 327 AA, with 34.89Kd molecular weight (MW) and 8.696 isoelectric point . The coding region and the 5’ flanking sequences of gC/EBPβ gene also had a high G+C content and a high CpG-dinucleotide frequency , indicating that the region represent a CpG island, just like C/EBPβs from other species.

B. Sequence and Evolutionary analysis of C/EBPβ

A comparison of the gC/EBPβ promoter sequence with that from rat, mouse, human, and chicken reveals an overall sequence identity between 25 and 62% (data not shown).

Despite lower overall sequence similarity was observed, a computer search of the gC/EBPβ promoter region for the presence of consensus recognition sequences of known transcription factors in the TRANSFAC database showed the existence of putative sites for several factors that was important for the expression of C/EBP genes (e.g., C/EBP, Sp1, MZF1, CREB, AP2 ).

Compared the predicted AA sequences with the sequences from other species available in the GenBank by DNAMAN software, the results was showed in Table 2 . The Percent similarity of AA sequences is from 64.6% to 99.4% among the 17 species. The similarity between goose and chicken is 93.7% at nucleotide level and 93.6% at protein level. There is higher
similarity between goose and Zebra Finch, which is 93.8% at nucleotide level and 97% at protein level.

In order to further evaluate the evolutionary relationships of C/EBPβ, a phylogenetic tree of the orthologous peptides for vertebrate C/EBPβ was constructed based on their amino acid sequence homologies (figure 2). In this tree, the two clusters were found: one cluster consisted of five kind of fish, another consisted of Mammalians, Amphibians and Birds. These distances revealed the evolutionary relationship of various species.

![Figure 2. Phylogenetic tree constructed by the neighbor-joining method based upon the amino acid sequences of C/EBPβ proteins in the 15 species above. The length of branch indicates evolutionary distance. Numbers above the line indicate percent bootstrap confidence values derived from 1000 replications.](image)

C. Tissue distribution of gC/EBPβ mRNA

The expression of gC/EBPβ mRNA in various tissues of normal landes goose was detected by real-time PCR. The result showed that gC/EBPβ mRNA were present in all the tissues analysed. However, the relative abundance of transcripts in the different tissues varied in the order: adipose tissue > liver > lung > heart > muscle > spleen / hypothalamus > kidney > stomachus muscularis. (figure 3)

D. mRNA expression level of C/EBPβ in Landes goose livers under different treatment

The real time PCR results for the relative abundance of mRNA transcripts of the C/EBPβ gene are shown in Table 3.

IV. DISCUSSION

The expression of this gene in the overfed geese was higher than that in the geese that were fed the control diet. Compared to the overfeeding groups, the geese that were fed the betaine diet showed decreased the expression of C/EBPβ mRNA in the fatty livers.

![Figure 3. The relative expression profile of gC/EBPβ mRNA analyzed by real-time PCR: The relative expression profile of gC/EBPβ mRNA was normalized by β-actin on to standardise the results. gC/EBPβ was analyzed in various tissues, including kidney (K), muscle (M), lung (L), liver (LI), stomachus (St), spleen (Sp), hypothalamus (Hy), adipose tissue (Ad) and heart (He). Each sample was repeated three.](image)
goose and mammals, indicating the function of the C/EBPβ in goose was similar in avian.

Indeed, expression analyses showed that the expression of gC/EBPβ mRNA in lipid metabolism important organs (adipose tissue and liver) has a high level. Indicates that it related with lipid metabolism closely.

The sequence of the gC/EBPβ gene region reveals that the gC/EBPβ coding region and the contiguous upstream regulatory region include a CpG island. Methylation groups may interfere with the binding of transcription factors to cognate target sites, thereby preventing the formation of an active transcription complex on the promoter (Comb and Goodman, 1990). Methylation of DNA has been shown to inhibit gene expression in vitro and in vivo (Eden et al., 1994; Razin et al., 1991). The methylation status of CpG islands was believed to play an important role in the regulation of gene expression during development and tissue differentiation (Becker et al., 1987; Li et al., 1992). And betaine can enhance the synthesis of methylated compounds including carnitine and phospholipids (Carter et al., 1995; Chiang et al., 1996). Because carnitine is required for transport of long-chain FAs into mitochondria, where they are degraded via β-oxidation, thus, betaine might be integrally involved in lipid metabolism via its role in phosphatidylcholine synthesis and in FA oxidation (Carter et al., 1995). In addition, the methylation of high GC content containing sequences may be correlated with gene mRNA expression and the suppression of transcription (Kawane et al., 2005; Le and Maizel, 2007). What’s more betaine is a hepatoprotective agent against alcoholic (Barak et al., 1997) and non-alcoholic steatosis (Neuschwander-Tetri et al., 2001).

Based on the reasons mentioned above, we determined the effects of dietary betaine supplementation on the mRNA expression level of C/EBPβ in landes goose fatty liver.

Our laboratory has reported effect of overfeeding on the transcriptional expression of ap2 gene in Landes Goose liver (Su et al., 2009). And the effect of betaine supplementation on the generation and development of fatty livers in geese, identified several significant differences between the livers of Landes geese fed an overfeeding diet (+) betaine and the livers of geese that were fed an overfeeding diet (−) betaine (Su et al., 2009). Experiments presented in this article demonstrated that overfeeding could increased the transcriptional expression of C/EBPβ gene in Landes Goose liver, was consistent with ap2 gene and Spot14 gene. However, it was found that dietary betaine supplementation could increase the expression of Spot14 mRNA, just opposite to the result of C/EBPβ gene in this article. This result means that the regulatory mechanisms of their might be different. Thus, epigenetic analysis of the C/EBPβ gene would be required to a further study of lipid metabolism in geese.

### TABLE I. PRIMERS USED FOR THE CLONING AND EXPRESSION OF THE LANDES GOOSE C/EBPβ GENE

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Anneal. T (°C)</th>
<th>Fragment Length</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F: 5’- ACCACGGATTTGTTATGGACT-3’ R: 5’- TTGAAGGTGTCCTCGTGGAT-3’</td>
<td>65</td>
<td>398bp</td>
<td>M26111</td>
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<tr>
<td></td>
<td>F: 5’- GCTTCTCTCCACAACTCAC-3’ R: 5’- AAAATGCTAGCTGCTGTAT-3’</td>
<td>62</td>
<td>816bp</td>
<td>GU068582</td>
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<tr>
<td></td>
<td>F: 5’- CTGTTGTATGGGATTCGTTA-3’ R: 5’- GGCTGCTGGGATGTGCTAA-3’</td>
<td>60</td>
<td>546bp</td>
<td>GU068582</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>F: 5’- CGGACTGTGTGGCTGCTGTC-3’ R: 5’- GCTGCTGACACCCCTTCTTC-3’</td>
<td>63</td>
<td>812bp</td>
<td>GU068582</td>
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<tr>
<td></td>
<td>F: 5’- GACAAAGTCCTGGAAGTCTGTA-3’ R: 5’- CACTTTCGCCCTGGCTGTC-3’</td>
<td>60</td>
<td>403bp</td>
<td>GU068582</td>
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<tr>
<td></td>
<td>F: 5’- GACAACACACGGACGAGAT-3’ R: 5’- AGCTGCTCCACCTTCTTC-3’</td>
<td>64</td>
<td>158bp</td>
<td>AY212285</td>
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</table>

### TABLE II. AMINO ACID SIMILARITIES OF GOOSE C/EBPβ WITH THOSE OF C/EBPβs OF OTHER SPECIES

<table>
<thead>
<tr>
<th>source</th>
<th>Length of protein</th>
<th>Sequence similarities(%)</th>
<th>GenBank Accession NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimpanzee [Pan troglodytes]</td>
<td>345</td>
<td>62.07</td>
<td>XP_525353</td>
</tr>
<tr>
<td>Human [Rattus norvegicus]</td>
<td>345</td>
<td>62.07</td>
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<tr>
<td>Rat [Rattus norvegicus]</td>
<td>297</td>
<td>59.36</td>
<td>NP_077039</td>
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<tr>
<td>Mouse [Mus musculus]</td>
<td>296</td>
<td>58.91</td>
<td>NP_034013</td>
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<tr>
<td>Cattle [bos taurus]</td>
<td>348</td>
<td>60.00</td>
<td>NP_789745</td>
</tr>
<tr>
<td>African clawed frog [Xenopus laevis]</td>
<td>288</td>
<td>52.71</td>
<td>NP_001089384</td>
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<tr>
<td>Monodelphis [Monodelphis domestica]</td>
<td>364</td>
<td>65.12</td>
<td>XP_001369325</td>
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<td>Zebrafish [Danio rerio]</td>
<td>280</td>
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<tr>
<td>Zebra Finch [Taeniopygia guttata]</td>
<td>324</td>
<td>96.04</td>
<td>XP_002187599</td>
</tr>
<tr>
<td>Chicken [gallus gallus]</td>
<td>328</td>
<td>96.66</td>
<td>NP_990584</td>
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### TABLE III. EFFECT OF BETaine TREATMENT ON C/EBPβ GENE EXPRESSION IN LIVER OF LANDES GEESE

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Overfeeding treatment</th>
<th>Overfeeding+betaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPβ</td>
<td>20.07±4.66</td>
<td>0.22±0.05</td>
<td>0.24±0.11</td>
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</table>

* Mean ± standard error of the relative abundance of gene transcripts in Landes goose liver. Each point represents an average of 6 observations (n=6).
* The values in table 2 stand for expression of the specialized gene in liver for Within a row, Capital letter notify the very significantly different (P<0.01), small letter is significantly different (P<0.05)
REFERENCES


