[BIO19] Molecular cloning and characterisation of the 5'-untranslated region and promoters in human peroxisome proliferator-activated receptor alpha (hPPARα)

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Introduction

The peroxisome proliferator activated receptors (PPARs) are members of the nuclear hormone receptor gene superfamily of ligand-activated transcription factors [Issemann and Green, 1990]. So far, three distinct subtypes of PPARs have been identified, PPARα, PPARγ and PPARβ/δ, with each subtype encoded by a separate gene and has a specific tissue distribution [Issemann and Green, 1990; Braissant et al, 1996]. The PPARα, the first isoform to be identified, is expressed mainly in tissues that have high ratio of fatty acid oxidation such as liver, heart and kidney [Issemann and Green, 1990; Braissant et al., 1996]. PPARα has attracted considerable attention since it was demonstrated to be regulators of lipoprotein metabolism [Staels et al., 1998], vascular inflammation [Chinetti et al., 2000], atherosclerosis [Chinetti et al., 2000; Pineda-Torra et al, 2002] and carcinogenesis [Neve et al., 2000; Collett et al., 2000]. Moreover, recent studies suggested important roles for PPARα in skin wound healing, and epidermal maturation and repair [Wahli, 2002].

In order to understand the structure and molecular mechanisms governing hPPARα regulation, we carried out experiments to identify and characterise the 5'-untranslated region (UTR) and promoter regions of the hPPARα gene. In this study, we managed to identify six differentially spliced variants in the 5'-untranslated region (UTR) of human PPARα gene, due to four different promoter usages and alternative splicing [Chew et al., 2003]. Since promoter A has been characterised [Pineda-Torra et al., 2002], three other promoters designated as promoter B, C and D were cloned, sequenced and their promoter activities analysed. By carrying out transient transfection analysis in human hepatocarcinoma HepG2 cells, we proved these three promoters are functional promoters, capable of inducing the basal luciferase gene expression, with promoter B being the most potent and strongest promoter of the three promoters.

Materials and Methods

Isolation of total cellular RNA

Total cellular RNA was isolated from the human hepatoma HepG2 cell line using Tri-Reagent LS (Molecular Research Center) according to the manufacturer's instruction. The concentration of each total cellular RNA was then quantified using GeneQuant (Amersham Pharmacia Biotech).

Rapid amplification of cDNA ends (5'RLM-RACE) and sequencing of RACE products

5'RLM-RACE was performed on 5 µg total RNA using the GeneRacer™ Kit (Invitrogen® Life Technologies) according to the manufacturer's instruction. The first strand cDNA was generated using primers supplied by the manufacturer and subjected to two PCR reactions. Specifically, the synthesised cDNA was used as the template in the amplification process using the internal gene-specific primer GSP1 (5'-GCAGG AACTCTTCAGATAACGG-3'), located at the 5' end hPPARα exon 3 and the GeneRacer™ 5' Primer. PCR cycling parameters were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of 1 min at 94°C, 1 min at 65°C, 1 min at 60°C, and 1 min at 72°C with a final elongation at 72°C for 10 min. The primary PCR mix was then subjected to a nested PCR under the same conditions with the GeneRacer™ 5' Nested Primer and GSP2 (5'-GCAGAGTGGGCTTTCCGTGT-3') designed immediately upstream of GSP1. Oligonucleotides GeneRacer™ 5' Primer and GeneRacer™ 5' Nested Primer were provided in the GeneRacer™ Kit. The PCR products were size fractionated on 1.5% (w/v) agarose gel and selected bands were then gel purified using QIAquick gel extraction kit (QIAGEN). The purified bands were directly ligated into

pGEM-T Easy vector (Promega) and transformed into JM109. Recombinant plasmids were then isolated and purified for DNA sequencing using Plasmid Midi Kit (QIAGEN). Sequencing reactions were carried out using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem).

Cloning and construction of reporter-plasmids

The promoter regions were amplified from human genomic DNA as template with Platinum® *Pfx* DNA polymerase (Invitrogen® Life Technologies) to minimise PCR generated mutations. These products were then used as template for generating promoter fragments which represent different regions of these three promoters. The promoters' fragments with different sizes were amplified from the cloned promoter as template using primers containing *Xho*I and *Hin*dIII to facilitate the cloning into the pGL3-Basic vector (Promega) in the correct orientation. For promoter B, reporter-promoter plasmids, pGL3alphB1 (carrying regions -341/+34), pGL3alphB2 (-765/+34) and pGL3alphB3 (-1147/+34), were constructed. For promoter C, reporter-promoter constructs were cloned and named pGL3alphC1 (carrying regions -413/+41), pGL3alphC2 (-967/+41), pGL3alphC3 (-1364/+41), while in promoter D, reporter-promoter plasmids designated pGL3alphD1 (-395/+28), pGL3alphD2 (-672/+28) and pGL3alphD3 (-934/+28) were constructed.

Cell culture and transient transfection assays

Human hepatoma HepG2 cell line were grown in Eagle's Minimum essential medium with Earle's BSS medium (Gibco) supplemented with 10% (v/v) fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Hyclone). The cultures were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in air. The cells were transfected with individual promoter fragment, together with pRL-TK containing *Renilla* luciferase (Promega) which served as internal control, using Lipofectin transfection reagent (Invitrogen® Life Technologies) according to the manufacturer's instructions for 36 hrs. The luciferase activities in the cell extracts were then determined using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalised to the *Renilla* luciferase activity, with each transfection being carried out in triplicate and measurements were repeated at least twice.

Results and Discussion

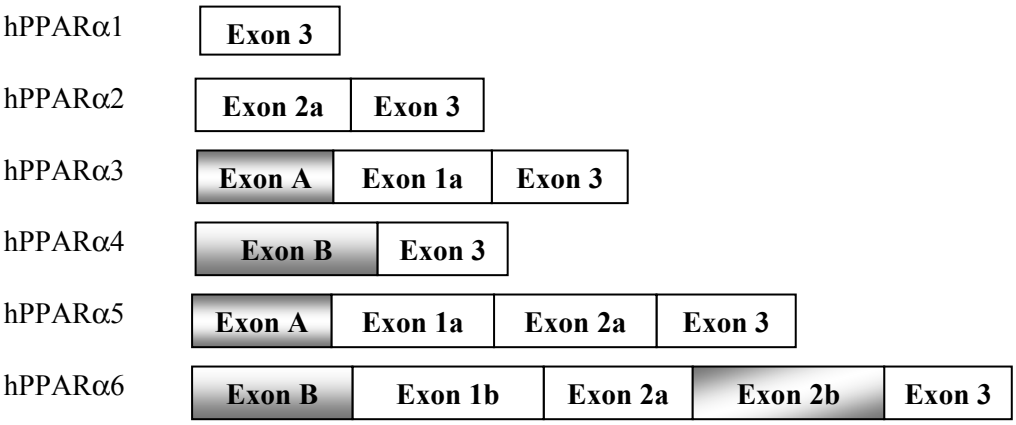
Identification of Alternatively Spliced Variants of the 5' UTR of hPPARα gene

Using RLM-RACE PCR approach, six RACE-PCR products of different sizes (100bp, 200bp, 230bp, 300bp, 330bp and 600bp) were successfully amplified (figure not shown). Sequencing analysis showed that the 3' end of all RACE-PCR products shared 100% identity with the 5' end of Exon 3 of human PPARα gene, where the gene-specific primers were designed, indicating that all RACE-PCR products were specific and represent the 5'-UTR of the human PPARα. Interestingly, sequence analysis amongst the six RACE-PCR products revealed different combination of exons which strongly indicates the existence of 6 differentially spliced variants in the 5'-UTR of the hPPARα gene, designated as hPPARα1, hPPARα2, hPPARα3, hPPARα4, hPPARα5 and hPPARα6 (Figure 1A) [Chew et al., 2003].

In addition to these six alternatively spliced variants, we have also successfully identified three new novel exons of the human PPARα gene, designated Exon A, Exon B and Exon 2b (Figure 1B) [Chew et al., 2003].

The presence of alternatively spliced variants in human PPARα mRNA is a common phenomenon amongst PPAR isoforms. For example, four variants resulting from four different promoters usages and alternative splicing at the 5'-UTRs have been identified in human PPARγ mRNA [Fajas et al., 1997, Fajas et al., 1998, Sundvold and Lien, 2001], while seven alternatively spliced variants were detected in monkey PPARγ mRNA [Zhou et al., 2002]. In addition, four differentially spliced variants induced by four differential promoter usages were also detected in mouse PPARβ mRNA [Larsen et al., 2002]. Meanwhile, in porcine PPARα mRNA, two alternatively spliced mRNA were found in the coding region of PPARα [Sundvold et al., 2001].

(A)



(B)

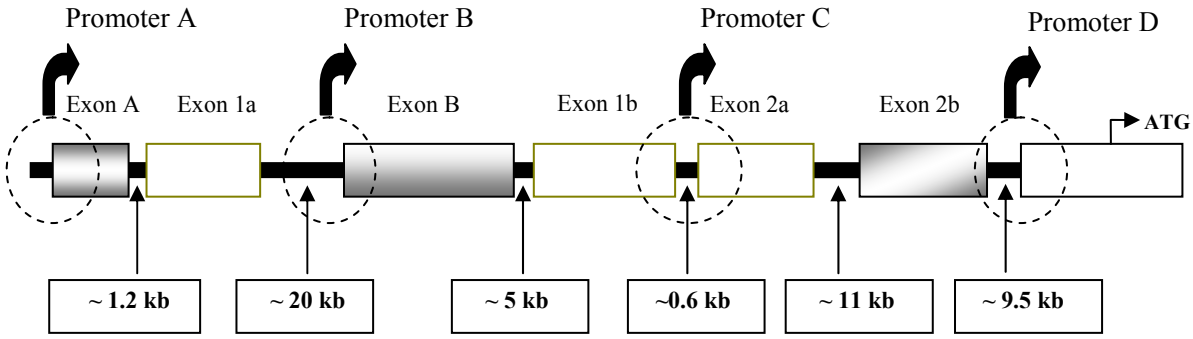


FIGURE 1 (A) The six alternatively spliced variants identified from this study. Alternative splicing mechanisms and different promoters usage give rise to variants with unique combinations of exons. (B) Schematic representation of the 5'-UTR of hPPARα gene. The new exons identified are indicated as shaded boxes. Numbers in bold and in boxes represents the sizes of the introns which span the exons. Arrows indicate the presence of promoter in front of Exon A, Exon B, Exon 2a and Exon 3.

Determination of transcriptional start sites

RLM-RACE method allows only full length 5'-capped transcripts to be amplified via the elimination of truncated mRNA, thereby rendering it a safe, reliable and accurate method for mapping of the 5' ends of transcripts and transcriptional start sites of genes [Maruyama et al., 1994, Volloch et al., 1994]. The transcriptional start sites of Exon 3 and Exon 2a were situated 31 bp and 124 bp upstream of the ATG initiation start codon in the processed mRNA, respectively, while exons A has two different transcriptional start sites, each individually marks the start site variants hPPARα3 and hPPARα5. The transcriptional start site of hPPARα3 and hPPARα5 are situated 172 bp and 264 bp upstream of the ATG initiation start codon, respectively; with the hPPARα5 transcriptional start site located 8 nucleotides upstream of the transcriptional start site of variant PPARα3 in Exon A. Meanwhile, Exon B also has two transcriptional start sites (variant PPARα4 and variant PPARα6) mapped to the positions 251 bp and 540 bp upstream of their respective ATG start codon. The transcriptional start site for PPARα4 is 48 bp upstream of the transcriptional start site in PPARα6 [Chew et al., 2003].

Multiple transcriptional start sites within the same promoter region are in fact a common phenomenon and frequently observed PPAR family, i.e. in the mouse PPARα and PPARβ gene [Larsen et al., 2002, Gearing et al., 1994].

Cloning and analysis of the promoter regions B, C and D

We determined the transcriptional start sites for each variant of the human PPARα gene, which suggested the presence of four promoters responsible for transcribing these variants. Since promoter A was identified and characterised (Pineda-Torra et al., 2002), promoters B, C and D were chosen to be further characterised. Direct PCR amplifications using human genomic DNA as template and gene-specific primers were carried out to amplify these putative promoter regions. Products with sizes of 1.2 kb (promoter B), 1.5 kb (promoter C) and 1.0 kb (promoter D) were successfully amplified.

A computer search of the putative promoter regions using the Matisnspector programme in TRANSFAC database (http://transfac.gbf.de /TRANSFAC/index.html) revealed the existence of multiple potential binding sites for transcription factors that may regulate the expression of the human PPARα gene. Binding sites for transcription factors such as Oct-1, AP-1, Sp1, C/EBP, STAT, GATA-1, GATA-2, HNF-3β and RORα1 were identified within the promoters B, C and D regions of human PPARα gene (figures not shown).

Characterisation and analysis of promoters B, C and D activities

The relative strength of the different promoters and different fragments of the promoters was compared by transient transfection in Hep3B and HepG2 cells, using various fragments of promoters' sequences. Fragments containing regions -341/+34 (pGL3alphB1), -765/+34 (pGL3alphB2) and -1147/+34 (pGL3alphB3) of promoter B, fragments bearing the regions -413/+41 (pGL3alphC1), -967/+41 (pGL3alphC2) and -1364/+41 (pGL3alphC3) of promoter C and fragments -395/+28 (pGL3alphD1), -672/+28 (pGL3alphD2) and -934/+28 (pGL3alphD3) of promoter D were cotransfected into the cells with pRL-TK plasmid which served as an internal control for transfection efficiency. Results obtained from this study proved that all three promoters B, C and D are functional promoters capable of driving the basal transcription of the luciferase gene.

Promoter activities for promoter B were the highest in HepG2 cells compared to promoter C and D, thereby indicating that promoter B is the highest promoter of the three, followed by promoter D being a stronger promoter than promoter C (Figure 2).

Overall, the smallest promoter fragments of the three promoters, pGL3alphB1 (-341/+34), pGL3alphC1 (-413/+41) and pGL3alphD1 (-395/+28), proved to have the strongest activity compared to their respective fragments in HepG2 cells (Figure 2). This could indicate the absence of elements conferring transcriptional repression upstream of the start sites, or alternatively, the presence of an element enhancing transcription between positions -341 to +34 of promoter B, positions -413 to +41 of promoter C, and positions -395 to +28 of promoter D, or a combination of both.

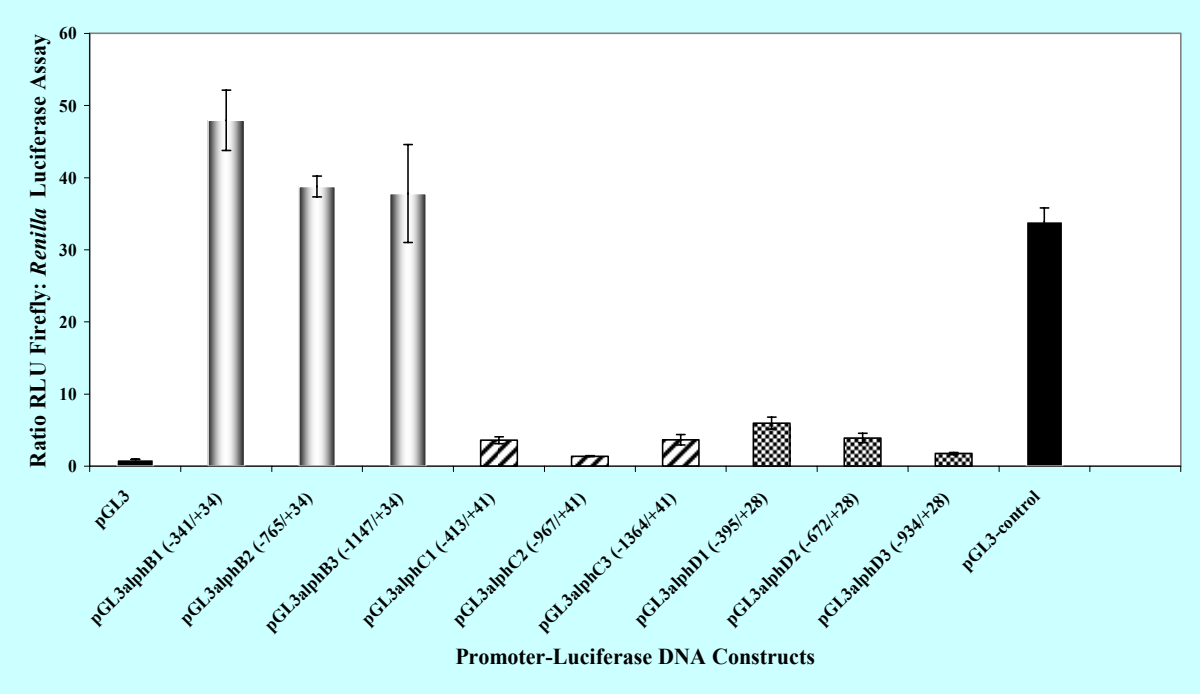


FIGURE 2 Analysis of promoters B, C and D activities using transient transfection assays. The promoter fragments of promoters B, C and D were fused into pGL3 basic vector and co-transfected with pRL-TK into HepG2. Values (mean ± SD) represent firefly luciferase activity normalised to a *Renilla* luciferase activity which served as internal control. Luciferase activities are shown relative to the activity of the pGL3 basic vector.

Conclusions

Six variants, named hPPARα1, hPPARα2, hPPARα3, hPPARα4, hPPARα5 and hPPARα6, were successfully identified at the 5'-UTR of human PPARα mRNA as a result of alternatively splicing and the use of four different promoters. Each of the transcriptional start sites of the variant was determined and mapped, leading to the discovery of four promoters in the human PPARα gene which are responsible for transcribing these alternatively spliced variants. In addition, three new novel exons designated Exon A, Exon B and Exon 2b were identified in this study. Three new novel human PPARα promoters were also cloned and characterised. Demonstrated by transient transfection assay, these putative promoters B, C and D were all proven to be functional promoters, with promoter B being the strongest of the three promoters.

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