Identification and characterization of transcript variants of chicken peroxisome proliferator-activated receptor gamma

Kui Duan,[*,†] Yingning Sun,[*,†,‡] Xiaofei Zhang,[*,†] Tianmu Zhang,[†,1] Wenjian Zhang,[*,†] Jiyang Zhang,[*,†] Guihua Wang,[*,†] Shouzhi Wang,[*,†] Li Leng,[*,†] Hui Li,[*,†] and Ning Wang[*,†,1]

[*]*Key Laboratory of Chicken Genetics and Breeding, Ministry of Agriculture;* [†]*Key Laboratory of Animal Genetics, Breeding and Reproduction, Education Department of Heilongjiang Province. College of Animal Science and Technology, Northeast Agricultural University, Harbin, Heilongjiang 150030, China; and* [‡]*College of Life Science and Agriculture Forestry, Qiqihar University, Qiqihar, Heilongjiang 161006, China*

ABSTRACT Peroxisome proliferator-activated receptor gamma regulates adipogenesis. The genomic structure of the chicken peroxisome proliferator-activated receptor gamma (cPPARγ) gene has not been fully characterized, and only one cPPARγ gene mRNA sequence has been reported in genetic databases. Using 5′ rapid amplification of cDNA ends, we identified five different cPPARγ mRNAs that are transcribed from three transcription initiation sites. The open reading frame analysis showed that these five cPPARγ transcript variants (cPPARγ1 to 5) could encode two cPPARγ protein isoforms (cPPARγ1 and cPPARγ2), which differ only in their N-terminal region. Quantitative real-time RT-PCR analysis showed that, of these five cPPARγ transcript variants, cPPARγ1 was ubiquitously highly expressed in various chicken tissues, including adipose tissue, liver, kidney, spleen and duodenal; cPPARγ2 was exclusively highly expressed in adipose tissue; cPPARγ3 was highly expressed in adipose tissue, kidney, spleen and liver; cPPARγ4 and cPPARγ5 were ubiquitously weakly expressed in all the tested tissues, and comparatively, cPPARγ5 was highly expressed in adipose tissue, heart, liver and kidney. The comparison of the expression of the five cPPARγ transcript variants showed that adipose tissue cPPARγ1 expression was significantly higher in the fat line than in the lean line from 2 to 7 wk of age ($P < 0.05$ or $P < 0.01$). Adipose tissue cPPARγ3 expression was significantly higher in the fat line than in the lean line at 3, 5 and 6 wk of age ($P < 0.01$, $P < 0.05$), but lower at 4 wk of age ($P < 0.05$). Adipose tissue cPPARγ5 expression was significantly higher in the fat line than in the lean line at 3, 4, and 6 wk of age ($P < 0.01$) and at 2 and 7 wk of age ($P < 0.05$). This is the first report of transcript variants and protein isoforms of cPPARγ gene. Our findings provided a foundation for future investigations of the function and regulation of cPPARγ gene in adipose tissue development.

Key words: chicken, PPARγ, adipose, transcript variant

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INTRODUCTION

The peroxisome proliferator activated receptors gamma (**PPARγ**) are a member of the nuclear receptor family of ligand-activated transcription factors. PPARγ, the primary regulator of adipogenesis and lipogenesis in mammals and birds, plays important roles in the development of obesity (Barak et al., 1999; Rosen and Spiegelman, 2001; Rosen et al., 2002; Farmer, 2006; Rosen and MacDougald, 2006). The PPARγ protein forms obligate heterodimers with the retinoid X receptor to regulate the transcription of genes involved in adipocyte differentiation and glucose and lipid metabolism (Tontonoz et al., 1994a; DiRenzo et al., 1997; Rosen et al., 1999; IJpenberg et al., 2004). The PPARγ protein is activated by various fatty acid metabolites, and its activity is regulated by post-translational modifications, including phosphorylation and sumoylation (Rocchi and Auwerx, 1999; Rosen et al., 1999; Imai et al., 2004).

Multiple mRNAs are transcribed from mammalian *PPARγ* genes due to differential promoter usage and alternative splicing (Sundvold and Lien, 2001). These PPARγ transcript variants share exons 1 to 6, but differ in their 5′-terminal regions, including their 5′-untranslated region (5′-UTR). The transcription of exons A1, A2, and B is driven by different promoters (Fajas et al., 1997; Fajas et al., 1998; Sundvold and Lien, 2001). Four mRNA variants are transcribed from the human *PPARγ* gene (***hPPARγ***). The hPPARγ1 transcript variant consists of exons A1 and A2 and exons 1 to 6. The hPPARγ2 transcript variant consists of exon B and exons 1 to 6. The hPPARγ3 and hPPARγ4

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[1]Corresponding author: wangning@neau.edu.cn

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transcript variants are unique to humans, and consist of exon A2 spliced with exons 1 to 6 and exons 1 to 6 only, respectively. Three mRNA variants are transcribed from the mouse *PPARγ* gene (***mPPARγ***). The mPPARγ1, mPPARγ2, and mPPARγ3 transcript variants consist of exons A1 and A2, exon B1, and exons C and A2 spliced with exons 1 to 6, respectively (Zhu et al., 1995; Takenaka et al., 2013).

Two protein isoforms, PPARγ1 and PPARγ2, are encoded by the mammalian *PPARγ* gene. The N-terminal amino acid sequences and functions of the PPARγ1 and PPARγ2 protein isoforms differ. Compared with the PPARγ1 protein isoform, the PPARγ2 protein contains an additional 28 or 30 amino acids at the N-terminus. PPARγ1 is ubiquitously expressed in tissues including adipose tissue, whereas the mammalian PPARγ2 protein is exclusively expressed in adipose tissue. PPARγ1 and PPARγ2 in vivo drive adipose tissue development, and in addition, PPARγ1 has been shown to function in energy metabolism (Yu et al., 2003; Sasaki et al., 2014). PPARγ2 has stronger transcription activation activity than PPARγ1, and plays the dominant role in adipogenesis (Tontonoz et al., 1994b). The mammalian PPARγ2 protein expression is regulated primarily by nutritional state (Tontonoz et al., 1994b). Gene knockout and mutational studies have shown that the PPARγ2 protein is important for maintaining insulin sensitivity (Werman et al., 1997; Zhang et al., 2004a).

Although *PPARγ* has been intensively studied in mammals, the chicken *PPARγ* gene (***cPPARγ***) has not been fully characterized. To date, only one cPPARγ mRNA sequence has been deposited in The National Center for Biotechnology Information (NCBI) database (accession no. NM_001001460.1). This cPPARγ transcript sequence consists of six exons, all of which are common to the mammalian PPARγ transcript variants. Previous studies have not detected cPPARγ2 in the chicken adipose tissues (Sato et al., 2004; Takada and Kobayashi, 2013), suggesting that chickens may lack a PPARγ2 protein isoform. However, recent studies in several laboratories, including ours, have identified at least two bands in Western blots of chicken adipose tissue lysates using an anti-PPARγ antibody (Matsubara et al., 2008). Based on these observations, we hypothesized that multiple transcript variants and protein isoforms, including a PPARγ2 protein, are expressed from *cPPARγ*, in a manner similar to that observed for the mammalian orthologs. In our current study, we identified and characterized a total of five cPPARγ transcript variants and two cPPARγ protein isoforms. Our findings create a foundation for future studies of the function and regulation of *cPPARγ* in adipose tissue development.

MATERIALS AND METHODS

Experimental Animals and Tissue Collection

Our animal experiments were conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (approval no. 2006–398), and were approved by the Laboratory Animal Management Committee of Northeast Agricultural University (Harbin, Heilongjiang, PRC). One group of 56 chickens from the 18th generation of the fat NEAUHLF broiler breeding line and one group of 56 chickens from the 18th generation of the lean NEAUHLF broiler breeding line were used in our study. The two broiler lines had been generated previously by divergent selection based on abdominal fat content and plasma level of very low-density lipoprotein at Northeast Agricultural University (Guo et al., 2011)

These birds were housed under identical environmental conditions with free access to food and water. They were fed a commercial and soybean-based diet that met all of the NRC requirements (Applegate and Angel, 2014). The birds received a starter diet of 3000 kcal ME/kg and 210 g/kg CP until they reached 3 wk of age. They were fed a grower diet of 3100 kcal ME/kg and 190 g/kg CP from 3 to 7 wk of age (Wang et al., 2006). Once each week, five to eight birds from each group were sacrificed after fasting for 6 h, and samples of abdominal adipose tissue were collected from each bird. For the 7-wk-old birds, samples were also collected from the heart, liver, spleen, lung, kidney, duodenal, brain, pectoralis muscle, pancreas, and proventriculus. After washing with a solution containing 0.75% NaCl, the tissues were snap-frozen in liquid nitrogen, and stored at -80°C.

RNA Extraction

Total RNA was extracted from 100 mg of frozen tissue using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The quality of the purified total RNA was assessed by electrophoresis in a denaturing formaldehyde agarose gel, based on visual comparison with the 18S and 28S ribosomal RNAs. The concentration of the total RNA was determined using an ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany), based on the ratio of the optical density at 260 nm to that at 280 nm (OD260/280).

Determination of the 5′ Terminal Sequences of cPPARγ mRNAs

Only RNA with an OD260/280 of 1.8 to 2.1 was used for reverse transcription (RT) and a polymerase chain reaction (PCR). Complementary DNA (**cDNA**) was reverse transcribed using oligo (**dT**) anchor primer, random hexamer primer, reverse transcription (Roche Molecular Systems, Pleasanton, CA, USA), and total RNA as template at 25°C for 30 min. The RNA was degraded by incubation at 50°C for 1 h, and the enzyme was inactivated by heating at 85°C for 5 min. The 5′-terminal sequences of the cPPARγ transcripts were obtained using rapid amplification

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Table 1. The primers used in the present study.

Name	Accession number	Sequence
5′RACE primer		
GSP1-R1	NM_001001460.1	R:5′ TGTCTGTCGTCTTTCCTGTC 3′
GSP1-R2	NM_001001460.1	R:5′ GCTTTTCTTATGGATGC 3′
GSP1-R3	NM_001001460.1	R:5′ CACGACATTCAATAGCCA 3′
GSP2-R1	NM_001001460.1	R:5′ CTGCTTTTCTTATGGATGCGACAAT 3′
GSP2-R2	NM_001001460.1	R:5′ CCACACACACGACATTCAATAGCGATA3′
GSP2-R3	NM_001001460.1	R:5′ TGAAAAATCAACAGTGGTAAATGGCTT3′
AAP		R:5′ GGCCACGCGTCGACTAGTACGGGI IGGGIIGGGIIG3′
Real-time primer		
NONO	NM_001031532.1	F: 5′ AGAAGCAGCAGCAAGAAC3′ R: 5′ TCCTCCATCCTCCTCAGT3′
cPPARγ1-F/R	KP736526	F: 5′ GGAGTTTATCCCACCAGAAG 3′ R: 5′ AATCAACAGTGGTAAATGGC 3′
cPPARγ2-F/R	KP736527	F: 5′ TGCGTTAGGAGAAGGGAGAGAA 3′ R: 5′ TCTGTGTCAACCATTGTAATCTCTCTT 3′
cPPARγ3-F/R	KP736528	F: 5′ GAGCCTGCAACAAATTACAATGG 3′ R: 5′ AGAAAAATAGGGAGGAGAAGGAGG 3′
cPPARγ4-F/R	KP736524	F: 5′ GTGAGATGCCGTGTTGAAATGTA 3′ R: 5′ GAAAAATCAACAGTGGTAAATGGC 3′
cPPARγ5-F/R	KP736525	F: 5′ ATCTTGGGCTCAAACACATTACAG 3′ R: 5′ AAAATAGGGAGGAGAAGGAGGC 3′
RT-PCR primer		
PPARγ1TGA	KP736526	F: 5′ CCCACCAGAAGGGAACAGTT 3′ R: 5′ ACACACGACATTCAATAGCC 3′
PPARγ5RT	KP736525	F: 5′ ATCTTGGCTCTTTGTGCA 3′ R: 5′ ATTTGTCTGTCGTCTTTCCT 3′

The cPPARγ gene-specific antisense primer sequences (GSP) and anchor primer (invitrogen) were used for 5′RACE analysis. Primer sequences (F = forward, R = reverse) for real-time RT-PCR and RT-PCR analyses of the five cPPARγ transcript variants (cPPARγs 1 to 5).

of cDNA ends (5′-RACE). The procedure was performed using the 5′-RACE system, version 2.0 (Invitrogen), according to the manufacturer's instructions. Three gene-specific antisense primers, GSP1-R1, GSP1-R2, and GSP1-R3, and the corresponding gene-specific nested antisense primers, GSP2-R1, GSP2-R2, and GSP2-R3, were designed based on the previously reported cPPARγ mRNA sequence (Table 1). The primers were synthesized by Invitrogen. The RT procedure was performed using GSP1-R1, GSP1-R2, GSP1-R3, and 5 μg of total RNA from chicken abdominal adipose tissue. The 5′-RACE PCR was performed using the AAP sense abridged anchor primer (Invitrogen) and the GSP2-R1, GSP2-R2, and GSP2-R3 nested antisense primers. Thermal cycling was performed using an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 61°C (or 63°C) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min in a T-gradient thermal cycler (Biometra, Göttingen, Germany). The PCR products were separated by electrophoresis in a 1.5% agarose gel, and purified using the Gel DNA Purification Kit (Corning Life Sciences-Axygen, Union City, CA, USA). The purified cDNA fragments were inserted into the pEasy-T1-simple plasmid (TransGen, China), and competent *Escherichia coli* were transformed using the ligation product. The DNA sequences of recombinant plasmids were determined by automated DNA sequencing.

Quantification of cPPARγ Expression in Various Tissues

Quantitative RT-PCR (**qRT-PCR**) was performed in a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using Roche FastStart Universal SYBR Green Master Mix (Roche Molecular Systems). To determine the relative expression of the cPPARγ transcript variants, the five cPPARγ transcript variant-specific primer pairs were designed. All primers used in the present study are listed in Table 1. Thermal cycling was performed using an initial incubation at 50°C for 2 min, followed by denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The dissociation curve for each PCR reaction was analyzed using the Dissociation Curve 1.0 software (Applied Biosystems) to detect and eliminate possible primer-dimer artifacts. The qRT-PCR analysis was performed in triplicate. The non-POU domain containing octamer-binding mRNA (NONO) was used as the internal mRNA control, and the relative expression of the target mRNA to that of the NONO mRNA was determined using the ΔCT method (Schmittgen and Livak, 2008), in which ΔCT = CT (target gene) − CT (NONO). The data are presented as the mean ± SD (Standard Deviation). Intergroup differences in target gene expression were evaluated based on the results of two-tailed unpaired Student t-tests using the Graph Pad Prism, version 5.0, software (San Diego, CA).

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Results with $P < 0.05$ were considered statistically significant, and those with $P < 0.01$ were considered highly significant.

Bioinformatics Analysis

The NCBI Sequence Read Archive (SRA) databases were searched with the previously reported cPPARγ mRNA sequence, using the method described in references (Trapnell et al., 2012; Trapnell et al., 2013). The retrieved sequences were aligned with the cPPARγ mRNA sequence using the DNAMAN, version 6.0, software (Lynnon, Quebec, Canada), and analyzed using the ClustalW2 computational tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Gene structure was analyzed using the UCSC genome browser (http://genome.ucsc.edu/). The open reading frames (ORFs) were analyzed using the StarORF computational tool (http://star.mit.edu/index.html). The amino acid sequences and protein domains were analyzed based on comparisons to those in the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and other external databases (Pfam, SMART, COG, PRK, and TIGRFAM). The CpG islands were predicted using the CpG Island searcher (http://cpgislands.usc.edu/) and Emboss CPGplot (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) programs. Transcription factor binding sites were predicted using the JASPAR program (http://jaspar.genereg.net), and promoter homology was analyzed using the Mulan program (http://mulan.dcode.org).

RESULTS

Identification of cPPARγ Transcript Variants

To determine the 5′-terminal sequence of the cPPARγ mRNAs, based on the known cPPARγ mRNA sequence (NM_001001460.1), we performed 5′-RACE on the total RNA extracted from chicken abdominal adipose tissue with three cPPARγ gene-specific primers, respectively. The 5′-RACE PCR products were cloned into T vector and transformed into E *coli.* A total of 167 recombinant plasmids were sequenced, and five distinct PCR products were identified that were 587 bp (107 colonies), 497 bp (17 colonies), 638 bp (20 colonies), 602 bp (3 colonies), and 375 bp (20 colonies) in size. Comparisons of these sequences with the NM_001001460.1 transcript showed that, as expected, the 3′-terminal sequences of the 5′-RACE PCR products contained sequences of exons 1 to 3, exons 1 and 2, or exon 1, respectively, of the known chicken mRNA sequence (NM_001001460.1). We assembled these 5′-RACE product sequences and the known chicken mRNA sequence, and generated a total of five different cPPARγ transcript variants, designated as cPPARγ1, cPPARγ2, cPPARγ3, cPPARγ4, and cPPARγ5 (Figure 1), the nucleotide sequences of which only differed in their 5′-terminus.

Characterization of cPPARγ Genomic Structure

To reveal the molecular events of the expressional regulation of *cPPARγ* transcript variants, we

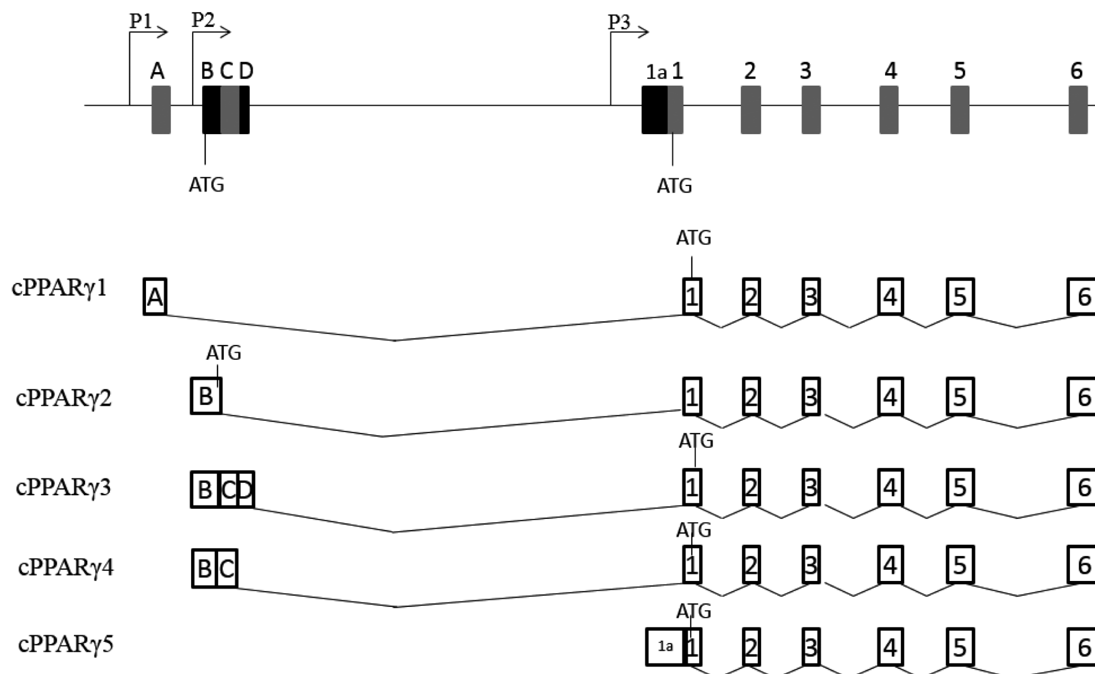

Figure 1. The exons of five cPPARγ transcript variants on chicken chromosome 12. The initiation codon ATGs are marked, cPPARγ1 consists of exon A spliced together with 1 to 6 exons; cPPARγ2 consists of exon B and exons 1 to 6; cPPARγ3 consists of exon B/C/D spliced together with 1 to 6 exons; cPPARγ4 consists of exon B/C spliced together with exons 1 to 6; cPPARγ5 consists of exon 1a and exons 1 to 6.

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determined the genomic structure of the *cPPARγ* gene. We aligned these five PPARγ transcript variant sequences against chicken genome sequence, respectively, using the blat search tool at the UCSC genome browser. As expected, all these five transcript variants were mapped to the chicken *PPARγ* locus on chromosome 12 in the Gallus gallus genome database (ICGSC Gallus_gallus-4.0/galGal4). The alignment showed that *cPPARγ* gene spans a region approximately 51 kb in size. The four cPPARγ transcript variants (cPPARγs 1 to 4) consisted of seven exons, whereas cPPARγ5 consisted of six exons only (Figure 1). These five cPPARγ transcript variants only differed in their 5′-most exons. The names and sizes of the identified 5′-most exons were as follows: exon A (109 bp); exon B (150 bp); exon B/C/D (292 bp); exon B/C (256 bp); and exon 1a (244 bp), which is fused to exon 1 (472 bp).

The cPPARγ1 transcript variant consisted of exon A spliced with exons 1 to 6. The cPPARγ2 transcript variant consisted of exon B spliced with exons 1 to 6. The cPPARγ3 transcript variant consisted of exons B/C/D spliced with exons 1 to 6. The cPPARγ4 transcript variant consisted of exon B/C spliced with exons 1 to 6, and the cPPARγ5 transcript variant consisted of exon1a and exons 1 to 6. All the introns followed the GT-AG rule. The 5′-UTRs of cPPARγs 1 to 5 were 117, 141, 300, 264, and 252 bp in size, respectively. As shown in Figure 1, the 5′UTR of cPPARγ1 consisted of exon A and a portion of exon 1. The 5′UTR of cPPARγ2 consisted of a portion of exon B. The 5′UTR of cPPARγ3 consisted of a portion of exon B/C/D and a portion of exon 1. The 5′UTR of cPPARγ4 consisted of exon B/C and a portion of exon 1, and the 5′UTR of cPPARγ5 consisted of exon 1a and a portion of exon 1.

Sequence Analysis of cPPARγ Transcript Variants

First, based on the above identified genomic structure of cPPARγ, the five variant-specific primer pairs, cPPARγ1TGA-F/R, cPPARγ2-F/R, cPPARγ3-F/R, cPPARγ4-F/R, and cPPARγ5RT-F/R (Table 1) were designed, and their locations are shown in Figure 2. Using these five variant-specific primer pairs, respectively, we performed RT-PCRs on total RNA extracted from chicken abdominal adipose tissue, and the resultant

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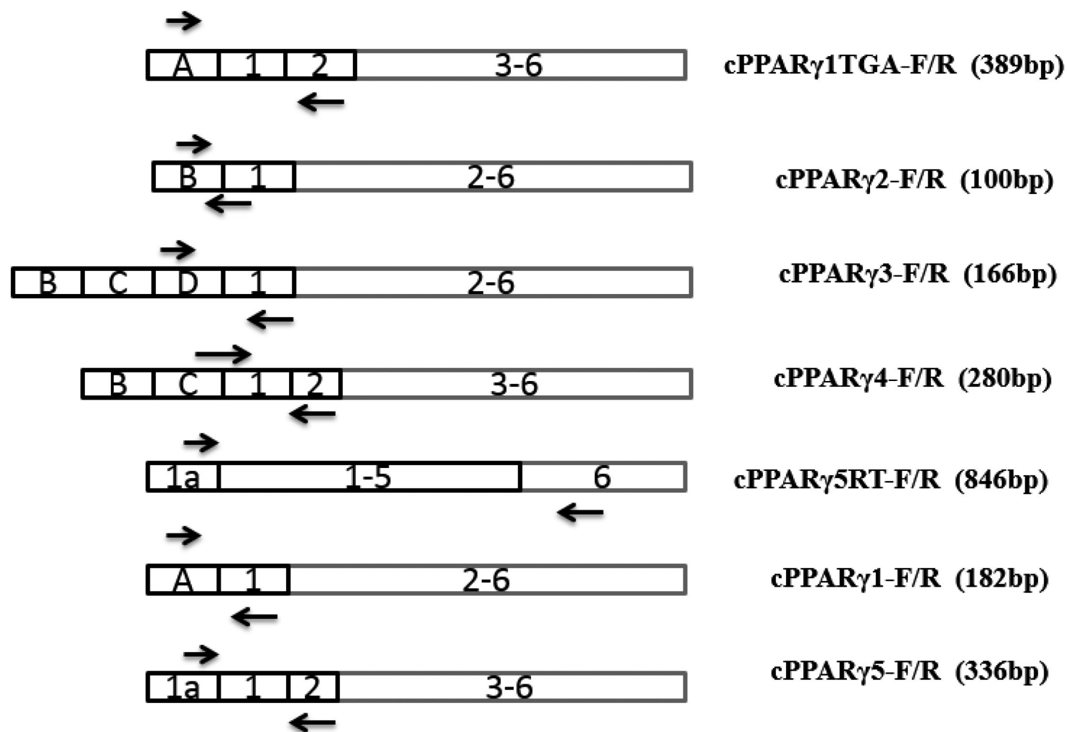

Figure 2. Schematic locations of PCR primers used in the present study. The cPPARγ1TGA-F/R primer pair was designed to identify the cPPARγ1 transcript. The cPPARγ1TGA-F primer is complementary to exon A, and the cPPARγ1TGA-R primer complementary to exon 2. The cPPARγ2-F/R primer pair was designed to validate the cPPARγ2 transcript. The cPPARγ2-F is complementary to exon B, and the cPPARγ2-R primer is complementary to the junction of exons B and 1. The cPPARγ3-F/R primer pair was designed to validate the cPPARγ3 transcript. The cPPARγ3-F is complementary to exon D, and the cPPARγ3-R is complementary to exon 1. The cPPARγ4-F/R primer pair was designed to validate the cPPARγ4 transcript. The cPPARγ4F is complementary to the junction of exons C and 1, and the cPPARγ1-R is complementary to exon 2. The cPPARγ5RT-F/R primer pair was designed to validate the cPPARγ5 transcript. The cPPARγ5RT-F primer is complementary to exon 1a and the cPPARγ5RT-R is complementary to exon 6. The cPPARγ1-F/R primer pair was designed to detect the relative expression of the cPPARγ1 transcript. The cPPARγ1F primer is complimentary to exon A, and the cPPARγ1-R is complimentary to exon 1. The cPPARγ5-F/R primer pair was used to detect the cPPARγ5 transcript expression. The PPARγ5-F is complimentary to exon 1a, and the PPARγ5-R is complimentary to exon 2. The primer pairs cPPARγ2-RT-F/R, cPPARγ3-RT-F/R, cPPARγ4-RT-F/R were also used to detect the relative expression of cPPARγ2, cPPARγ3 and cPPARγ4, respectively. The primer locations are shown as horizontal arrows. The eight exons are shown as rectangles labeled A, B, C, D, 1a, and 1 to 6.

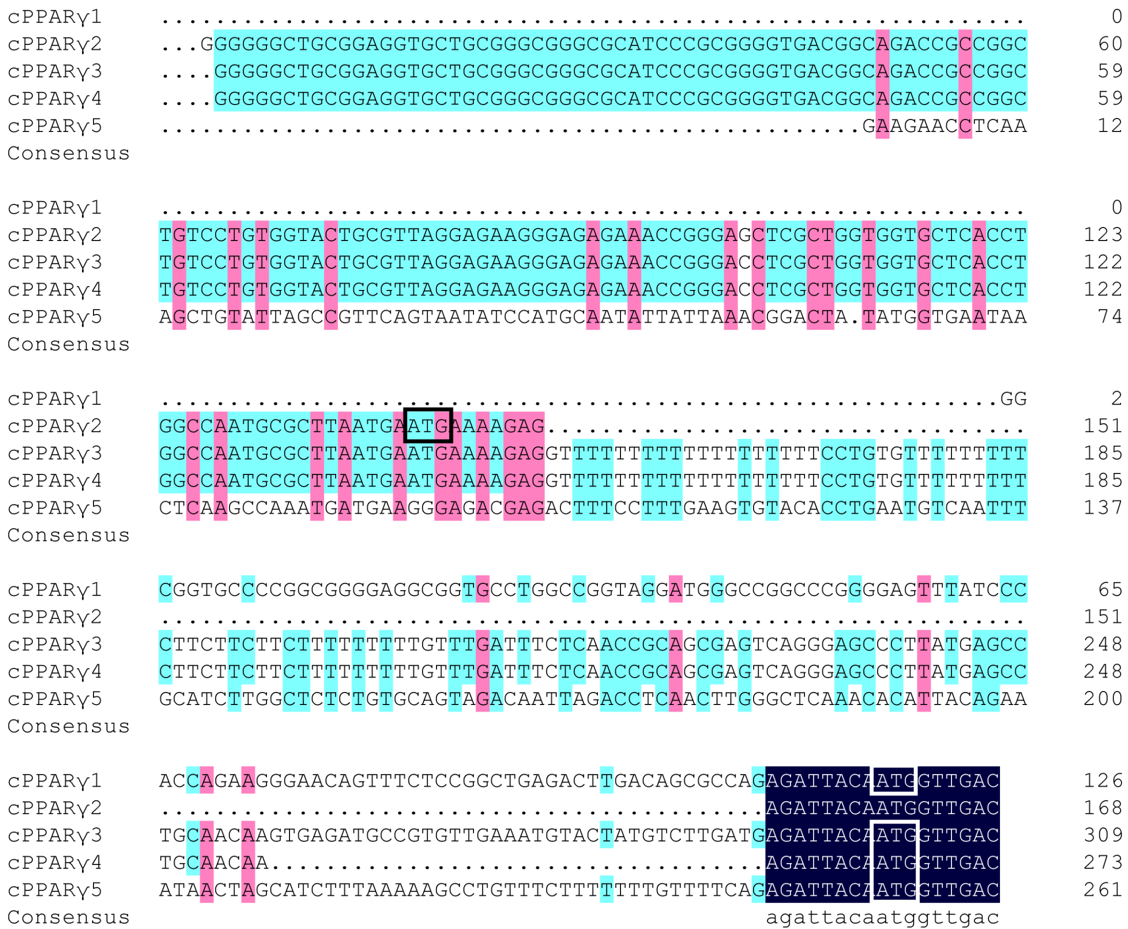

Figure 3. The alignment of 5′ terminal sequences of the five cPPARγ transcript variants (cPPARγs 1 to 5). The 5′ terminal sequences that are identical among the five cPPARγ transcript variants are marked in purple. The 5′ terminal sequences that are identical among four of the five cPPARγ transcript variants are marked in pink. The 5′ terminal sequences that are identical among three of the five transcript variants are marked in light-green; The start codon ATG of transcript variants cPPARγ1, cPPARγ3, cPPARγ4, cPPARγ5 are marked by a white box. The start codon ATG of cPPARγ2 is marked by a black box.

amplified PCR products were cloned and sequenced. All these five RT-PCRs yielded a single product band with the expected size (389, 100, 166, 280, and 846 bp, respectively), and their sequences were identical to the expected sequences of the five chicken PPARγ transcript variants, respectively. These data suggest that these five identified cPPARγ transcript variants do exist and our identified genomic structure of cPPARγ is correct.

These five cPPARγ transcript variants had identical sequences in exons 1 to 6, which are highly conserved in mammals and chicken, but differed in their upstream exons whose sequences are not conserved in mammals and chicken (Figure 3). The clustalw2 alignment showed that the cPPARγ1 sequence had high homology (71.79% and 69.23%, respectively) with the hPPARγ1 and mPPARγ1 transcripts. The cPPARγ2 sequence had high homology (62.0% and 55.91%, respectively) with the hPPARγ1 and mPPARγ2 transcripts. The cPPARγ3 transcript variant had high homology (62.40%) with the mPPARγ3 transcript. The cPPARγ4 sequence had high homology (59.06%) with the mPPARγ2 transcript. The cPPARγ5 sequence had high homology (66.17% and 66.14%, respectively) with the mPPARγ3 and mPPARγ2 transcripts.

Characterization of cPPARγ Promoter Sequences

The transcript variants of a gene differ in the first exons, suggesting alternative promoter usage (Ayoubi et al., 1996). Based on our identified cPPARγ transcript variant sequences, it is concluded that the cPPARγ gene has three different promoters and transcription start sites. One promoter, designated P1, was located upstream of exon A. The second promoter, designated P2, was located upstream of exon B, and the third promoter, designated P3, was located upstream of exon 1a,

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which had been confirmed in our previous study (Ding et al., 2011; Sun et al., 2014). The P1 and P3 promoters drive the transcription of the PPARγ1 and PPARγ5 transcript variants, respectively, whereas the P2 promoter drives the transcription of a transcript that is alternatively spliced to yield the cPPARγ2, cPPARγ3, and cPPARγ4.

We analyzed the 2-kb sequences of the three cPPARγ promoter regions upstream from their transcription initiation sites, using bioinformatics software (mulan, TFsearch and jaspar). Both P1 and P2 promoter regions were GC-rich, and contained GC boxes at positions −42 and −69, respectively, relative to their respective transcription initiation sites, but they contained no canonical TATA box. Furthermore, both promoters contained C/EBP binding sites and multiple Sp1 and AP1 binding sites. By contrast, the P3 promoter contained a TATA box, but no GC box. Similar to P1 and P2 promoters, the P3 promoter contained a C/EBP binding site and multiple Sp1 and AP1 binding sites. The CpG island analysis showed that P1 and P2 promoters had a proximal CpG island, whereas the P3 promoter had no CpG island.

Protein Isoforms of cPPARγ

Based on the five identified cPPARγ transcript variant sequences, two cPPARγ protein isoforms (cPARγ1 and cPPARγ2) were predicted using ORF finder software. The 3′ end of exon 1 and exons 2 to 6 in all these five cPPARγ transcript variants encoded an identical amino acid sequence. The cPPARγ1, cPPARγ3, cPPARγ4, and cPPARγ5 transcript variants encoded the cPPARγ1, which is 475 amino acids in size and uses a start codon in exon 1. The cPPARγ2 transcript variant, which has the shortest 5′ UTR, encoded the cPPARγ2, which is 481 amino acids in size and uses a start codon in exon B. The protein structural domain analysis showed that both cPPARγ protein isoforms consisted of the primary structural domains: the N-terminal activation function 1 (AF1), a DNA binding domain, and a ligand binding domain, which are similar to the human and mouse PPARγ protein isoforms.

In addition, surprisingly, ORF analysis revealed that there was one small upstream open reading frame (uORF) in the 5′-UTRs of the cPPARγ1 and cPPARγ3 transcript variants, which contained 54 and 12 nucleotides, respectively, and two uORFs, which were 15 and 51 bp in size, in the 5′UTR of the cPPARγ5.

Expression Patterns of cPPARγ Transcript Variants in Various Tissues

To determine the tissue distribution of these five identified cPPARγ transcript variants, we measured the relative expression of these five cPPARγ transcript variants in various tissues of the fat chicken line of NEAUHLF ($n = 3$) using quantitative real-time RT-PCR. The variant-specific primer pairs cPPARγ1-F/R, PPARγ2-F/R, PPARγ3-F/R, PPARγ4-F/R, and cPPARγ5-F/R were used for real-time RT-PCR expression analysis of cPPARγs 1 to 5, respectively. As shown in Figure 4, cPPARγ1 was detected in all the tested tissues of the 7-wk-old chickens, with the highest level of expression observed in the abdominal adipose tissue and the lowest level of expression observed in the pectoralis muscle. The cPPARγ2 expression was higher in the abdominal adipose tissue than in other tissues tested. The cPPARγ3 expression was higher in the abdominal adipose, spleen, kidney, and liver than in the other tissues tested. The cPPARγ4 and cPPARγ5 expression was low in all tested tissues. Comparatively, cPPARγ4 expression was higher in the abdominal adipose tissue and proventriculus, but cPPARγ5 expression was higher in heart, liver, kidney, and abdominal adipose tissue than in the other tissues tested. On the whole, the expression of these five cPPARγ transcript variants was high in the abdominal adipose tissue (Figure 4).

Expression Patterns of cPPARγ Transcript Variants During Adipose Development

To understand the roles of the five identified cPPARγ transcript variants in chicken adipose tissue development and adipogenesis, we measured the relative expression of the five cPPARγ transcript variants in the abdominal adipose tissue of lean and fat chicken lines of NEAUHLF using qRT-PCR and compared their individual expression in the abdominal adipose tissue between lean and fat chicken lines. As shown in Figure 5, the cPPARγ1 expression in the fat line was significantly higher from 2 to 7 wk of age ($P < 0.05$ or $P < 0.01$), compared with the lean line. The cPPARγ2 expression in the fat line increased from 1 to 6 wk of age, but an opposite trend was observed in the lean line. The cPPARγ2 expression was significantly lower in the fat line than in the lean line at 4 wk of age ($P < 0.01$), but its expression was significantly higher in the fat line than in the lean line at 2 and 6 wk of age ($P < 0.01$). The cPPARγ3 expression was significantly higher in the fat line at 3, 5, 6 wk of age ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively), but significantly lower at 4 wk ($P < 0.05$), compared with that in the lean line. The cPPARγ4 expression was significantly higher in the fat line than in the lean line at 2, 3, and 6 wk of age ($P < 0.01$). The cPPARγ5 expression was significantly higher in the fat line than in the lean line at 2, 3, 4, 6, and 7 wk of age ($P < 0.05$ or $P < 0.01$). Interestingly, for all of the transcript variants in the fat line, their expression peaked at 6 wk of age. The differential expression of these cPPARγ transcript variants in the abdominal adipose tissue may account for the fatness difference between the lean and fat broiler lines.

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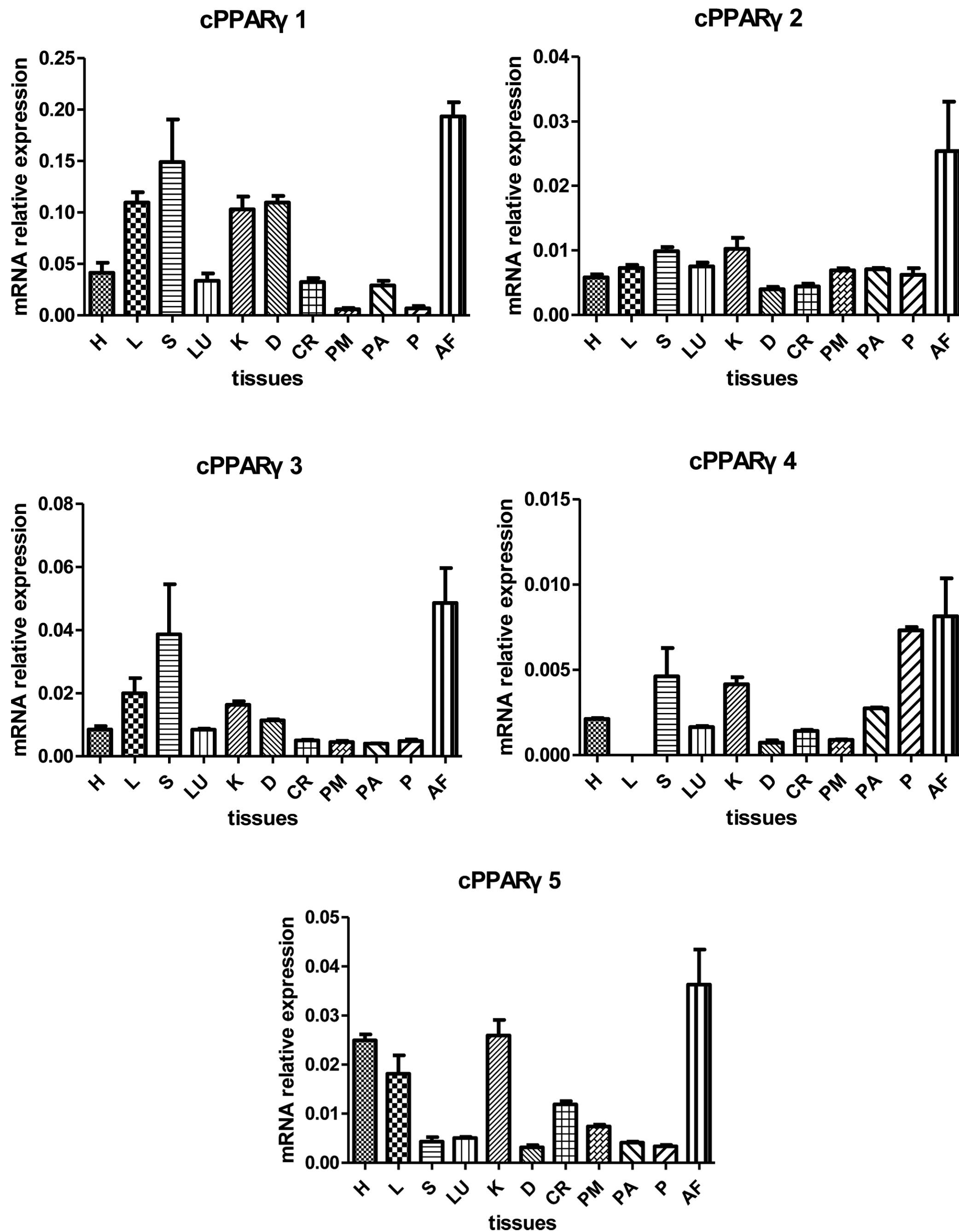

Figure 4. The tissue expression distribution of cPPARγs 1 to 5. Expression was detected using real-time RT-PCR, NONO (non-POU domain containing, octamer-binding) was used as an internal control. Diagrams show the relative expression (mean ± SD) of cPPARγs 1 to 5. The tissues, heart (H), liver (L), spleen (S), lung (LU), kidney (K), duodenal (D), brain (CR), Pectoralis muscle (PM), pancreas (PA), proventriculus (P), abdominal adipose (AF) were from the 7-wk-old fat broiler line of 18th generation of NEAUHLF. The relative expression of cPPARγs 1 to 5 is normalized to that of the NONO gene in the same sample. The Y axis represents the relative expression ratios expressed in arbitrary units.

DISCUSSION

In our current study, we determined the 5′-terminal sequences of cPPARγ transcripts, and identified a total of five cPPARγ transcript variants, which are predicted to produce two protein isoforms. Our results confirmed our hypothesis that *cPPARγ* encodes multiple transcript variants and protein isoforms.

These five different cPPARγ transcript variants were validated by RT-PCR and DNA sequencing. Our findings are, in part, supported by bioinformatics analysis of NCBI SRA and EST databases. Based on the NCBI SRA data (SAMN01831569 to SAMN01831592 and GSM1054359 to GSM1054382), we generated three different cPPARγ mRNA sequences that contained an additional 63, 106, and 133 bp in their 5′-terminal sequence, compared with the known cPPARγ mRNA (NM_001001460.1). These three additional sequences overlapped with the cPPARγ1, cPPARγ2 and cPPARγ5, respectively. An EST

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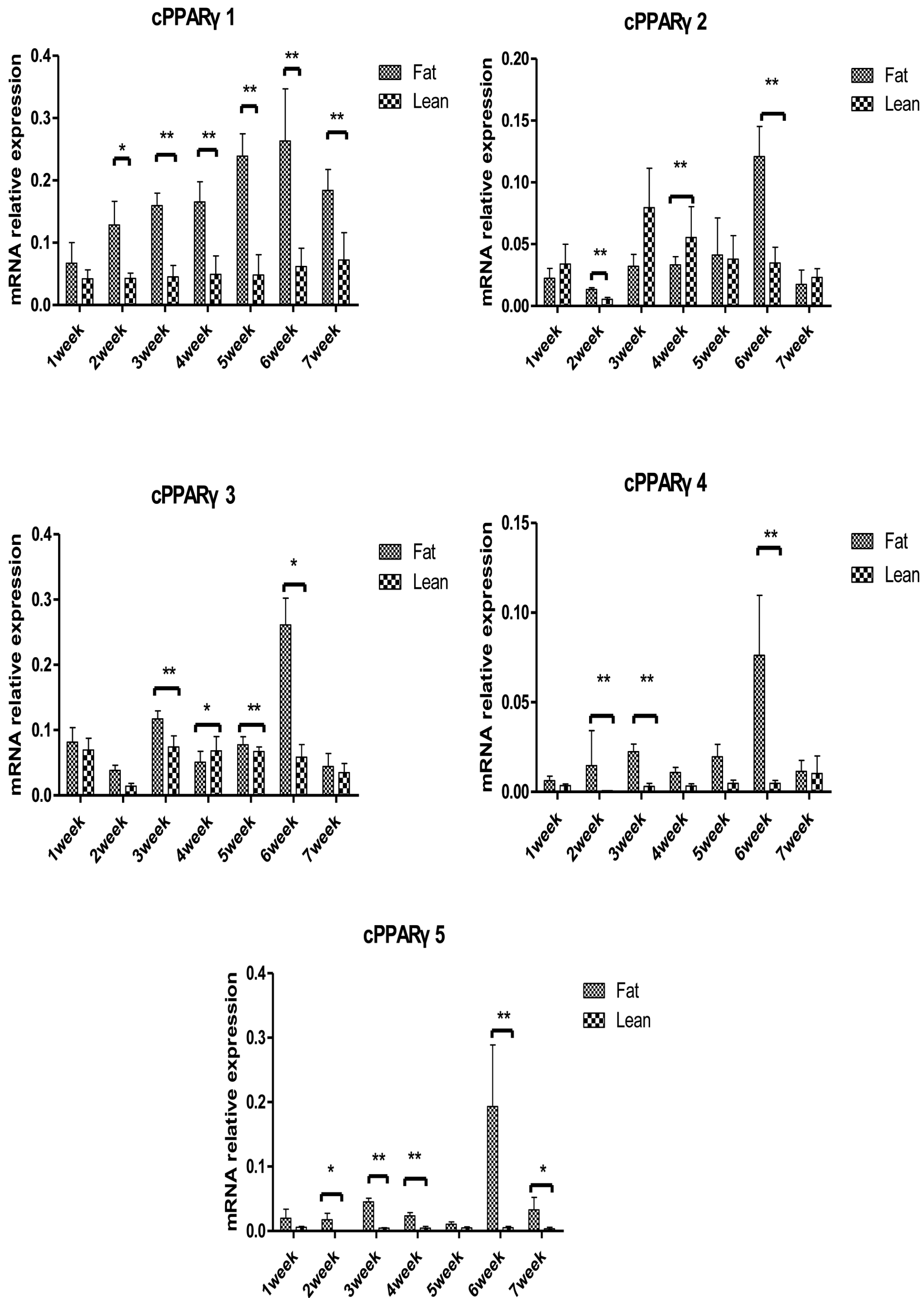

Figure 5. Comparison of the expression of the five cPPARγ transcript variants in abdominal adipose tissue between lean and fat chicken lines from 1 to 7 wks of age. The expression of cPPARγ1, cPPARγ2, cPPARγ3, cPPARγ4, and cPPARγ5 transcript variants was detected using real-time RT-PCR in abdominal adipose tissues of the lean and fat chicken lines of the 18th generation of NEAUHLF (each line $n = 8$), NONO (non-POU domain containing, octamer-binding) was used as an internal control. The relative expression of cPPARγs 1 to 5 is normalized to that of NONO gene in the same sample. The Y axis represents the relative expression ratios expressed in arbitrary units. Asterisks indicate significant different expression between fat and lean chicken lines (unpaired Student's t-test) $P < 0.05(^{*})$ or $P < 0.01(^{**})$.

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sequence (BU359396.1) contains an additional 33 bp in the 5′-end compared with the known cPPARγ mRNA sequence (NM_001001460.1), and this additional 33-bp sequence overlapped with our identified 5′-terminal sequence of cPPARγ1.

Although for these five cPPARγ transcript variants, the sequences of exons 1 to 6 were highly conserved, the sequences of their upstream exons, which encode the 5′UTRs are diverse in sequence and length, and are not conserved in mammals and chickens. The 5′UTRs of mRNAs play important roles in the posttranscriptional regulation of gene expression. Various cis-regulatory elements of 5′UTRs have been identified, including a 5′-cap structure, a translation initiation motif, upstream AUGs, uORFs, internal ribosome entry sites, terminal oligopyrimidine tracts, secondary structure, and G-quadruplexes. The length and sequence diversity in the 5′UTRs of the cPPARγ transcript variants may suggest that 5′UTRs may play important roles in regulating cPPARγ gene expression post-transcriptionally. The uORFs have been shown to regulate gene expression (Sachs and Geballe, 2006; Wethmar et al., 2010; Barbosa et al., 2013; Ferreira et al., 2014). The chicken *CCK-CHR* gene has one uORF in the 5′UTR of the CCK-CHR mRNA (Nilsson et al., 2003). No uORFs have been identified in the 5′UTRs of human and mouse PPARγ transcript variants; however, in our present study, we identified one small uORF in the 5′UTRs of cPPARγ1 and cPPARγ3 and two uORFs in the 5′UTR of cPPARγ5. These results suggest that the posttranscriptional regulatory mechanisms of PPARγ gene expression differ between mammals and chicken. Future studies are warranted to determine whether the 5′UTRs in cPPARγ transcript variants regulate cPPARγ gene expression.

It has been shown that four transcript variants are transcribed from the *hPPARγ* gene as the result of the alternative promoter usage and different splicing (Fajas et al., 1997; Fajas et al., 1998). The *mPPARγ* gene contains two distinct promoters that drive expression from three transcription start sites (Zhu et al., 1995; Takenaka et al., 2013). In the present study, our data indicated that the cPPARγ gene contains three promoters and three transcription start sites. Taken together, these data suggest that alternative promoter usage of PPARγ gene is conserved in mammals and chickens.

Our results showed that chickens express two cPPARγ protein isoforms (cPPARγ1 and cPPARγ2) that differ only in their N-terminal regions. The cPPARγ1 is identical to the previously reported cPPARγ protein (NP_001001460.1). The cPPARγ2 contain six additional N-terminal amino acids, compared with the cPPARγ1. The protein homology analysis showed that the cPPARγ1 protein shared a high level of amino acid identity with the human and mouse PPARγ1 proteins. However, the cPPARγ2 protein isoform did not share a high level of homology with human or mouse PPARγ2, and displayed substantial variation in its N-terminus. The AF1 domain, which is located in the N-terminal region of PPARγ, is a ligand-independent activation domain that regulates the specificity of PPARγ transcriptional activity during adipogenesis. Previous studies have shown that the hPPARγ2 protein isoform is a more potent ligand-independent activator than the hPPARγ1 protein (Werman et al., 1997) and plays the dominant role in the adipogenesis (Zhang et al., 2004b). Whether cPPARγ1 and cPPARγ2 have different functions in adipogenesis and adipose development remain unknown.

Tissue distribution analysis showed that, although the cPPARγ1 is ubiquitously expressed in chicken tissues, it is predominantly expressed in abdominal adipose tissue compared with other cPPARγ transcript variants, which is similar to human and mouse PPARγ1, suggesting that the function and regulation of the cPPARγ1 in adipogenesis and adipose development is similar to that of human and mouse PPARγ1. The level of cPPARγ1 expression was significantly lower in the lean line than in the fat line from 2 to 7 wk of age, indicating that this difference might be involved in the mechanism underlying the difference in the percentage of abdominal fat between the lean and fat chicken lines. The cPPARγ1 protein isoform can be encoded by the cPPARγ1, cPPARγ3, cPPARγ4, and cPPARγ5 transcript variants, but cPPARγ1 is the only transcript expressed at a high level in abdominal adipose tissue, suggesting that the cPPARγ1 transcript variant maybe is primarily responsible for the expression of the PPARγ1 protein isoform.

In mammals, both PPARγ1 and PPARγ2 protein isoforms are essential for adipocyte differentiation and adipose tissue development (Mueller et al., 2002). However, although the level of PPARγ1 mRNA expression is substantially higher than that of the PPARγ2 mRNA in mammalian adipocytes, the PPARγ2 protein is the predominant regulator of adipocyte differentiation (Yanase et al., 1997). The human and mouse PPARγ2 protein isoforms have an additional 28 and 30 amino acids, respectively, in the AF1 domain in the N-terminal region of each protein, compared with the AF1 domain of the PPARγ1 protein. However, our results showed that the cPPARγ2 protein isoform had only six additional amino acids in its N-terminal region, compared with the cPPARγ1 protein. Future studies are warranted to determine the specific roles of cPPARγ protein isoforms in adipocyte differentiation and adipose tissue development.

In the present study, we identified five cPPARγ transcript variants that encode two protein isoforms, cPPARγ1 and cPPARγ2. Previous attempts to isolate cPPARγ transcript variants using 5′-RACE had failed (Sato et al., 2004), possibly because the sensitivity of the 5′-RACE technology used in previous studies was insufficient. Differences in sample preparation, primer designing, and PCR conditions might also have contributed to differences between our results and those of

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previous studies. To the best of our knowledge, this is the first report of transcript variants and protein isoforms of cPPARγ. Our findings provide the basis for future investigations of the roles of the cPPARγ1 and cPPARγ2 proteins in chicken adipogenesis, adipose tissue development, and fat accumulation.

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