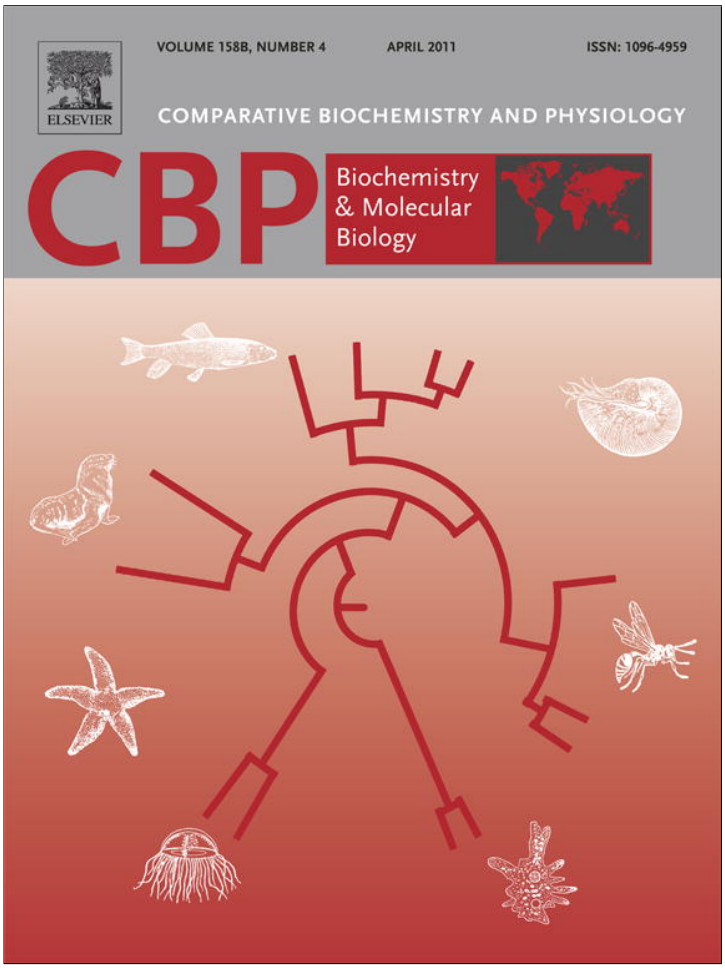

Comparative Biochemistry and Physiology, Part B 158 (2011) 297–303

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Functional analysis of the chicken PPARγ gene 5′-flanking region and C/EBPα-mediated gene regulation

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ARTICLE INFO

Article history:
Received 21 October 2010
Received in revised form 3 January 2011
Accepted 4 January 2011
Available online 14 January 2011

Keywords:
Chicken
CCAAT/enhancer binding protein-α
Peroxisome proliferator-activated receptor-γ
Transcriptional regulation

ABSTRACT

Peroxisome proliferator-activated receptor-γ (*PPARγ*) and CCAAT/enhancer binding protein-α (C/EBPα) are the master regulators of adipogenesis. The regulatory mechanism of *PPARγ* and *C/EBPα* gene expression is clear in mammals, however, little is known in chicken. The aim of the present study was to characterize chicken PPARγ promoter and investigate whether *PPARγ* could be regulated by C/EBPα in chickens. A 2-kb nucleotide sequence upstream of the start codon of chicken *PPARγ* gene was cloned and characterized by using bioinformatics and experimental approaches. This 2-kb promoter region exhibited strong promoter activity in DF1 cells. The reporter gene assay showed that the chicken C/EBPα could activate *PPARγ* gene promoter. Further study by electrophoretic mobility shift assay and mutational analysis revealed that the chicken C/EBPα could directly bind to and regulate the *PPARγ* gene promoter. Our results demonstrate that *PPARγ* can be directly regulated by C/EBPα in chickens.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear hormone receptor superfamily, and include three distinct isoforms (α, β or δ, γ) (Issemann and Green, 1990; Dreyer et al., 1992; Michalik et al., 2006). PPARγ is the most frequently studied PPAR isoform, which is involved in adipose development and function (Gray et al., 2005; Lefterova et al., 2008), including insulin sensitivity, lipid storage, energy dissipation, and adipokine secretion (Rosen et al., 2000; He et al., 2003; Koutnikova et al., 2003; Rangwala and Lazar, 2004). It is conceivable that the *PPARγ* gene can participate in regulation of adipose metabolism in many ways (Lehrke and Lazar, 2005; Heikkinen et al., 2007).

PPARγ gene is the master regulator of adipogenesis in mammals. No factor has been discovered that promotes adipogenesis in the absence of PPARγ. Crucial signalling pathways in adipogenesis converge on the regulation of PPARγ expression or activity (Rosen and Dougald, 2006). A series of studies have shown that PPARγ and C/EBPα, another key transcription factor in adipogenesis, could cross-regulate each other and mediate adipocyte differentiation (Rosen et al., 2002; Farmer, 2006; Yu et al., 2006). Lack of PPARγ greatly reduces the expression of C/EBPα (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999). Similarly, lack of C/EBPα also decreases PPARγ expression (Rosen et al., 2002). Further studies have shown that in human and mouse PPARγ2 promoters, there were C/EBPα binding sites located respectively at −56 and −120, and C/EBPα could bind to these sites to induce and maintain PPARγ2 expression in human and mouse (Saladin et al., 1999; Elberg et al., 2000; Tang et al., 2004).

Most of our knowledge of adipogenesis is derived from studies of mammalian, but not avian adipogenesis. There are some significant differences in lipogenesis between mammals and avian species (Han et al., 2009). For example, lipogenesis in chickens is active mainly in the liver like human (O'Hea and Leveille, 1969a; Patel et al., 1975), whereas in some mammals like pig, dog and cat, it is active mainly in adipose tissue (O'Hea and Leveille, 1969b; Stangassinger et al., 1986; Richard et al., 1989), or in some mammals like rodent and rabbit it is active in both adipose tissue and liver (Pullen et al., 1990). This raises the question of whether our knowledge of adipogenesis could apply to avian species. Therefore, chicken adipogenesis has been investigated in recent years, and the role of chicken *PPARγ* (*cPPARγ*) in adipocyte differentiation has also been widely investigated. *cPPARγ* is highly expressed in adipose tissue (Meng et al., 2005). During chicken preadipocyte differentiation, the *cPPARγ* gene expression begins in the early differentiation stage of preadipocyte differentiation (Matsubara et al., 2005), and abundantly expressed in highly differentiated preadipocytes as compared with less differentiated preadipocytes (Wang et al., 2008), suggesting that the early expression of cPPARγ is required for chicken adipocyte differentiation. The knockdown of chicken *PPARγ* led to the inhibition of chicken preadipocyte differentiation (Wang et al., 2008). In contrast, overexpression of *cPPARγ* gene in chicken embryo fibroblasts (CEFs) leads to an adipocyte phenotype of cytoplasmic lipid droplet accumulation, and higher expression of the *A-FABP* gene (Liu et al., 2010). When chicken

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doi:10.1016/j.cbpb.2011.01.001

fibroblasts are induced by oleate to transdifferentiate into adipocyte-like cells, chicken *C/EBPα* (*cC/EBPα*) and *cPPARγ* expression is simultaneously increased (Liu et al., 2009). All these results suggest that *cPPARγ*, similar to mammalian *PPARγ*, acts as a key regulator in chicken adipogenesis.

In mammals, *PPARγ* gene has two promoters, three mRNA isoforms, and two protein isoforms (Fajas et al., 1997; 1999; Zhu et al., 1995). PPARγ2 is more adipose specific than PPARγ1 (Saladin et al., 1999; Elberg et al., 2000), and could be regulated by C/EBPα by directly binding to its promoter in human and mouse (Saladin et al., 1999; Elberg et al., 2000; Tang et al., 2004). In chickens, *PPARγ* gene has only one mRNA and one protein isoform (Sato et al., 2004), and it is not yet known whether cC/EBPα can bind to *cPPARγ* promoter and regulate its expression. In this study, we aimed to characterize the *cPPARγ* gene promoter and investigate whether cC/EBPα can directly regulate *cPPARγ* gene.

2. Materials and methods

2.1. Plasmids and constructs

The 5′-flanking region (1.8 kb) upstream of the translation start site of the *cPPARγ* gene (GenBank accession no. AB045597) was amplified from chicken genomic DNA by PCR with a pair of specific primers (forward, 5′-GCGGTACCCCATCTTAGGCAACATAA-3′; reverse, 5′-GCGA GCTCGTCTACTGCACAAAGAGC-3′), which contained *Kpn I* and *sac I* sites, respectively. Takara LA Taq™ Polymerase (TAKARA, Dalian, China) was used for the PCR reaction, and the PCR product was cloned into pGL3 basic vector (Promega, Madison, WI, USA), generating the promoter reporter construct pGL3-cPPARγ (−1985/−89).

The −1985/−89 fragment was also screened for putative transcriptional factor binding sites and promoter elements by the following softwares and databases: TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html); TRANSFAC Database (http://www.gene-regulation.com/pub/databases.html); PromoterScan (http://www-bimas.cit.nih.gov/molbio/proscan/); and CpG Island Searcher (http://cpgislands.usc.edu/).

A total of 5 truncation mutations of *PPARγ* gene promoter were prepared using two methods. One was PCR-based method, and the primer sequences used in this experiment were shown in Table 1. All of the forward primers contained a *KpnI* site, and the reverse primers contained a *SacI* site. The PCR products were cloned into pGL3 basic vector (Promega) to generate the promoter deletion constructs pGL3-cPPARγ (−1261/−89), pGL3-cPPARγ (−1026/−89) and pGL3-cPPARγ (−327/−89). The other method was to use restriction digestion as described by Kockar and Foka (2001). The promoter construct pGL3-cPPARγ (−1985/−89) was double digested with restriction enzymes *KpnI* and *PstI* to generate promoter deletion constructs pGL3-cPPARγ (−1520/−89), and *KpnI* and *SpeI* to generate promoter deletion construct pGL3-cPPARγ (−520/−89). All the constructs were sequenced to confirm their identity.

2.2. Luciferase reporter assay

The DF-1 chicken fibroblast cells (Harbin Veterinary Research Institute, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Invitrogen) at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. At 90% confluence, the cells were passaged and plated in 12-well plates, and transfection was carried out by using FuGENE 6 reagent according to the manufacturer's instructions (Roche, Mannheim, Germany). In the experiment group, cells were transfected with the *cPPARγ* promoter/reporter plasmid (1 μg) and a Renilla luciferase plasmid (2 ng). In the control group, the cells were transfected with the basic pGL3 empty plasmid (1 μg) and Renilla luciferase plasmid (2 ng) (Promega). Transfected cells were cultured for 48 h, and then used for luciferase reporter gene expression assays.

The luciferase assay was carried out according to the manufacturer's instructions of the dual luciferase assay system (Promega) on an Orion MPL2 luminometer (Berthol Detection System, Germany). Each transfection was repeated at least five times.

To detect the effect of cC/EBPα on cPPARγ deletion mutant promoter activity, C/EBPα expression plasmid (pCMV-HA-C/EBPα) was used for cotransfection, which was generated by our laboratory. Cotransfection was performed with 0.6 μg cPPARγ promoter/reporter plasmid, 0.4 μg pCMV-HA-C/EBPα plasmid and 1.2 ng Renilla luciferase plasmid. The control cells were transfected with the cPPARγ promoter/reporter plasmid, Renilla luciferase plasmid and empty expression vector (pCMV-HA vector). Transfected cells were cultured for 48 h, and then used for luciferase reporter gene expression assays.

2.3. Electrophoretic mobility shift assay (EMSA)

For EMSA, nuclear extracts were prepared from DF1 cells by overexpressing chicken C/EBPα. Briefly, DF1 cells were transfected with chicken C/EBPα expression plasmid (pCMV-HA-C/EBPα). At 48 h after transfection, the cells were harvested using enzyme digestion with trypsogen (Invitrogen), and washed twice with PBS. The nuclear extracts were prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Florida, USA) with Protease Inhibitor Cocktail Set I (Merck, Darmstadt, Germany). Expression analysis of chicken C/EBPα was analyzed by western blotting. The primary antibody used was HA-Tag polyclonal antibody (1:200, Clontech, Palo Alto, CA, USA), and the horseradish peroxidase-conjugated secondary antibody was mouse anti-rabbit IgG (1:5000, Abcam, Cambridge, MA, USA).

Oligonucleotide probes, corresponding to the sequence −350 to −270, −285 to −215, −235 to −155, and −171 to −89 of *cPPARγ* promoter, were obtained from annealed ssDNA synthesized by Invitrogen and labeled with biotin (Biotin 3′ End DNA Labeling Kit, Pierce). Mutational probes were designed with mutations in the cC/EBPα binding-site sequence (ATTTG to GATGT) (Table 2).

EMSAs were performed using a lightshift chemiluminescent EMSA kit (Pierce) according to the manufacturer's standard protocol. The basic binding reactions contained 2 μL nuclear extracts, 50 fmol biotin-labeled probes, 2 μL binding buffer, 1 μL poly (dI:dC) and 1 μL NP-40. For the competition reactions, the mixtures were combined with unlabeled probes, and for super shift assay, rabbit anti-chicken C/EBPα

Table 1
Primer sequences for *cPPARγ* promoter by PCR.

Primer name	Sequence	Product size (bp)
PPAR −1261/−89	F 5′-gc ggtacc TTCATTTCGTAACAACGG-3′ R 5′-gc gagctc GTCTACTGCACAAAGAGC-3′	1172
PPAR −1062/−89	F 5′-gc ggtacc TGAATCAGTTAGCGGAATA-3′ R 5′-gc gagctc GTCTACTGCACAAAGAGC-3′	937
PPAR −327/−89	F 5′-gc ggtacc AGGGTAGCCTGAGCACTGGG-3′ R 5′-gc gagctc GTCTACTGCACAAAGAGC-3′	238

Table 2
Oligonucleotide probe sequences for EMSA.

Probe name	Sequence
Probe −350/−270	5′-CCTATGACATGCTATTCAGTTCCAAGGGTAGCCTGAGCACT GGGTG CCAACAAAACATCATTTGTTCTCATGATGTGTATG-3′
Probe −285/−215	5′-CTCATGATGTGTATGATATCAGAGCCTGTGTATGAAGAACC TCAAAGCTG TATTAGCCGTTCAGTAATATC-3′
Probe −235/−155	5′-TATTAGCCGTTCAGTAATATCCATGCAATATTATTAAACGG ACTATATGG TGAATAACTCAAGCCAAATGATGAAGGGAGA-3′
Probe −171/−89	5′-CAAATGATGAAGGGAGACGAGACTTTCCTTTGAAGTGTACA CCTGA ATGTCA ATTTG CATCTTGGCTCTTTGTGCAGTAGAC-3′
Mut probe −171/−89	5′-CAAATGATGAAGGGAGACGAGACTTTCCTTTGAAGTGTAC ACCTGA ATGTCA GATGT CATCTTGGCTCTTTGTGCAGTAGAC-3′

antiserum (prepared in our laboratory by immunizing rabbits with purified recombinant chicken C/EBPα expressed in *E. coli*) was added to reactions.

2.4. Mutagenesis

The mut-pGL3-PPARγ(−327/−89) construct was generated by mutating the C/EBPα binding-site sequence from TTG to ACA using the mut-primer (5′-CTACTGCACAAAGAGCCAAGATGTGTATTGACAT TCAGG-3′) according to the instructions of the Quick Change Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

2.5. Statistical analysis

JMP 4.0 software (SAS Institute, Cary, NC, USA) was used for all statistical analysis, and P values <0.05 were considered statistically significant, and P values <0.01 were highly significant. All data are expressed as mean ± SD. Student's *t* tests were used to determine the significant differences between groups.

3. Results

3.1. Sequence analysis of the 5′ flanking region of the cPPARγ gene

To study the regulation of the *cPPARγ* gene, a 1895 bp fragment (−1985 to −89) upstream of the translation start site of *cPPARγ*, was amplified from chicken genomic DNA and sequenced. Sequence analysis showed that the amplified fragment had an overall identity of 99.1% to the corresponding sequence from chicken genome. The sequence was then analyzed for the presence of putative transcriptional factor binding sites by using the TRANSFAC and TFSEARCH programs, which revealed that there were many transcription factor binding sites. Notably, 17 C/EBPs binding sites were found in the 5′ flanking region of the *cPPARγ* gene (−1896, −1609, −1582, −1503, −1483, −1444, −1399, −1387, −1364, −1207, −1182, −1106, −884, −667, −447, −432, and −150). Two GATA-2 binding sites were identified at −1656 and −1351, and one GATA-3 binding site at −1761 upstream of the translation start site of the *cPPARγ* gene. In addition, this fragment also contained several binding sites for activator protein-1 (AP-1) (−1718, −1218, −937, −431), Sp1 (−1341), and Oct-1 (−1178, −495 and −119). Software Promoter Scan predicted a core promoter region from −915 to −665 upstream of the translation start site of *cPPARγ*, with a TATA box at −686/−681. The transcription initiation site of *cPPARγ* was predicted to locate at −656. CpG island analysis showed that no GC-rich region existed in this fragment (Fig. 1).

We also analyzed both human and mouse *PPARγ1* and *PPARγ2* promoter sequences for the presence of putative transcriptional factor binding sites by using the TRANSFAC and TFSEARCH programs. The results showed that C/EBP, Sp1 and AP1 binding sites are conserved in mammalian *PPARγ1 and PPARγ2* and chicken *PPARγ* promoters. However, there were some differences between mammalian *PPARγ1* and *PPARγ2* promoters. For example, mammalian *PPARγ2* promoters have a TATA box, but no SRE and GC-rich region, different from *PPARγ1*. Chicken *PPARγ* promoter region is similar to mammalian *PPARγ2* promoters in structure (Fig. 1).

3.2. Promoter analysis of the 5′ flanking region of cPPARγ

To confirm and characterize 5′-flanking region of *cPPARγ*, the report gene construct was made by inserting the −1985/−89 fragment into pGL3 basic vector, and a total of five promoter/reporter fusion plasmids were constructed. Their promoter activities were measured (Fig. 2A). In contrast to the negative control, the promoter (−1985/−89) and its truncation mutants all had promoter activity. The pGL3-cPPARγ (−1261/−89) promoter construct compared favorably with other promoter constructs, which caused a six-fold induction of luciferase activity as compared with the negative control ($P<0.01$). Removal of the nucleotides from region −1261 to −1026

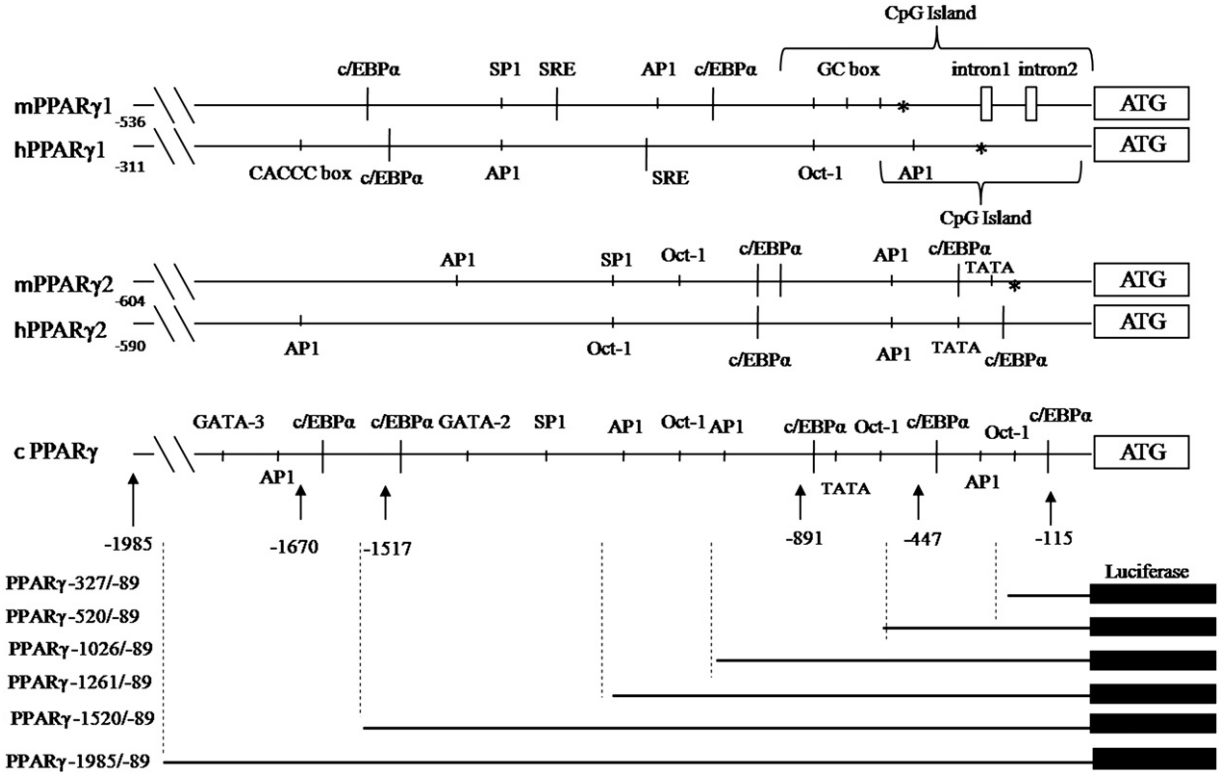

Fig. 1. Schematic diagram of human, mouse and chicken PPARγ promoters and sequential deletion promoter of chicken PPARγ. The relative position of the putative TATA box, CpG island and binding sites for the transcription factors C/EBPα, GATA-2, GATA-3, Sp1, Oct-1, and AP-1 are shown in the figure with indication that the consensus sequence occurs in 5′-flanking region of hPPARγ1, hPPARγ2, mPPARγ1, mPPARγ2 and cPPARγ. The transcription start site is indicated by an asterisk.

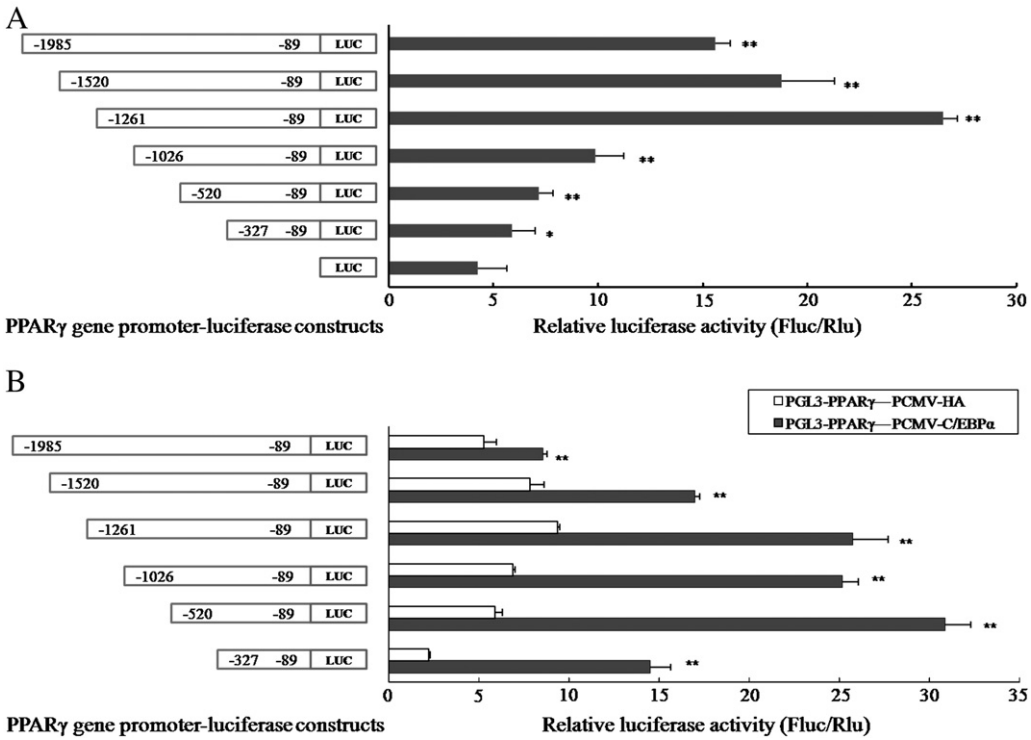

Fig. 2. Activity of the *cPPARγ* promoter. A, the *cPPARγ* promoter constructs (−1985/−89, −1520/−89, −1261/−89, −1026/−89, −520/−89 and −327/−89) were transfected into DF1 cells, and pGL3-Basic vector was used as a negative control. B, DF1 cells were co-transfected with *PPARγ*–luciferase constructs and a C/EBPα expression vector (pCMV-HA-C/EBPα) (black bar). DF1 cells were co-transfected with *PPARγ* promoter constructs and pCMV–HA vector as a negative control (white bar). The promoter activity was expressed as relative luciferase activity (Fluc/Rluc). The left of the figure shows the *cPPARγ* promoter and its sequential deletion promoters inserted upstream of the luciferase gene in the reporter plasmid pGL3-Basic, and the right of the figure shows the relative promoter activity. The data are the mean of five individual values ± SD. *P<0.5, **P<0.01.

led to a drastic reduction of promoter activity (P<0.01), indicating that negative regulatory elements exist in the −1261/−1026 region. Of these six different length promoters, the −327/−89 promoter seemed to have the lowest promoter activity in DF1.

3.3. Transcription regulation of cPPARγ by cC/EBPα

The human and mouse *PPARγ2* promoters have been shown to be activated by C/EBPα (Lefterova et al., 2008; Tang et al., 2004; Elberg et al., 2000). However, it is not known whether the *cPPARγ* promoter can be activated by C/EBPα, therefore, we investigated the effect of cC/EBPα on the *cPPARγ* gene promoter in chickens. The six *cPPARγ* promoter/reporter constructs were co-transfected into DF1 cells with pCMV-HA-C/EBPα, and luciferase activity was measured. pCMV-HA vector was used instead in the negative control group. As shown in Fig. 2B, all the *cPPARγ* promoters could be significantly activated by the expression of *cC/EBPα* compared with the negative control (P<0.01). The expression of *cC/EBPα* could improve the promoter activity of the shortest promoter region (−327/−89) seven-fold (Fig. 2B). We conclude that cC/EBPα could activate the promoter of the *cPPARγ* gene, and there is at least one C/EBPα regulation site in the −327/−89 region of *cPPARγ* gene promoter.

3.4. Determination of specific cC/EBPα binding site in the proximal cPPARγ promoter

To locate cC/EBPα binding site in the −327/−89 region of cPPARγ promoter, 4 probes, corresponding to sequences −350 to −270, −285 to −215, −235 to −155 and −171 to −89, were generated. The probes were incubated with the similar amount of nuclear extracts (Fig. 3A, lane 2). As shown in Fig. 3B (lane 4), one complex appeared exclusively with the probe −171/−89, but not the other three probes.

To confirm the sequence specificity of the DNA binding probe, competition experiments were performed with an excess of the unlabelled double stranded probe −171/−89. As shown in Fig. 3B, compared to the control group (Fig. 3C, lane 2), the addition of the excess of unlabelled probe −171/−89 reduced the binding signal (Fig. 3C, lanes 3 and 4), and the binding could be abrogated by a 50-fold molar excess of unlabeled probes (Fig. 3C, lane 5), suggesting that the DNA binding is sequence-specific.

To fine-map the C/EBPα binding site, the −171/−89 region of *cPPARγ* promoter was analyzed by TRANSFAC and TFSEARCH softwares. The results showed that a C/EBPα binding site existed at −150/−154 in this promoter region. To determine whether this site is a real C/EBPα binding site, a mutated probe was generated, which contain an ATTTG to GATGT mutation in this putative C/EBPα binding site. The EMSA result showed that the mutated probe had lost the ability to bind C/EBPα (Fig. 4A, lane 2), and the excess unlabeled mutated probes were not able to compete with the wild-type probes (Fig. 4B). These data indicate that the C/EBPα binding site is located at −150/−154 of the *cPPARγ* promoter.

To confirm that cC/EBPα specifically binds to the mapped binding site, a super shift assay was performed using the cC/EBPα antibody. In contrast with addition of A-FABP antibody (negative control), addition of cC/EBPα antibody resulted in a retarded band that corresponded to DNA–protein–antibody complexes (Fig. 3D). These results showed that the cC/EBPα protein could specifically bind to the C/EBPα binding site at –150/−154 of the *cPPARγ* promoter.

3.5. Effect of the cC/EBPα binding site mutation on the regulation of cPPARγ by cC/EBPα

To investigate the importance of this C/EBPα binding site in the *cPPARγ* promoter further, the mut-pGL3-PPARγ (−327/−89) was constructed with C/EBPα binding-site mutant. The promoter activity

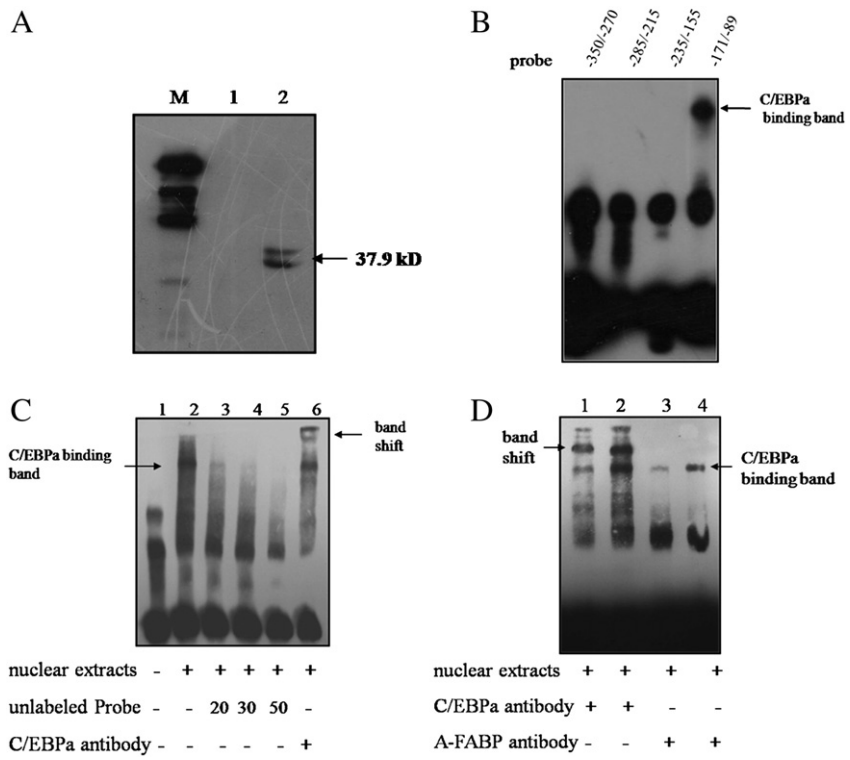

Fig. 3. Analyses of C/EBPα binding sites in the −327/−89 region using EMSA. A, Nuclear extracts were analyzed by western blotting using chicken C/EBPα antibody. Lane M, protein marker; lane 1, nuclear extracts from normal cells; lane 2, nuclear extracts from cells transfected with pCMV-HA-C/EBPα plasmid. B, The −327/−89 region was subdivided into four parts (−350/−270, −285/−215, −235/−155 and −171/−89), and four probes, corresponding to these four parts, were synthesized and labeled with biotin. The nuclear extracts were prepared from cells transfected with pCMV-HA-C/EBPα plasmid. A specific DNA–protein complex was formed only when the probe −171/−89 was incubated with nuclear extracts. C, Competition EMSA with 20-, 30- and 50-fold molar excess of unlabeled probe (lanes 3–5). Super shift was performed with chicken C/EBPα antibody (lane 6). D, Super shift was performed with the probe −171/−89. Lanes 1 and 2, cC/EBPα antibody; lanes 3 and 4, A-FABP antibody as a negative control.

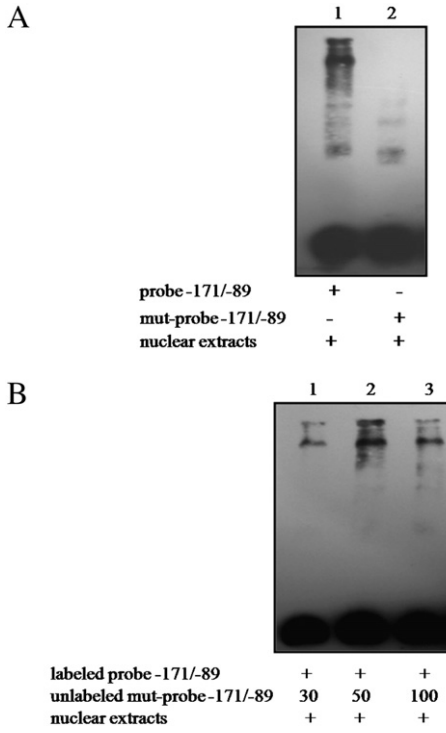

Fig. 4. Mutation analysis of the C/EBPα binding site at −150/−154. The EMSAs were performed with probe −171/−89 and its mutant probe, whose C/EBPα binding site (−150/−154) was mutated. The nuclear extracts were prepared from the cells transfected with pCMV-HA-C/EBPα plasmid. (A) Lane 1, probe −171/−89; lane 2, the mutated probe −171/−89. (B) Competition experiments with the unlabeled mutant probes −171/−89. Lanes 1−3, 20-, 30- and 50-fold molar excess of unlabeled mutated probes, respectively.

of the mut-pGL3-PPARγ (−327/−89) and its corresponding wild-type promoter/reporter construct, pGL3-PPARγ (−327/−89), were compared by using luciferase assay in the presence or absence of *cC/EBPα* expression. As shown in Fig. 5, in the absence of cC/EBPα (*cC/EBPα* gene is not expressed in DF1 cells, unpublished data), the normal promoter construct pGL3-PPARγ (−327/−89) and mut-pGL3-PPARγ (−327/−89) had similar promoter activity. However, in the presence of cC/EBPα, the promoter activity of pGL3-PPARγ (−327/−89), but not mut-pGL3-PPARγ (−327/−89), increased (Fig. 5). These data indicate that this C/EBPα binding site is required for the transcription regulation of cC/EBPα on the *cPPARγ* promoter (−327/−89).

Together, our finding indicates that cC/EBPα can directly bind to *cPPARγ* promoter, and activate its expression.

4. Discussion

Lipogenesis in mammals occurs in adipose tissue and liver, whereas in avian species, the liver is the main lipogenic site. Gene expression analysis shows that expression patterns of adipogenic transcription factors are different *in vitro* and *in vivo* between mammals and avian species (Matsubara et al., 2005). These evidences suggest that mammalian adipogenesis is different from avian adipogenesis.

In mammal and chicken, *PPARγ* play an important role in adipogenesis. However, accumulating evidences show the regulational mechanism of *PPARγ* gene is different between mammal and chicken. Gene structure analysis shows that human and mouse *PPARγ* genes have two separate promoters which can generate three different mRNA isoforms (Fajas et al., 1997; 1999; Zhu et al., 1995), whereas *cPPARγ* gene has only one mRNA isoform and one protein isoform (Sato et al., 2004). In addition, *PPARγ* gene is expressed in chicken kidney but not in other

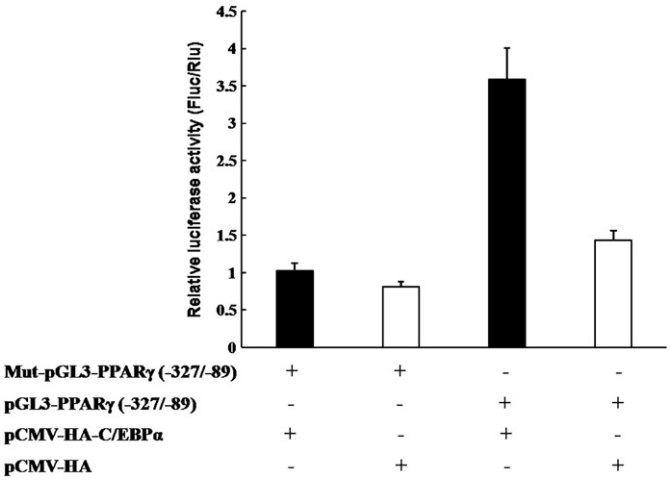

Fig. 5. Reporter gene analysis of the C/EBPα binding site in the *cPPARγ* promoter (327/−89) region. DF-1 cells were co-transfected as indicated with 0.6 μg pGL3-PPARγ (−327/−89) or mut-pGL3-PPARγ (−327/−89), 0.4 μg pCMV-HA-C/EBPα or pCMV-HA, and 1.2 ng Renilla luciferase plasmid in each well. Promoter activity was expressed as relative luciferase activity (Fluc/Rluc). The data are the mean of five individual values ± SD.

species (Meng et al., 2005). Based on these evidences, it is worth further study into regulational mechanism of c*PPARγ* gene.

Promoter nucleotide sequence comparison of *PPARγ* among the human, mouse and chicken showed *PPARγ* promoters were not conserved at the nucleotide level between chicken and mammals. The human and mouse *PPARγ2* promoters shared 75% identity at the nucleotide level, and the human and mouse *PPARγ1* promoter shared 26% identity at the nucleotide level. But the *cPPARγ* promoter showed a very low level of identity (less than 15%) to both human and mouse PPARγ1 and PPARγ2 at the nucleotide level.

Comparison of bioinformatically identified transcription factor binding sites among human, mouse and chicken *PPARγ* gene promoters showed that *cPPARγ* promoter is similar to the mammalian *PPARγ2* promoter rather than *PPARγ1* promoter, suggesting that chicken *PPARγ* and mammalian *PPARγ2* might share a similar regulation mechanism. However, cPPARγ, like mammalian PPARγ1, has no additional amino acids in the N-terminal. Therefore, we speculate that during evolution from birds to mammals, *PPARγ* gene may evolve from one promoter and one mRNA isoform to two promoters and three mRNA isoforms, and chicken *PPARγ* may have the combinational function of mammalian *PPARγ1* and *PPARγ2*.

Promoter deletion analysis showed that the deletion from −1985 to −1261 resulted in a marked increase in promoter activity, suggesting the presence of negative regulatory elements in this region (Fig. 2A). Actually, bioinformatics analysis showed that this region (−1985 to −1261) contained some negative regulatory elements, such as GATA2/3 binding sites which inhibit *PPARγ* expression by binding to the *PPARγ* gene promoter (Tong et al., 2000, 2005). However, further deletions from −1261 to −1026 significantly decreased the promoter activity (Fig. 2A). Sequence analysis showed that there were some elements important for promoter function in this region, such as AP1, Oct1, and TFIIB, etc. The absence of these sites might reduce the transcription activity of the *cPPARγ* promoter. These data suggests the regulatory elements important for chicken *PPARγ* promoter activity are contained between −1261 to −1026 and *PPARγ* promoter activity is negatively regulated by the sequence −1985 to −1261.

Previous studies have shown that mouse C/EBPα can bind to the promoter of the *PPARγ2* and activate its expression (Tang et al., 2004; Elberg et al., 2000). Human C/EBPα and C/EBPβ can also specifically bind to the *PPARγ2* promoter, and regulate adipocyte differentiation (Saladin et al., 1999). We found that cC/EBPα could directly bind to the *cPPARγ* promoter, and activate its expression. The C/EBPα binding site was identified at −150/−154 in *cPPARγ* promoter, and its central sequence is TTG (Fig. 5). Compared with previous studies (Clarke et al., 1997; Saladin et al., 1999), this C/EBPα binding site is conserved among human, mouse *PPARγ2*, and chicken *PPARγ* promoters. Furthermore, this C/EBPα binding site has been functionally verified in humans and mice. These results indicate that mechanism of regulation of *PPARγ* by C/EBPα is evolutionarily conserved between mammals and birds. Apart from this C/EBPα binding site, there are several putative C/EBPα binding sites in the *cPPARγ* promoter region, and we only confirmed one of these C/EBPα binding sites. Because cooperative binding of transcription factors to cis-regulatory elements is essential for gene expression regulation, we can't rule out that C/EBPα can bind to other C/EBPα binding sites to regulate the *cPPARγ* promoter.

In summary, the chicken *PPARγ* promoter was characterized in the present study, and it was found that chicken *PPARγ* gene promoter is similar to mammalian *PPARγ2* promoter, but not *PPARγ1* promoter, and chicken *PPARγ* could be directly regulated by cC/EBPα.

Acknowledgements

The authors gratefully acknowledge the members of the Poultry Breeding Group of the College of Animal Science and Technology in the Northeast Agricultural University for help in culturing the cells and collecting the data. This research was supported by National 973 Project of China (No. 2006CB102105), National 973 Project of China (No. 2009CB941604), Postdoctoral Science Foundation of China (No. 20070410254) and Natural Science Foundation Key Project of Hei Longjiang Province (No. ZJN0604–01).

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