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Identification of Functional Peroxisome Proliferator-Activated Receptor α Response Element in the Human *Ppsig* Gene

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Abstract—Peroxisome proliferator-activated receptor α (PPARα), one of the key ligand-activated nuclear receptors interacting with PPAR response elements (PPREs), may trigger the expression of PPAR-responsive genes and be involved in the transcriptional regulation of lipid metabolism, energy balance, and some diseases. Previous studies have demonstrated that the mouse *Ppsig* gene is a novel PPARα target gene taking a pivotal role in maintaining energy balance during fasting. Disparity between humans and rodents in their PPAR systems requires corroborating experiments to determine whether the *hPpsig* gene (*Ppsig* homologous gene in human) is also a PPARα target gene. In this work, eight putative PPREs in the promoter and first intron of *hPpsig* were identified. However, only one intronic PPRE could respond to PPARα by transient transfection. Furthermore, the binding activity of PPARα with this intronic PPRE was confirmed by electrophoretic mobility shift assay *in vitro*. This investigation might help to elucidate the transcriptional regulatory mechanisms of *Ppsig* in humans.

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Key words: EMSA, human *Ppsig*, intronic PPREs, PPARα, transcriptional regulation

Peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear hormone receptor subfamily, was first identified in 1990. PPARα regulates biochemical reactions including lipid metabolism, inflammation, oxidative stress, and amino acid metabolism in a very complex way [1]. Also, PPARα has been related to a series of diseases such as hyperlipidemia, atherosclerosis, alcoholic fatty liver, and diabetes [2]. Similar to other nuclear hormone receptors, the physiological actions of PPARα depend on forming a heterodimer with retinoid X receptor (RXR), binding to a peroxisome proliferator response element (PPRE), and then activating/repressing transcription of target genes. PPRE was also named direct repeat-1 (DR-1) with the sequence AGGTCAnAGGTCA [3]. Since the major role of PPARα is to regulate energy balance, many functional PPREs have been reported in the promoter or first intron of genes encoding relevant enzymes, such as adipose differentiation-related protein [4], carnitine-acylcarnitine translocase [5], myocardial lipid droplet protein [6], hormone-sensitive lipase [7], glycogen synthase-2 [8], and others.

It has recently been demonstrated that mouse *Ppsig*, a PPARα target gene with one functional PPRE located in the first intron, may play an important role during fasting [9]. Because humans and rodents are dissimilar in their PPAR systems, conclusions from rodent models may not be directly applied to humans. The data have shown that wild-type (WT) mice exhibit significant hepatomegaly and hepatocyte proliferation after treated with fenofibrate, while a similar phenomena has not been observed in PPARα-humanized mice. Also, different expressions of a series of genes regulated by PPARα were found between the WT and PPARα-humanized mice treated with Wy-14,643 [(4-chloro-6-(2,3-xylidino)-2-pyrimidinyl-thio) acetic acid] [10]. In addition, the PPREs in the promoters of human acyl-CoA oxidase (AOX) and cynomolgus AOX lack sensitivity to PPARα; but in rat AOX promoter, the PPRE shows high response to PPAR [11]. So corroborating experiments need to be done to prove whether the *hPpsig* gene, the *Ppsig* homologous gene in humans, is also a PPARα target gene. Therefore, the investigation in this research was to determine whether functional PPREs might exist in the *hPpsig*

Abbreviations: AOX, acyl-CoA oxidase; DIG, digoxin; DR-1, direct repeat-1; PPARα, peroxisome proliferator-activated receptor α; PPREs, PPAR response elements; RXR, retinoid X receptor; WT, wild type.

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gene by *in silico* analyses, transient transfection, and electrophoretic mobility shift assay.

MATERIALS AND METHODS

Construction of vectors. Human genomic DNA was isolated and purified from peripheral blood mononuclear cells. The 2614 bp region within the *hPpsig* promoter (−3142/−529) containing seven putative PPREs (named PPRE1 to PPRE7) was amplified by nest PCR with NF (5′-CCCCTCCAGTGTCCAAAGACTTCCC-3′) and NR (5′-TCCTACGCATTTCTGTGCTGCTCCC-3′) as the outer primers, 5UF (5′-CCTCTCGAGTGGGTGGGCAAAGTAG-3′) and 5UR (5′-GCCAAGCTTCCACGGGGATTACAAG-3′) as the nest primers. A 207 bp fragment flanking the putative PPRE (named PPRE8) within intron 1 (+273/+479) of the *hPpsig* gene was amplified using IF (5′-CAGCTCGAGCAGCCGCTACCAGACG-3′) and IR (5′-GCCAAGCTTCCACGGGGATTACAAG-3′) with the same first round PCR product as template. After double digestion with *Xho*I and *Hind*III, the two fragments were subcloned into luciferase reporter vectors, respectively. The promoter region (−3142/−529) of *hPpsig* was subcloned into pGL4.23 (Promega, USA) to construct pGL4.23/5UTR. And the intron 1 region (+273/+479) of *hPpsig* was used to generate pGL4.23/intron 1 reporter.

Three kinds of the intronic PPRE8 mutant reporters were constructed by overlap-extension PCR. The MA fragment was generated with four primers, the forward primer MAF (5′-GTCGATAGCCTTTttttTAGTAGGCCCCGGGAATAG-3′) and the reverse primer MAR (5′-CTATTCCCGGGGCCTACTAaaaaAAAGGCTATCGAC-3′) containing mutant nucleotides (shown in lower case), and the outer primers IF/IR. After the overlap-extension, the PCR product was double digested with *Xho*I and *Hind*III, and then cloned into the pGL4.23 vector to construct pGL4.23/MA. Another PPRE8 mutant reporter pGL4.23/MX was generated in the same way, using the inner primers MXF (5′-CAACACTGTCGATttttTTTGCCCTAGTAGGCCCC-3′) and MXR (5′-GGGGCCTACTAGGGCAAAaaaaATCGACAGTGTTG-3′). Primers MTF (5′-CAACACTGTCGATttttTTTttttTAGTAGGCCCCGG-3′) and MTR (5′-CCGGGGCCTACTAaaaaAAAaaaaATCGACAGTGTTG-3′) were used to construct pGL4.23/MT.

Plasmids pIRES1neo/huPPARα and hRXRα/pSVL were gifts from Prof. P. Chambon. The open reading frames of hPPARα and hRXRα were subcloned into pSG5 vector (Stratagene, USA), respectively.

Cell culture, transfection, and luciferase assays. HepG2 and Bel-7402 cells were cultured in 24-well plates with Dulbecco's Modified Eagle's Medium (DMEM, high glucose) plus 10% (w/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C. The PPRE-driven luciferase plasmid, hPPARα expression plasmid, and hRXRα expression plasmid were transiently co-transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Plasmid pRL-TK (Promega) was used as the internal control for transfection efficiency. Fenofibrate (Sigma-Aldrich, USA), a specific PPARα ligand to activate the heterodimeric complex of hPPARα and hRXRα, was prepared as 50 mM stock solution in dimethyl sulfoxide (DMSO) [12]. After transfection, the cells were incubated for 24 h in the presence or absence of 50 μM fenofibrate. Subsequently the cells were assayed for luciferase expression using the Dual-Luciferase reporter assay system (Promega). Bel-7402 cells were transfected and studied as described previously.

***In vitro* transcription and translation.** The proteins of hPPARα and hRXRα were transcribed and translated *in vitro* using the TNT T7 Quick Coupled Transcription/Translation system (Promega) according to the manufacturer's protocols. The *in vitro*-translated proteins were labeled with Transcend non-radioactive translation detection system (Promega) and verified by Western blot according to the manufacturer's protocols.

EMSA. Oligonucleotides for EMSA were 5′-end-labeled with digoxin (DIG) and annealed with antisense oligonucleotides in annealing buffer (10 mM Tris, 1 mM EDTA, 0.1 mM NaCl, pH 8.0). Double-stranded oligonucleotides (designated as probes) including PPRE8, mutPPRE8-MA, mutPPRE8-MX, and mutPPRE8-MT were used for binding analysis. For competition analysis, the unlabeled probes of all the above four kinds were utilized. The probes, hPPARα and hRXRα proteins, were incubated in EMSA/Gel-Shift Binding buffer (Beyotime, China) for 30 min at 25°C. Then the DNA–protein complexes were loaded on a 6% non-denaturing polyacrylamide gel and electrophoresed. The resolved products were transferred onto a positively charged nylon membrane (GE Healthcare, USA) by electroblotting. After prehybridization, the membrane was incubated in hybridization solution containing anti-DIG-AP (Roche, Germany), and then the signals were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche) as substrates.

Statistical analysis. Numerical data were analyzed by Student's *t*-test using SPSS 16.0 software. Analyses with $p \le 0.05$ were considered significant. Data are represented as means ± SD of three independent experiments.

RESULTS

Eight putative PPREs found in the *hPpsig* gene. The genomic sequence of the *hPpsig* gene (Accession number NW_927719) was retrieved from the GenBank database. Previous studies showed that functional PPREs might be

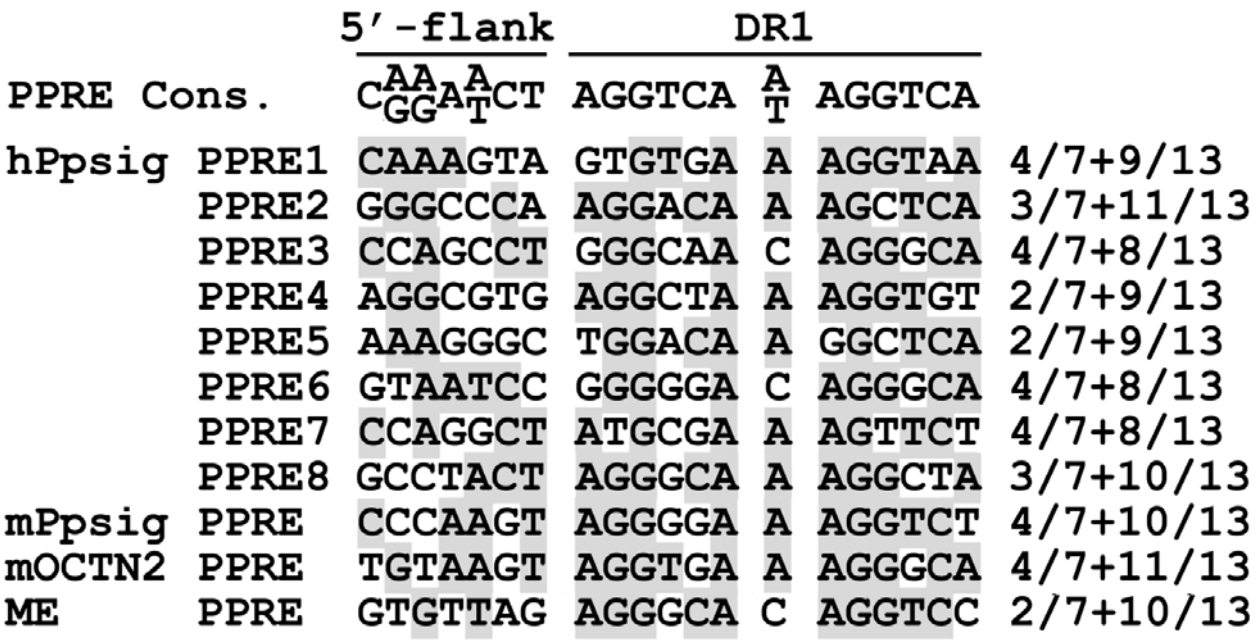

Fig. 1. Alignment of the PPREs from *hPpsig* and other PPARα target genes. PPREs from *hPpsig*, *Ppsig*, mOCTN2, and ME were compared to the consensus PPRE sequence [14]. On the right side the numbers of matches compared to consensus in the 5′-flanking region and the core DR-1 are shown.

present in the promoter or first intron of many genes [4-8]. So, in order to predict the PPRE motifs in the *hPpsig* gene, the 5′-UTR region (−3342/−1) and the intron 1 region (+198/+2670) were analyzed using the online MatInspector program (http://www.genomatix.de/cgi-bin/matinspector_prof). The results revealed that seven putative PPREs were found in the 5′-UTR region, named PPRE1 to PPRE7, respectively. In addition, only one putative PPRE, named PPRE8, was found in the region of intron 1. PPRE1, PPRE4, PPRE5, and PPRE6 were in the forward direction, and the other PPREs were in the reverse direction. After sequence alignment, all the eight putative PPREs were confirmed to be consistent with the classical PPRE motif, of which PPARα could bind to the 5′ half-site and RXRα bind to the 3′ half-site (Fig. 1).

PPREs in the 5′-UTR have no function. The pGL4.23/5UTR reporter containing seven PPREs was constructed as described previously. As the modified coding region of firefly luciferase is located downstream from the *hPpsig* 5′-UTR region, the activity of luciferase is examined to represent the transcription level triggered by the interactions between hPPARα/hRXRα and the PPREs. The results from transient transfections in HepG2 cells showed that there was almost no increase in relative luciferase activity in the treatment group compared with the control group (Fig. 2a). Even in the group treated with fenofibrate, a strong peroxisome proliferator, only a slightly enhanced luciferase activity could be found. The results were similar in Bel-7402 cells. This indicated that the seven putative PPREs from the *hPpsig* 5′-UTR region (−3142/−529) might not be functional PPARα response elements.

Intronic PPRE8 is a functional PPRE. To determine whether the intronic PPRE8 of *hPpsig* gene was functional, pGL4.23/intron 1 reporter was transiently transfected into HepG2 cells and Bel-7402 cells as described previously. The results showed that the relative luciferase activities were clearly different among the three groups (Fig. 2b). The lowest relative luciferase activity was found in the control group, which was transfected with pGL4.23/intron 1 only. Compared with the control group, a 2-fold higher relative luciferase activity was observed in the test group that was co-transfected with pGL4.23/intron 1, pSG5-hPPARα, and pSG5-hRXRα plasmids. And a significantly increased relative luciferase activity, nearly 4-fold higher, was found in another test group treated with fenofibrate. Similar results were observed in Bel-7402 cells. These data suggest that the putative PPRE8 located in the first intron of *hPpsig* gene is a PPARα response element.

To verify this supposition, three reporters were constructed respectively with the mutations in PPARα binding site, RXRα binding site, or in both sites. So these mutant reporters eliminated the binding capabilities to hPPARα/hRXRα. As anticipated, the results indicated that compared with the wild-PPRE group, the relative luciferase activities in the groups transfected with the mutant reporters decreased significantly, regardless of being treated with or without fenofibrate (Fig. 2c), which further confirmed that the putative PPRE8 of *hPpsig* gene is a functional PPRE motif.

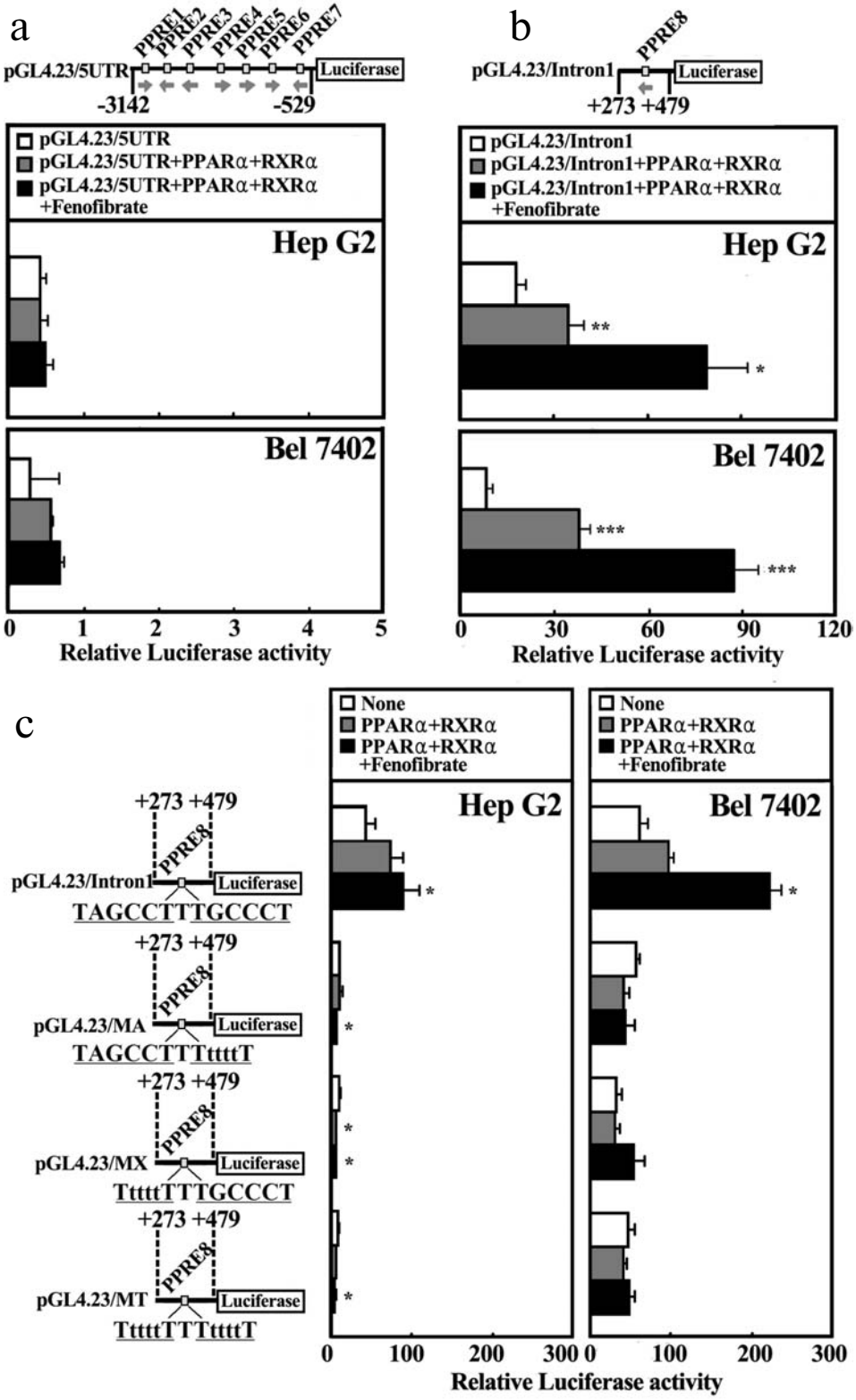

Fig. 2. Intronic PPRE8 is the functional PPRE motif in *hPpsig*. The pGL4.23/5UTR (a), pGL4.23/intron 1 (b), and mutant reporters (c) were transiently transfected into HepG2 cells and Bel7402 cells with or without hPPARα and hRXRα expression vectors. After transfection, the cells were incubated in the presence or absence of 10 μM fenofibrate and subsequently assayed for luciferase expression. The pRL-TK was co-transfected in all of the tested groups for normalization. The data represent the means ± SD of at least three independent experiments; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Binding of PPARα/RXRα heterodimer to PPRE8 in the *hPpsig* gene. EMSA was performed to determine whether the hPPARα/hRXRα heterodimer indeed bound to PPRE8 *in vitro*. The 5′-DIG-labeled probes corresponding to the PPRE8 were incubated with *in vitro* transcribed/translated hPPARα and hRXRα, separately or simultaneously. As shown in Fig. 3, a unique band was distinguished in the presence of both hPPARα and hRXRα proteins (lane *3*). But the same band was not observed in the presence of either hPPARα or hRXRα alone (lanes *1* and *2*). In competition experiments, unlabeled PPRE8 probes were added prior to the labeled probes to interact with the heterodimer, which resulted in weakening of the specific retarded band in a dose-dependent manner along with 5- to 100-fold increasing excess of unlabeled probes (lanes *4-6*). To confirm the binding sequence in PPRE8, mutant PPRE8 probes carrying mutations in the PPARα binding site, RXRα binding site, and in both sites were synthesized, respectively. The competition assay was designed as in the previous experiment. The signals of the unique band were not disturbed even using 100-fold superabundant mutant probes (lanes *7-9*). Compared with the competitive groups, no retarded bands were displayed in the groups where the 5′-DIG-labeled mutant probes were incubated with the hPPARα/hRXRα heterodimer directly (lanes *10-12*). All of these results led to the conclusion that the hPPARα/hRXRα heterodimer binds specifically to PPRE8 of the *hPpsig* gene.

DISCUSSION

Previous studies showed that the molecular mechanisms of PPAR in the regulations of homologous target genes in different species might be distinct. For example, the intronic PPREs of ACBP genes are conserved in rats, mice, and humans. While the rat ACBP gene has another functional PPRE located in the promoter region, the human and mouse ACBP genes lack the PPRE in the promoter region [13]. That was why it was needed to determine which PPRE is functional in the *hPpsig* even though it was known in mouse *Ppsig* [9]. There are seven upstream PPREs and one intronic PPRE predicted in the *hPpsig* gene located on human chromosome 2. Transient transfection assays and EMSA demonstrated that only the putative PPRE8 located in the first intron really showed significant response to the hPPARα/hRXRα heterodimer. It is similar to the mouse *Ppsig* gene located on mouse chromosome 6, as only one PPRE located in the first intron of the mouse *Ppsig* gene is active [9]. Alignment of the functional PPREs from *hPpsig* and mouse *Ppsig* shows that the two PPREs are highly homologous and maintained regardless of evolutionary pressure (Fig. 1). Thus, this functional PPRE may be the important regulatory element for the *Ppsig* gene.

The conservative sequence of PPRE was summarized as DR-1 (AGGTCAnAGGTCA) at one time [3]. The optimized PPRE motif was longer as it contained not only the DR-1 motif, but also seven nucleotides (termed as 5′-flanking region) located upstream of the DR-1 sequence [14] (Fig. 1). Several sequence characteristics of the PPREs may contribute to their activities. For instance, when the spacing nucleotide of the DR-1 motif was "A", the strongest binding activity was found [15]. Besides, studies on the AOX gene showed that the four bases located in the 5′-flanking region of the PPRE motif provide high-affinity binding activity of AOX PPRE with PPARα/RXRα [16]. In this study, eight PPREs were predicted in *hPpsig*. Among them, the PPRE2 and PPRE8 have the highest similarity compared with the PPREs of mouse *Ppsig* and mouse organic cation transporter 2 (mOCTN2) exhibiting as positive controls [17]. Based on our experiments, only the intronic PPRE8 is confirmed as the functional one in *hPpsig*. However, PPRE2 has 11 out of 13 matches in the core DR-1 motif; and PPRE8 has only 10 out of 13 matches. This phenomenon might be explained by differences in the 5′-flanking regions between PPRE2 and PPRE8. It was reported that MEd is the PPRE of the malic enzyme (ME) gene, but it did not have binding activity. When the 5′-flanking region of PPRE in the MEd was changed from "GTGTTAG" to "GTGTTCT", the binding activity of the PPARα/RXRα heterodimer to ME gene was significantly enhanced [15]. The PPRE8 has the constant "ACT" at the 3′ end of the 5′-flanking region and shows more conservativeness than PPRE2. So PPRE8 might bind to PPARα/RXRα heterodimer more easily and stably.

Previous studies have shown that the mouse *Ppsig* might play an important role in lipid metabolism regulated by PPARα [9]. As the intronic PPREs located in mouse *Ppsig* and *hPpsig* gene are sensitive to PPARα/RXRα heterodimer, we presume that *hPpsig* might be involved in a similar pathway under the control of PPARα. In addition, the mouse *Ppsig* was identified to be highly homologous with gene of retinol saturase (*Retsat*). The Retsat protein belongs to the family of oxidoreductases, specifically those acting on the CH-CH group of donor with other acceptors, and catalyzes reaction of all-*trans*-retinol to produce (R)-all-*trans*-13,14-dihydroretinol [18-20]. In recent years studies on mouse *Retsat* showed that the transcription of *Retsat* required for adipocyte in 3T3-L1 cells was controlled by PPARγ. And the PPARγ/RXRα heterodimer was found to bind to the same PPRE, which had been reported to be the PPARα/RXRα binding site located in the first intron of the mouse *Ppsig* gene [9, 21]. Also, it had been demonstrated that there was a close link between the adiposity of *Retsat*-null mice and PPARγ [22]. All the results showed that mouse *Ppsig* (*Retsat*) is a dual-PPAR target gene that might be regulated by both PPARα and PPARγ. And thus, it was speculated that *hPpsig* might participate in

a

PPRE8
+362 5′-CAACACTGTCGATAGCCTTTGCCCTAGTAGGCCCC-3′ +396
3′-GTTGTGACAGCTATCGGAAACGGGATCATCCGGGG-5′

mutPPRE8-MA
+362 5′-CAACACTGTCGATAGCCTTTttttTAGTAGGCCCC-3′ +396
3′-GTTGTGACAGCTATCGGAAAaaaaATCATCCGGGG-5′

mutPPRE8-MX
+362 5′-CAACACTGTCGATttttTTTGCCCTAGTAGGCCCC-3′ +396
3′-GTTGTGACAGCTAaaaaAAACGGGATCATCCGGGG-5′

mutPPRE8-MT
+362 5′-CAACACTGTCGATttttTTTttttTAGTAGGCCCCGG-3′ +398
3′-GTTGTGACAGCTAaaaaAAAaaaaATCATCCGGGGCC-5′

b

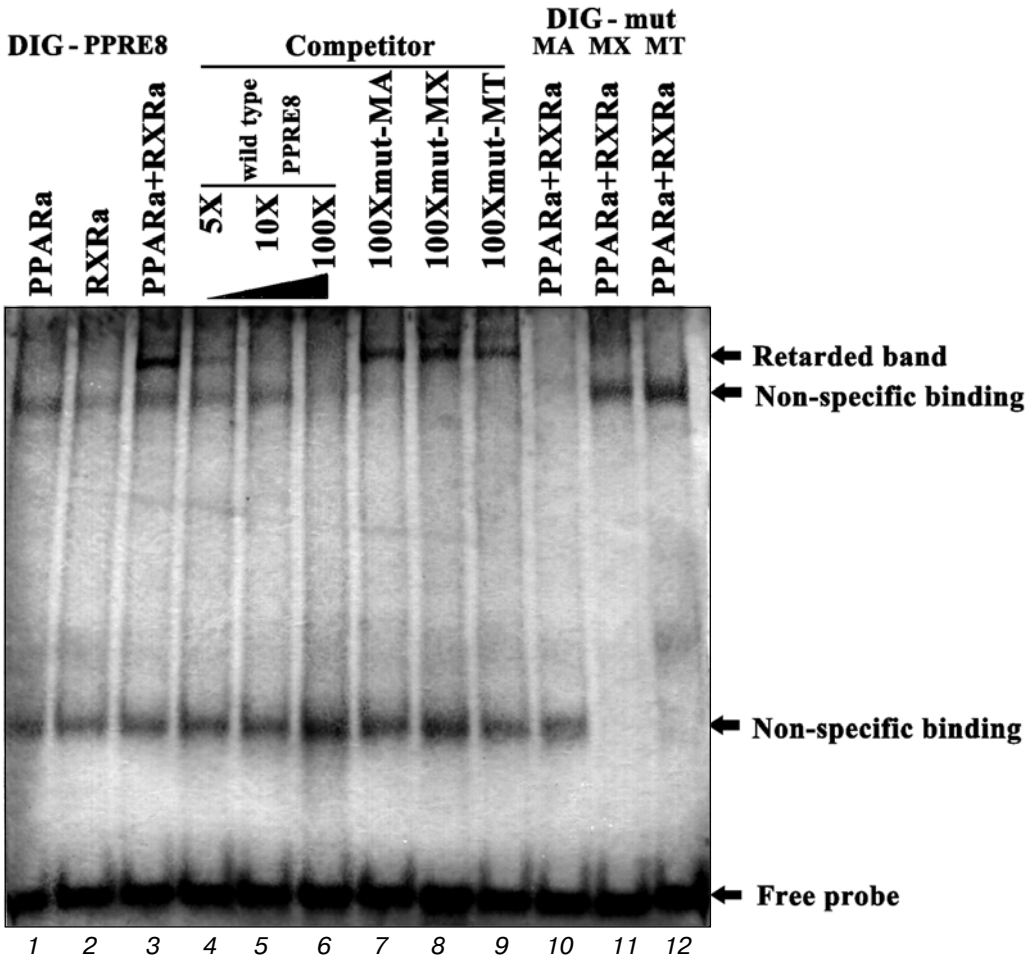

Fig. 3. PPARα/RXRα heterodimer is bound to PPRE8 in *hPpsig in vitro*. a) Sequences of double-stranded probes used in EMSA. Mutant nucleotides are shown in lower case. b) EMSA was performed with DIG-labeled PPRE8 probes and *in vitro*-translated hPPARα and/or hRXRα proteins (lanes *1-3*). In the competitive group, competitors were added into the reactions before the interaction of DIG-labeled PPRE8 probes and hPPARα/hRXRα. 5-, 10-, and 100-fold molar excess of unlabeled wild-type PPRE8 probes and 100-fold molar excess of unlabeled mutant PPRE8 probes for competition are indicated (lanes *4-9*). In the mutant groups, the hPPARα/hRXRα proteins and the DIG-labeled mutant probes were used for the binding reactions (lane *10-12*). The retarded band represents the PPRE8/hPPARα/hRXRα complex. Shift of the PPRE8 probe was observed in at least three independent experiments.

differently regulated pathways in various tissues. For one thing, *hPpsig* perhaps took part in catabolism in liver and kidney triggered by PPARα; for another, it might be involved in lipogenesis in adipose tissue regulated by PPARγ. However, further studies should be done to determine the mechanisms of *hPpsig* transcription regulation and to answer the question whether *hPpsig* is also a one or a dual-hPPAR target gene

In summary, the *hPpsig* gene with one PPRE located in the first intron is a novel PPARα/RXRα target gene as indicated by transient transfection and electrophoretic mobility shift assay *in vitro*. And it is suggested that *hPpsig* may be relevant to different regulation pathways in various tissues in the body.

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