[BIO27] Molecular characterization of peroxisome proliferator activated receptor gamma2 (PPARγ2) promoter from bovine

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Introduction

Peroxisome proliferator activated receptor gamma (PPARγ) is a member of the PPAR subfamily of nuclear hormone receptors (Schoonjans *et al.*, 1996a). So far, two PPARγ isoforms; γ1 and γ2, have been cloned in bovine and produced by alternative promoter usage and differential splicing of a single gene (Sundvold *et al.*, 1997). PPARγ functions by forming heterodimeric complexes with 9-*cis*-retinoic acid X receptors (RXRs). This heterodimer regulates transcription by binding to PPRE, known also as DR-1 response element in the regulatory regions of target genes (Tontonoz *et al.*, 1994a).

PPARγ is a critical transcription factor in the regulation of adipocyte differentiation. It is specifically expressed at high levels in mammalian adipose tissues (Tontonoz *et al.*, 1994b). It was found that forced expression of PPARγ in fibroblasts in the presence of weak PPARγ activators resulted in differentiation of the cells to adipocytes (Tontonoz *et al.*, 1994c). Adipocyte differentiation is accompanied by the induction of several fat-specific marker genes involved in lipid homeostasis such as adipocyte fatty acid binding protein (aP2) (Tontonoz *et al.*, 1994b) and lipoprotein lipase (Schoonjans *et al.*, 1996b). These genes have been shown to contain PPAR response elements in their regulatory regions. PPARγ has also been shown to up-regulate the expression of the fatty acid transporters FATP-1 and CD36 in adipocytes (Martin *et al.*, 1997). These data demonstrated that PPARγ plays a pivotal role in the adipogenic signaling cascade and also suggested that the receptor can influence the production and cellular uptake of its own activators.

Most of the past and present studies on PPARγ have been and are concentrating on human and mouse especially in the development of lipid-related diseases such as obesity, diabetes and atherosclerosis whereas studies on economical importance animals such as cow, chicken and sheep are very limited. All the above mentioned evidence proved that PPARγ plays an important role in regulating the level of fat production in animals, thus, the ratio of fat to lean meat. Such findings would have a significant influence in the meat industry and beneficial not only to the farmers but also to the consumers. Therefore, it is vital to carry out a study on bovine PPARγ because of its major overall economic value. PPARγ gene expression is mainly regulated at transcriptional level (Fajas *et al.*, 1997). Thus, it is crucial to characterize the promoter located at the 5' flanking region of the gene to further our understanding of the regulation of PPARγ2 gene, as well as the function/activities of the bovine PPARγ2 promoter.

Materials and methods

Amplification of PPARγ2 bovine promoter

Construction of genomic libraries. Bovine genomic DNA was extracted from fresh adipose tissue using Wizard® Genomic DNA Purification Kit (Promega) and digested separately with five different blunt-end restriction enzymes (*Dra*I, *EcoR*V, *Pvu*II, *Sca*I and *Stu*I). Purified digested products were ligated to blunt-end adaptor provided in the Universal GenomeWalker™ Kit (Clontech).

Amplification of the promoter. Primary PCR was carried out with each restriction digested library using forward (5'-GTAATACGACTCACTATAGGGC-3') and reverse (5'-CTTGTGAGGTCCTTGCAGACACTG-3') primers. Subsequently, nested PCR was carried out using 40-fold dilution of primary PCR product using forward (5'-ACTATAGGGCACGCGTGGT-3') and reverse (5'-TCCCAGAGTTTCACCCATCACAGC-3') primers. Both forward primers were provided in the Universal GenomeWalker™ Kit designed against the blunt-end adaptor (Clontech) while both reverse primers were

designed against the 5' end of the coding region of PPARγ2 gene. PCR mixtures contained 1.0 μl of template (adaptor ligated restriction digest library/primary PCR product), 1X buffer containing 20mM $MgSO_4$, 400 nM of each primer, 400 μM of dNTPs, and 1 unit of *Pfu* DNA polymerase (Promega) in 25 μl total reaction. The mixtures were incubated at 94ºC for 3 min before subjected to 7 cycles (5 cycles for nested PCR) of amplification at 94ºC for 30 sec, 65ºC for 1 min and 72ºC for 2 min 30 sec; followed by 32 cycles (23 cycles for nested PCR) of amplification at 94ºC for 30 sec, 60ºC for 1 min and 72ºC for 2 min 30 sec. The PCR products were further incubated at 72ºC for 4 min. The PCR products were gel purified, cloned into pGEM-T Vector (Promega) and sequenced.

Identification of transcriptional start sites of bovine PPARγ2 gene

Isolation of total cellular RNA. Total cellular RNA was isolated from bovine adipose tissue using Tri Reagent (Molecular Research Center) as described by the manufacturer and size fractionated on 1% (w/v) formaldehyde gel.

Construction of full-length cDNA. Total cellular RNA was treated with RQ1 DNase (Promega) to ensure the purity of RNA extracted from any contamination of genomic DNA. The GeneRacer™ Kit (Invitrogen) was utilized to obtain the full-length cDNA. First strand of cDNA was then synthesized by reverse-transcribing the ligated mRNA using Superscript™ II RT (Invitrogen) and oligo dT primer.

Identification of transcriptional start sites of bovine PPARγ2 gene. Primary PCR was performed with first strand cDNA using forward (5'-CGACTGGAGCACGAGGACCATGA-3') and reverse (5'-CATCTCTGTGTCAACCATGGT-3') primers. A nested PCR was performed with a 100-fold dilution of the primary PCR product using forward (5'-GGACACTGACATGGACTGAAGGAGTA-3') and reverse (5'-CTTGTGAGGTCCTTGCAGACACTG -3') primers. Both forward primers which were provided in the Gene Racer™ Kit (Invitrogen), were designed against the RNA

oligonucleotide that was ligated to the full-length decapped mRNA while the reverse primers were gene-specific primers that were designed against the 5' end of bovine PPARγ2 exon B. PCR mixtures contained 1ul of template (cDNA/primary PCR product), 2.5 mM $MgCl_2$, 400 nM dNTPs, 400 μM of each primers, and 1 unit of Taq DNA Polymerase (Promega) in 1X reaction buffer (25 μl final volume). The mixtures were incubated at 94ºC for 3 min before subjected to 7 cycles (5 cycles for nested PCR) of amplification at 94ºC for 30 sec, 65ºC for 1 min and 72ºC for 1 min; followed by 32 cycles (23 cycles for nested PCR) of amplification at 94ºC for 30 sec, 60ºC for 1 min and 72ºC for 1 min. The PCR products were further incubated at 72ºC for 4 min. The PCR-RACE products were gel purified, cloned into pGEM-T Vector (Promega) and sequenced.

Characterization of bovine PPARγ2 promoter

DNA-Promoter Construct. PCR was performed to generate four promoter constructs i.e. approximately 220 bp, 430 bp, 620 bp and 900 bp in size, respectively. Forward and reverse primers were designed based on the sequence of the cloned promoter with restriction sites of *Sac*I and *Hind*III at the 5' end of the primers, respectively. These fragments were then gel purified and subjected to a double digestion using *Sac*I and *Hind*III restriction endonucleases. The purified fragments were subsequently ligated to pGL3-Basic Vector (Promega), which was previously digested with the same restriction enzymes. Transformation was carried out and the transformants were screened using RVprimer3 and GLprimer2 (Promega). Recombinant plasmids containing respective promoter-luciferase constructs were further verified by sequencing.

Cell line. MDBK (NBL–1) which is *Bos taurus* (bovine) normal kidney cell line, was purchased from American Type Culture Collection (ATCC). MDBK cells were grown in Eagle's minimum essential medium in the presence of 2.0 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate. This medium was also supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-activated (56ºC, 30 minutes) fetal calf serum (FCS). The cells were

maintained in a humid incubator of 5% (v/v) carbon dioxide at 37ºC. The medium in the culture flask was replaced every 3 days.

Transient transfection. Transient transfection was carried out using three different ratios of pRL-TK to promoter luciferase construct i.e. 1:10, 1:20 and 1:40, whereby different amount of promoter-luciferase constructs (1 μg – 4 μg) were transfected into MDBK cells while the concentration of co-transfected pRL-TK which served as an internal control for transfection efficiency was kept constant at 0.1 μg. 5 μl of Lipofectin® Reagent (Invitrogen) was used in every transfection. Plasmid pGL3-Basic Vector and pGL3-Control Vector were used as negative and positive controls, respectively. The cells were incubated at 37ºC in CO_2 incubator for 4 hours. After incubation, the medium was replaced and the cells were further incubated for another 36 hours. Luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega) using a luminometer (TD-20/20 Turner Designs). Each transfection reaction was carried out in four replicates and the measurement of luciferase activity was done in duplicates.

Results and discussion

Bovine PPARγ2 promoter with the size of 1.1 kb was successfully amplified using GenomeWalking technique. The nucleotide sequence of the promoter is shown in Figure 1. A comparison of this regulatory sequence with that of other species using T-Coffee and Box Shade programmes (www.ch.embnet.org/software) revealed 67% identity with human PPARγ2 promoter (Accession No: AF 310249) and 62% identity with mouse PPARγ2 promoter (Accession No: AY 236531) (data not shown).

Sequencing analysis of the PCR-RACE products revealed two putative transcriptional start sites of bovine PPARγ2 gene, which located at 173bp and 143bp upstream the start codon of the gene, respectively (Figure 1 (+1 and +1*, respectively)). The presence of multiple transcriptional start sites are common in PPARγ gene in other species (Gearing *et al*, 1994; Fajas *et al*., 1997; Chew *et al*, 2003) as well as in other genes (Ballarino *et al*., 2002; Kherrouche *et al*., 2004). For example, Fajas

et al. (1997) identified two transcriptional start sites in human PPARγ1 gene and three transcriptional start sites in human PPARγ2 gene. In addition, Ballarino *et al*. identitfied two transcriptional start sites within the same promoter in *Xenopus laevis* βTrCP gene.

The promoter sequence was scanned for the putative *cis*-acting elements (transcription factors binding sites) using MatInspector programme in the Genomatix website (www.genomatix.de/cgi-bin/eldorado/main.pl). Several potential binding sites were identified in the bovine PPARγ2 promoter such as Oct-1, Sp1, GATA-1, GATA-2, GATA-3, CREB, IRF, STAT, Smad4 and C/EBP. Figure 1 (underlined) shows the locations of binding sites of transcription factors which presence in the promoter suggesting their potential roles in regulating the expression of PPARγ2 in bovine.

Four promoter constructs of different sizes, i.e. approximately 220 bp (**F1**), 430 bp (**F2**), 620 bp (**F3**) and 900 bp (**F4**) were successfully generated by PCR and verified by sequencing (data not shown). In transient transfection, it was demonstrated that a steady increase in the amount of the promoter constructs transfected into the cell produced a gradual increase in the promoter activities (Figure 2), indicates that the promoter was a functional promoter. F3 fragment, which consists of region -605/-5 exhibited the strongest transcriptional activity among the constructs. This could indicate the presence of elements that positively regulate the transcription, and/or, the absence of elements that repressed the transcription of the promoter in that region.

Conclusion

The presence of transcriptional activity in the promoter fragments proved that the 1.1 kb bovine PPARγ2 promoter is a functional promoter. Two transcriptional start sites were identified located at 173bp and 143bp upstream the start codon of the gene, respectively. In addition, several potential transcription factors binding sites which may involve in regulating the expression of PPARγ2 in bovine were determined.

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                                                     GATA1
-885 AAATCCACAA GTCACTGCAA TTCCATGAGG CCACTTGTGT GATAGGAGGA -836

                     OCT1                      IRF2 (-)
-835 GGCCTGCTCC CACATTGAAA TTTGGCACAG CTAGCGTTTC CCTTTGCCAA -786

           PPAR/RXR         CEBP
-785 AAAGGTCAAA GGCCTTGAGC AAGAAGCCAG CTTTTTCCTG ATTACAAAC -736

                                                    IRF4 (-)
-735 TGACCACAAT TCCTCTCCAA CCTATCCGTG TCAGTCTTTT TTTTTTTTTT -686

            STAT
-685 TCCTTTCTGG GAATGCATTA TTCCTCTCAG GGAGAATTCT ACTTTTCTCA -636

-635 TGATGATCTG GAATCTATCC TTCCAATGTT AACGAATGTT CACACATCAG -586

           OCT1P                    PAX4
-585 CTGATGTATA TGCTATGGAT GGATAAACTT AATAATTAAG TTTCTGAGCA -536

-535 GCAGACCCTG ATATATAGAA TCTGAGCTGT CTCAGGCCCT GTCTCCAAGT -486

             TATA (-)                            TATA (-)
-485 CTGTTAATGC TTCAGTTTTA GAGAATCTGG ATCGCTGTGT CATTTTTTTA -436

-435 CAGACTACAC AAAGTAAGAG AGTAAATACC TAAGGACAGA GAATTTCAGT -386

             TATA (-)         OCT1 (-)
-385 AGCTGATCGC AAATTTTATA AACACATACT TGAATTCAGA CATTTTCTTC -336

       GATA
-335 AGATATCAGA TGATATGACT AGAGATTCAA CCAAGGGTGG GCAGACAGAA -286

        IRF7                    CEBP β(-)                GATA3
-285 AAAAAAATTA TCCAAGTAAG TTTAGCTAGT ATTTCATAAC CCAGAGATGT -236

           IRF1 (-)            OCT1P (-)
-235 AAAGTTTTCC ATTTTGGAAT CAACTGAATA TTGAACCACG TCTGCCCTCA -186

                            SP1 (-)
-185 GAGTTCTAAG TACTGTCCTG CCCCCGCCCC TCACAAGACA CGAGGGCCAC -136

        Smad4 (-)                              Smad4
-135 CGGCGAGACG GTGTGGCAAT GTTTTCCTTG TAATGTACCA GGTCTTGCCA - 86

                            CREB
- 85 AAGCAGTGAA CAGCCTTATG ACGCAACAGC TTGTCACAGC TGGTCTCTAA - 36

                                              ┌─►+1

 -35  CAGGAGGATG CCAGCCAGTC CATCCAGGCC TGG TCCTCTG TGAGTTTATT  15

                    ┌─►+1*

  16  CCACCTCCT CCAAACATTT GGAAACGGAC GTCTTGACTC ATTGGTGCGT   65
  66  TCCCAAGTTT TACTGCCATG CATCTTTTTC TTTGAACGGA ACTGGCCTTT   115
 116  TGCAAGAAAT AGACCAAATA TCGGTGGGAG TCGTGGCAAA TCCCTGTTCC  165
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166   GTGCTGTG**AT G**

FIGURE 1   Nucleotide sequence of bovine PPARγ2 promoter with two transcriptional start sites and predicted transcription factors binding sites**.** Numbers of the nucleotides are relative to the first transcription start site (+1) of the gene. The second transcription start site of the gene is labeled as (+1*). The predicted sites underlined with (-) indicates the sequence occurs in the reverse orientation. The start codon of the gene (ATG) is in bold.
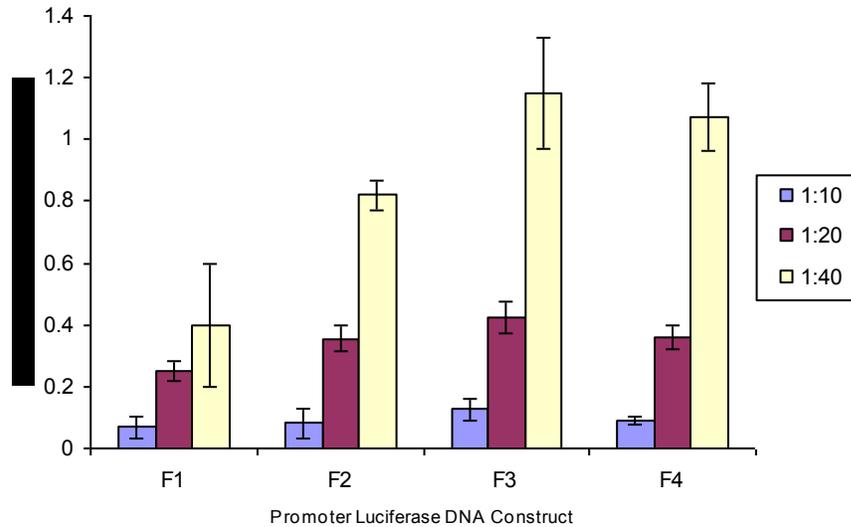

FIGURE 2   Analysis of bovine PPARγ2 promoter fragments activities in MDBK cell line. Promoter-constructs of bovine PPARγ2 were transfected transiently in MDBK cell line at different ratios. Each value is the results of four independent experiments (n=4). Ratio of 1:10, 1:20 and 1:40 represents the concentration ratio of pRL-TK: Luciferase-DNA construct.

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