

[BIO27] Molecular characterization of peroxisome proliferator activated receptor gamma2 (PPAR γ 2) promoter from bovine

Ida Shazrina Ismail, Yahya Mat Arip and Tengku Sifzizul Tengku Muhammad

School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

Introduction

Peroxisome proliferator activated receptor gamma (PPAR γ) is a member of the PPAR subfamily of nuclear hormone receptors (Schoonjans *et al.*, 1996a). So far, two PPAR γ isoforms; γ 1 and γ 2, have been cloned in bovine and produced by alternative promoter usage and differential splicing of a single gene (Sundvold *et al.*, 1997). PPAR γ 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).

PPAR γ 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 PPAR γ in fibroblasts in the presence of weak PPAR γ 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. PPAR γ 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 PPAR γ 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 PPAR γ 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 PPAR γ 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 PPAR γ because of its major overall economic value. PPAR γ 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 PPAR γ 2 gene, as well as the function/activities of the bovine PPAR γ 2 promoter.

Materials and methods

Amplification of PPAR γ 2 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, *Eco*RV, *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 PPAR γ 2 gene. PCR mixtures contained 1.0 μ l of template (adaptor ligated restriction digest library/primary PCR product), 1X buffer containing 20mM MgSO₄, 400 nM of each primer, 400 μ M of dNTPs, and 1 unit of *Pfu* DNA polymerase (Promega) in 25 μ l total reaction. The mixtures were incubated at 94°C for 3 min before subjected to 7 cycles (5 cycles for nested PCR) of amplification at 94°C for 30 sec, 65°C for 1 min and 72°C for 2 min 30 sec; followed by 32 cycles (23 cycles for nested PCR) of amplification at 94°C for 30 sec, 60°C for 1 min and 72°C for 2 min 30 sec. The PCR products were further incubated at 72°C for 4 min. The PCR products were gel purified, cloned into pGEM-T Vector (Promega) and sequenced.

Identification of transcriptional start sites of bovine PPAR γ 2 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 PPAR γ 2 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'-CTTGTGAGGTCCTTGACAGACTG-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 PPAR γ 2 exon B. PCR mixtures contained 1ul of template (cDNA/primary PCR product), 2.5 mM MgCl₂, 400 nM dNTPs, 400 μ M of each primers, and 1 unit of Taq DNA Polymerase (Promega) in 1X reaction buffer (25 μ l final volume). The mixtures were incubated at 94°C for 3 min before subjected to 7 cycles (5 cycles for nested PCR) of amplification at 94°C for 30 sec, 65°C for 1 min and 72°C for 1 min; followed by 32 cycles (23 cycles for nested PCR) of amplification at 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min. The PCR products were further incubated at 72°C for 4 min. The PCR-RACE products were gel purified, cloned into pGEM-T Vector (Promega) and sequenced.

Characterization of bovine PPAR γ 2 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 *SacI* and *HindIII* at the 5' end of the primers, respectively. These fragments were then gel purified and subjected to a double digestion using *SacI* and *HindIII* 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 μ g/ml streptomycin and 10% (v/v) heat-activated (56°C, 30 minutes) fetal calf serum (FCS). The cells were

maintained in a humid incubator of 5% (v/v) carbon dioxide at 37°C. 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 37°C in CO₂ 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 PPAR γ 2 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 PPAR γ 2 promoter (Accession No: AF 310249) and 62% identity with mouse PPAR γ 2 promoter (Accession No: AY 236531) (data not shown).

Sequencing analysis of the PCR-RACE products revealed two putative transcriptional start sites of bovine PPAR γ 2 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 PPAR γ 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 PPAR γ 1 gene and three transcriptional start sites in human PPAR γ 2 gene. In addition, Ballarino *et al.* identified two transcriptional start sites within the same promoter in *Xenopus laevis* β TrCP 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 PPAR γ 2 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 PPAR γ 2 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 PPAR γ 2 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 PPAR γ 2 in bovine were determined.

166 GTGCTGTGAT G

FIGURE 1 Nucleotide sequence of bovine PPAR γ 2 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.

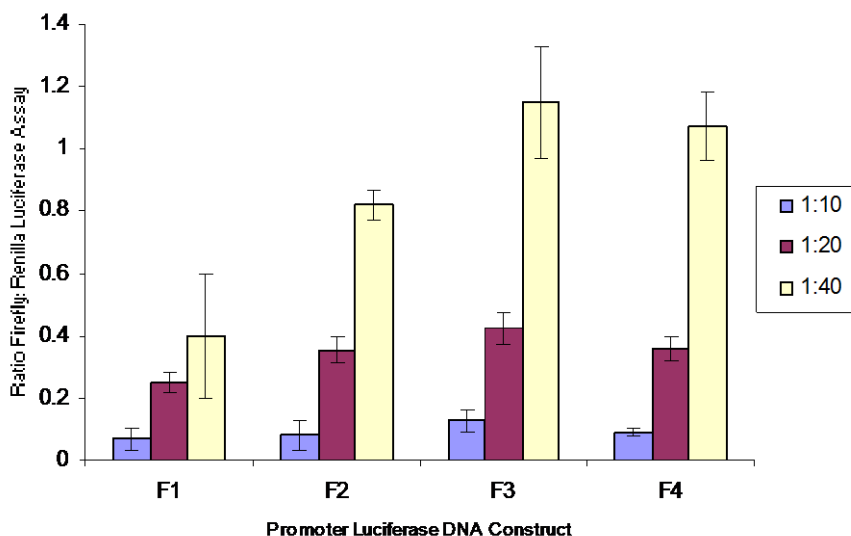


FIGURE 2 Analysis of bovine PPAR γ 2 promoter fragments activities in MDBK cell line. Promoter-constructs of bovine PPAR γ 2 were transfected transiently in MDBK cell line at different ratios. Each value is the results of four independent experiments (n=4). Ratio of 1:10, 1:20 and 1:40 represents the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

Acknowledgement

The authors wished to thank the Ministry of Science, Technology and Innovation (MOSTI) for the National Science Fellowship (NSF) awarded to Ida Shazrina Ismail and Malaysian Toray Science Foundation (MTSF) for the Science and Technology Research Grant awarded to Ida Shazrina Ismail and Tengku Sifzizul Tengku Muhammad.

References

Ballarino, M., Marchioni, M., Carnevali, F. (2002) The *Xenopus laevis* β TrCP gene: genomic organization, alternative splicing, 5' nad 3' region characterization and comparison of its structure with that of human *Xenopus laevis* β TrCP genes. *Biochem. Biophys. Acta* 1577, 81-92

Chew C. H., Samian, M. R., Najimudin, N., Tengku-Muhammad, T. S. (2003) Molecular characterization of six alternatively spliced variants and a novel promoter in human peroxisome proliferators-activated receptor α . *Biochem and Biophys. Res. Com.* 305, 235-243

Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., Najib, J., Laville, M., Fruchart, J. C., Deeb, S., Vidal-Puig, A., Flier, J., Briggs, M. R., Staels, B., Vidal, H. and Auwerx, J. (1997) The organization, promoter analysis and expression of human PPARgamma gene. *J. Biol. Chem.* 272, 18779-17789

- Gearing, K. L., Crickmore, A., Gustafsson, J. A. (1994) Structure of the mouse peroxisome proliferators-activated receptor α gene. *Biochem and Biophys. Res. Com.* 199, 255-263
- Kherrouche, Z., De Launoit, Y. and Monte, D. (2004) Human E2F6 is alternatively spliced to generate multiple protein isoforms. *Biochem. and Biophys. Res. Com.* 317, 749-760
- Martin, G., Schoonjans, K., Lefebvre, A. M., Staels, B. and Auwerx, J. (1997) Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase gene by PPAR α and PPAR γ activators. *J. Biol. Chem.* 272, 28210-28217
- Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B. and Auwerx, J. (1996b) PPAR α and PPAR γ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* 15, 5336-5348
- Schoonjans, K., Staels, B. and Auwerx, J. (1996a) The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochem. Biophys. Acta* 1302, 93-109
- Sundvold, H., Brzozowska, A. and Lien, S. (1997) Characterisation of bovine peroxisome proliferators-activated receptor γ 1 and γ 2: genetic mapping and differential expression of the two isoforms. *Biochem. and Biophys. Res. Com.* 239, 857-861
- Tontonoz, P., Erding, H., Graves, R.A., Budavari, A. I. And Spiegelman, B. M. (1994b) mPPAR gamma2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* 8, 1224-1234
- Tontonoz, P., Graves, R.A., Budavari, A., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P. and Spiegelman, B. M. (1994a) Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR γ and RXR α . *Nucleic Acids Res.* 22, 5628-5634
- Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994c) Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated-transcription factor. *Cell*, 79, 1147-1156