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Mouse OCTN2 is directly regulated by peroxisome proliferator-activated receptor α (PPARα) via a PPRE located in the first intron

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ABSTRACT

Recent studies provided strong evidence to suggest that organic cation transporter 2 (OCTN2) is a direct target gene of peroxisome proliferator-activated receptor α (PPARα). However, subsequent studies failed to demonstrate a functional peroxisome proliferator response element (PPRE) in the promoter region of the OCTN2 gene. In the present study we hypothesized that the OCTN2 gene is transcriptionally induced by PPARα via a functional PPRE located in the first intron. *In silico*-analysis of the first intron of mouse OCTN2 revealed 11 putative PPRE with high similarity to the consensus PPRE. In addition, reporter gene assays using a mouse OCTN2 intron reporter construct containing a cluster of three partially overlapping PPRE (PPREint-1-8-10) revealed a marked response to exogenous mouse PPARα/RXRα and subsequent stimulation with PPARα agonist WY-14,643. Introduction of a selective mutation in either PPRE8 or PPRE10 in the PPREint-1-8-10 reporter constructs caused a substantial loss of the responsiveness to PPARα activation, but a selective mutation in PPRE1 resulted in a complete loss of responsiveness to PPARα activation. Moreover, gel shift assays revealed binding of PPARα/RXRα heterodimer to the PPRE1 of mouse OCTN2 first intron. In conclusion, the present study shows that mouse OCTN2 is a direct target gene of PPARα and that transcriptional upregulation of OCTN2 by PPARα is likely mediated via PPRE1 in its first intron.

1. Introduction

Peroxisome proliferator-activated receptor α (PPARα) is a ligand-activated transcription factor that acts as an important regulator of lipid metabolism and energy homeostasis [1]. PPARα is abundantly expressed in tissues with high rates of fatty acid oxidation such as liver, heart muscle, skeletal muscle, and kidney [2]. Transcriptional regulation of genes by PPARα is mediated by binding of activated PPAR/retinoid X receptor (RXR) heterodimers to specific DNA sequences, called peroxisome proliferator response elements (PPRE), present in and around the promoter region of target genes [3–9], thereby stimulating the expression of those genes. Proteins encoded by these genes are involved in all aspects of fatty acid catabolism including cellular fatty acid uptake, intracellular fatty acid transport, fatty acid transport through the mitochondrial membrane, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis as well as gluconeogenesis [2,10]. PPARα can be activated by both endogenous and synthetic ligands. Endogenous ligands of PPARα are fatty acids and their derivatives (eicosanoids) [11,12]. Endogenous ligand-activation of PPARα is observed during fasting [10,13], since free non-esterified fatty acids are released from adipose tissue and taken up into tissues at increased levels during this state. Consequently, in the liver, where PPARα is most abundant, β-oxidation, ketogenesis as well as gluconeogenesis is dramatically increased as a consequence of the increased expression of PPARα target genes [2,10]. In addition to endogenous ligands, PPARα is also activated by a heterogenous group of synthetic compounds including WY-14,643 and the fibrate class of lipid lowering drugs (clofibrate, fenofibrate, bezafibrate, and gemfibrozil) [11,12].

Many years ago it has been shown that starvation or treatment of rats with clofibrate increases the hepatic concentration of carnitine [14–17], which is an essential metabolite that is required for the β-oxidation of long-chain fatty acids in the mitochondrial matrix [18–20]. Hence, all tissues that use fatty acids as a fuel source require carnitine for normal function. The reason for the fasting- and clofibrate-induced increase in hepatic carnitine concentrations became clear until recently we observed a marked, about 8-fold increase in the hepatic mRNA content of novel organic cation transporter 2 (OCTN2) in the liver of rats treated with the PPARα agonist clofibrate [21]. OCTN2 and other OCTN isoforms (OCTN1 and OCTN3) are polyspecific transporters mediating the transport of several cations and l-carnitine [22,23]. Due to its high binding affinity for carnitine and its wide expression, OCTN2 is the

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physiologically most important carnitine transporter, operating for the reabsorption of carnitine from the urine as well as playing a major role in tissue distribution, and intracellular carnitine homeostasis [24,25]. Subsequent studies in PPARα knockout mice clearly revealed that transcriptional up-regulation of hepatic OCTN2 by fasting or treatment with PPARα agonist WY-14,643 is dependent on PPARα [26,27]. In addition, studies in rats and pigs showed that OCTN2 is induced by fasting or clofibrate also in several other tissues with abundant PPARα expression including kidney, skeletal muscle, heart, and small intestine [28–32]. Moreover, *in silico*-analysis of the promoter region of rat OCTN2 revealed several putative PPRE upstream of the transcription start site [28]. Collectively, these findings provided strong evidence to suggest that OCTN2 is a direct PPARα target gene. Nevertheless, a more recent study employing reporter gene and gel shift assays revealed only a weak response of rat OCTN2 promoter gene constructs to fenofibrate or exogenous PPARα and a weak binding of PPARα to a proximal PPRE of the rat OCTN2 promoter, respectively [33]. Based on these results it has been concluded that the contribution of this proximal PPRE to the OCTN2 promoter activity is low and that a more functional PPRE might be located in other regulatory regions of the OCTN2 gene [33]. Since regulatory elements are frequently observed in intronic regions [7,8,34] we hypothesized that the OCTN2 gene is transcriptionally induced by PPARα via one or more functional PPRE located in intronic regions of the OCTN2 gene. In this study, we therefore performed *in silico*-analyses, reporter gene experiments and gel shift assays to investigate whether functional PPRE exist in intronic regions of the mouse OCTN2 gene, and to get more insights into molecular mechanism underlying PPARα-dependent up-regulation of the mouse OCTN2 gene.

2. Materials and methods

2.1. Chemicals

WY-14,643 was purchased from Sigma–Aldrich (Steinheim, Germany). RPMI1640 GlutaMax-l medium, fetal calf serum (FCS) and gentamycin were from Invitrogen (Karsruhe, Germany).

2.2. Cell culture

The human hepatoma cell line HepG2 (DSMZ, Braunschweig, Germany), which is commonly used for transient transfection assays [35], was used in the present study. HepG2 cells were cultured in RPMI1640 GlutaMax-l medium supplemented with 10% FCS and 0.05 mg/ml gentamycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Medium was changed every 2 days. After reaching a confluence of 70–80%, the cells were seeded in 96-well culture plate at a density of 4–5 × 10^4/well. The cells were used for transfection 24 h after reaching a confluence of 70%.

2.3. In silico-analysis of mouse OCTN2 promoter and first intron

Using cDNA and genomic sequences from NCBI GenBank (accession number BC031118 and CT571271) an approximately 2 kb fragment of mouse OCTN2 promoter from −1632 to +121 relative to transcription start site, and a 2 kb fragment of mouse OCTN2 first intron were scanned and analyzed for putative PPRE using NUBIScan (nuclear receptor binding site scanner [36]).

2.4. Generation of mouse OCTN2 promoter reporter gene constructs

Five mouse OCTN2 promoter-truncation constructs were designed. The 1753 bp promoter fragment pGL4.10-PPREprom-1753 (from −1632 to +121 relative to transcription start site) containing four identified putative PPRE was PCR amplified from mouse BAC clone RP23-303F24 (imaGene, Berlin, Germany). The generated PCR fragment with XhoI and HindIII restriction sites introduced at the 5′ and 3′ ends was subcloned into the XhoI and HindIII digested pGL4.10 [luc2] vector (Promega, Mannheim, Germany) upstream of the luciferase reporter gene. The OCTN2 promoter-truncation constructs pGL4.10-PPREprom-1043 (from −922 to +121) containing three putative PPRE, pGL4.10-PPREprom-750 (from −629 to +121) containing one putative PPRE, and pGL4.10-PPREprom-556 (from −435 to +121) containing no PPRE but a TATA box and three Sp1 sites, and pGL4.10-PPREprom-369 (from −248 to +121) containing no PPRE but a TATA box and one Sp1 site were PCR amplified from parental clone pGL4.10-PPREprom-1753 by using different 5′-primers flanking the putative PPRE and a common 3′-primer. The primer sequences and PCR product sizes are shown in Table 1. The generated PCR products containing two adapters of XhoI and HindIII site at the end were subcloned into the XhoI and HindIII digested pGL4.10 [luc2] vector upstream of the luciferase reporter gene. The cloned DNA fragments were sequenced to confirm the integrity of the constructs.

2.5. Generation of mouse OCTN2 first intron reporter gene constructs

Four DNA fragments containing the putative PPRE predicted from NUBIScan, namely PPREint-1-8-10 (280 bp from 1798 to 2079), PPREint-6-7 (179 bp from 1663 to 1843), PPREint-9-4 (504 bp from 1235 to 1739), and PPREint-2-3-5-11 (703 bp from 1 to 703) were PCR amplified from mouse BAC clone RP23-303F24 (imaGene) using specific primers. The primer sequences and PCR product sizes are shown in Table 1. The generated PCR products containing two adapters of KpnI and HindIII site in the end of the

Table 1
Oligonucleotides used for PCR amplification of promoter region and first intron of mouse OCTN2.

Oligonucleotide name	Oligonucleotide sequence (5′–3′)	PCR product size (bp)
PPREprom-F	ATAAAGCTTGCCGTCCTCAGCCTCCCAC	
PPREprom-369R	ATACTCGAGGTTCTGAAGCCCGGTTCAGCC	369
PPREprom-556R	ATACTCGAGCTGCTCCAGGAAGTGTCCTG	556
PPREprom-750R	ATACTCGAGCTGTCGTTGTCAATGAGACTC	750
PPREprom-1043R	ATACTCGAGCACCTTTCTCATCACCAGTCT	1043
PPREprom-1753R	ATACTCGAGCAGCATTTCTCTCTCCTGGCTT	1753
PPREint-1810F	ATAGGTACCCAATGGATCTGGATGCTATTG	
PPREint-1810R	ATAAAGCTTGCTGACTAGCTGCAGTTGTC	280
PPREint-67F	ATAGGTACCCTTCACTGTCGTCTTGCTCG	
PPREint-67R	ATAAAGCTTCTCTTACGAAACTGCTAAGATA	179
PPREint-94F	ATAGGTACCCTGGTCAGTGATGGAGCACA	
PPREint-94R	ATAAAGCTTTTGCTGATCAGCCAGCCTCT	504
PPREint-23511F	ATAGGTACCGTAGGTGACAGCCCCCCT	
PPREint-23511R	ATAAAGCTTCCAGCAGAGTGACTGAGAAAT	703

PCR fragments were subcloned into the KpnI and HindIII digested pGL4.23 [luc2/minP] vector (Promega) which contains the minimal promoter minP followed by the luciferase reporter gene luc2 (Promega). After cloning, fragments were sequenced to confirm the integrity of the constructs.

2.6. Site-directed mutagenesis

Three mutation constructs of the OCTN2-PPREint-1-8-10 construct were prepared by selectively introducing a mutation in either PPRE1, PPRE8 or PPRE10 of the OCTN2 first intron with Site-Directed Mutagenesis kit (Stratagene Europe, Amsterdam, Netherlands) using the following oligonucleotides: PPREint-10-mut (forward: 5′-GCCTCCTGCAAAATCTGCAGACCTGTAAGTAGGTG-3′, reverse: 5′-CACCTACTTACAGGTCTGCAGATTTTGCAGGAGGC-3′), PPREint-8-mut (forward: 5′-GCAAAAACTGCTGTCCTGAAAGTAGGTGAAAGGG-C-3′, reverse: 5′-GCCCTTTCACCTACTTTCAGGACAGCAGTTTTTGC-3′), PPREint-1-mut (forward: 5′-GACCTGTAAGTAGGTGTATGGGCA-TATAACTCTTA-3′, reverse: 5′-TAAGAGTTATATGCCCATACACCTACT-TACAGGTC-3′). The mutant constructs were controlled for the presence of the intended mutation and the absence of any unexpected mutations by DNS sequencing.

2.7. Transient transfection

HepG2 cells were transiently transfected with the generated reporter gene constructs and co-transfected with either mouse PPARα expression plasmid (pCMX-mPPARα) and mouse RXRα expression plasmid (pCMX-mRXRα) (both, generous gifts from R.M. Evans, Salk Institute for Biological Studies, San Diego, CA, USA) or empty vector (pCMX) using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Cells were also co-transfected with pGL4.74 Renilla luciferase (Rluc) (encoding the renilla luciferase reporter gene; Promega), which was used as an internal control reporter vector to normalize for differences in transfection efficiency. A 3X ACO-PPRE vector (containing three copies of consensus PPRE from the ACO promoter in front of a luciferase reporter gene; a generous gift from Dr. Sander Kersten, Nutrigenomics Consortium, Top Institute (TI) Food and Nutrition, Wageningen, Netherlands) and pGL4.10 (or pGL4.23) vector were used as positive and negative control vectors, respectively. Following transfection, cells were treated with either 50 μM WY-14,643 to achieve activation of PPARα or vehicle only (DMSO = control) for 24 h. Afterwards, cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Luciferase activities were determined with the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's instructions using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany) as described recently in more detail [37].

2.8. Electrophoretic mobility shift assay (EMSA)

The mouse PPARα and mouse RXRα proteins were generated from the expression vectors by *in vitro* transcription/translation using TNT® Quick Coupled Transcription/Translation Kit (Promega) according to the manufacturer's protocol. The following oligonucleotides were annealed with annealing buffer (10 mM Tris, 1 mM EDTA, 0.1 mM NaCl; pH 8.0): OCTN2-PPREint-1-8-10 (forward: 5′-TCGTAAGAGTTATATGCCCTTTCACCTACTTAC AGGT-CAGCAGTTTTTGCAGGAGG-3′, reverse: 5′-CTTGCCTCCTGCAAA-AACTGCTG ACCTGTAAGTAGGTGAAAGGGCATATAACTCTT-3′), for mutation OCTN2-PPREint-1-8-10-mut (forward: 5′-TCGTAAGAGT-TATATGCCCATACACCTACTTTCAGGACAGC AGATTTTGCAGGAGG-3′, reverse: 5′-CTTGCCTCCTGCAAAATCTGCTGTCCTGAAA GTAGGT-GTATGGGCATATAACTCTT-3′), PPREcont-spec (rat-ACO-PPRE) as specific control and for competition (forward: 5′-TTCCCGAACGT-GACCTTTGTCCTGGTCCCCTT TGAT C-3′, reverse: 5′-AAAGGGGAC-CAGGACAAAGGTCACGTTCGGGAAGATC-3′), PPREcont-non-spec as non-specific control (forward: 5′-GATCGTGACTCTTGTGGGGAT ACTCTCTGACTCTA-3′, reverse: 5′-AGCTTAGAGTCAGAGAGTATCC-CCACAAGAG TCAC-3′). For OCTN2-PPREint-10 (forward: 5′-GA-CTGCCTCCTGCAAAAACTGCTGA CCTGTAAGTAG-3′, reverse: 5′-AG-CTCTACTTACAGGTCAGCAGTTTTTGCAGGAGG C-3′), for mutation OCTN2-PPREint-10-mut (forward: 5′-GACTGCCTCCTGCAAAATCTG CAGACCTGTAAGTAG-3′, reverse: 5′-AGCTCTACTTACAGGTCTGCA-GATTTTGCAG GAGGC-3′). For OCTN2-PPREint-8 (forward: 5′-GACTAAAAACTGCTGACCTGTAAGT AGGTGAAAGGG-3′, reverse: 5′-AGCTCCCTTTCACCTACTTACAGGTCAGCAGTTTT T-3′), for mutation OCTN2-PPREint-8-mut (forward: 5′-GACTAAAAACTGCTG-TCCTGAA AGTAGGTGAAAGGG-3′, reverse: 5′-AGCTCCCTTTCACC-TACTTTCAGGACAGCAGT TTTT-3′). For OCTN2-PPREint-1 (forward: 5′-GACTCTGTAAGTAGGTGAAAGGGCAT ATAACTCTTA-3′, reverse: 5′-AGCTTAAGAGTTATATGCCCTTTCACCTACTTACAG-3′), for mutation OCTN2-PPREint-1-mut (forward: 5′-GACTCTGTAAGTAGGTG-TATGGG CATATAACTCTTA-3′, reverse: 5′-AGCTTAAGAGTTATAT-GCCCATACACCTACTTA CAG-3′). After annealing 100 ng of double-stranded DNA-probes were labelled with 0.05 mM DIG-ddUTP in 1× labelling buffer (0.2 M potassium cacodylate, 25 mM Tris–HCl, 0.25 ng/ml bovine serum albumin; pH 6.6), 5 mM $CoCl_2$, 20 U/μl Terminal transferase (Roche) and incubated for 15 min at 37 °C. Then 2 μl of each *in vitro*-translated PPARα and RXRα proteins were incubated with 4 ng DIG-labelled probes and 5-, 50- and 100-fold molar excess of unlabelled specific probes for competition in 1 μg poly d(I-C) and EMSA binding buffer (10 mM Tris–HCl, 120 mM KCl, 0.5 mM EDTA, 0.1% Triton-X-100, 12.5% glycerol, 0.2 mM DTT) for 30 min at RT. The protein-DNA complexes were subjected to electrophoresis on 6% native polyacrylamid gels, and transferred to a positive charged nylon membrane. The DIG-labelled DNA was detected by chemiluminescence using Anti-Digoxigenin-AP Conjugate and CSPD (both from Roche) according to the manufacturer's protocol (Roche), and a Bio-Imaging system (Biostep, Jahnsdorf, Germany).

2.9. Statistical analysis

Numerical data were analyzed by one-way ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). Differences of $P < 0.05$ were considered to be significant.

3. Results

3.1. In silico-analysis of mouse OCTN2 for the existence of putative PPRE

To investigate, which genomic region could be responsible for the PPARα-induced up-regulation of the mouse OCTN2 gene, we performed *in silico*-analysis of the promoter region and first intron of mouse OCTN2 for the existence of putative PPRE using NUBIScan software. According to this, four putative PPRE in the promoter region and 11 putative PPRE in the first intron were identified. The sequence alignment between the consensus PPRE AGGT-CAAAGGTCA (termed DR-1: direct repeat 1) known from the literature [2] and the putative PPRE identified in the promoter and first intron of mouse OCTN2 showed high similarity (Table 2).

3.2. Transcriptional activity of putative PPRE in the proximal promoter of mouse OCTN2

To evaluate whether the putative PPRE identified in the mouse OCTN2 promoter region are responsible for PPARα-mediated

Table 2
Alignment of consensus PPRE sequence with sequences of predicted putative PPRE as obtained from *in silico*-analysis of the promoter region and first intron of mouse OCTN2 using NUBIScan software.

PPRE denomination	Position	Sequence
Consensus PPRE	–	AGGTCA A AGGTCA
Putative PPRE in promoter region of mouse OCTN2		
PPRE1[b]	−1389 to −1401 (+)[a]	AGGGAA a GGGTGA
PPRE2	−749 to −761 (−)	AAGGCA t AGGGCA
PPRE3	−761 to −773 (−)	AGCTCA c AGGCCC
PPRE4	−459 to −471 (−)	AGGTCA a AGCTAC
Putative PPRE in intron 1 of mouse OCTN2		
PPRE1	1850 to 1862 (−)	AGGTGA a AGGGCA
PPRE2	226 to 238 (−)	GGGTCT c AGGGCA
PPRE3	302 to 314 (+)	AGAGAA g AGGCCA
PPRE4	1616 to 1628 (−)	AGGTCA g GGTAAA
PPRE5	309 to 321 (+)	AGGCCA c AGTTTT
PPRE6	1749 to 1761 (−)	AGGTCA a AAAGAA
PPRE7	1755 to 1768 (−)	ATGCAG t AGGTCA
PPRE8	1862 to 1874 (+)	TACTTA c AGGTCA
PPRE9	1438 to 1450 (+)	AGTCCA c AGAGAA
PPRE10	1869 to 1881 (+)	AGGTCA g CAGTTT
PPRE11	582 to 594 (+)	AGATCA c TGGAGA

[a] The direction of the PPRE is indicated in brackets with "+" or "−".
[b] PPRE are numbered by ascending *p* value as obtained from *in silico*-analysis for putative PPRE of promoter region and first intron of mouse OCTN2 using NUBIScan software.

transactivation of OCTN2, we generated a series of plasmid constructs containing 5′-deleted fragments of mouse OCTN2 promoter upstream of a firefly luciferase reporter gene. These promoter reporter constructs were transiently transfected into HepG2 cells with co-transfection of either pCMX-mPPARα and pCMX-mRXRα or empty vector (pCMX). As shown in Fig. 1, we did not observe an increase of luciferase activity by WY-14,643 and/or co-expression of mouse PPARα and RXRα in cells transfected with either of these OCTN2 promoter reporter constructs indicating that the putative PPRE in the promoter region are probably not functional.

3.3. Transcriptional activity of putative PPRE in the first intron of mouse OCTN2

Next, we evaluated whether the putative PPRE identified in the first intron of mouse OCTN2 are responsible for PPARα-mediated transactivation of OCTN2. As shown in Fig. 2A and B, some of the 11 putative PPRE identified in the first intron of mouse OCTN2 overlapped each other: PPREint-1, PPREint-8 and PPREint-10 overlapped with each other, PPREint-6 overlapped with PPREint-7, and PPREint-5 overlapped with PPREint-3. PPRE2, PPRE4, PPRE9, and PPRE11 did not overlap with the other PPRE. Therefore, we generated different OCTN2 intron reporter gene constructs each containing a cluster of these partially overlapping PPRE, and transiently transfected these OCTN2 intron reporter constructs into HepG2 cells with co-transfection of either pCMX-mPPARα and pCMX-mRXRα or empty vector (pCMX). As shown in Fig. 2B, the luciferase activity did not increase in response to WY-14,643 and/or co-expression of mouse PPARα/RXRα in cells transiently transfected with the reporter constructs PPREint-6-7, PPREint-9-4 and PPREint-2-3-5-11. However, in cells transiently transfected with the reporter construct PPREint-1-8-10 luciferase activity increased approximately 20-fold by co-expression of mouse PPARα/RXRα and about 100-fold by co-expression of mouse PPARα/RXRα and subsequent stimulation with WY-14,643 when compared to cells treated without WY-14,643 and without co-expression of mouse PPARα/RXRα ($P < 0.05$). These findings indicated that PPRE1, PPRE8, and/or PPRE10 in the first intron at positions 1850–1869 are probably functional, and responsible for PPARα-dependent regulation of mouse OCTN2.

To further explore which of PPRE1, PPRE8, and PPRE10 in the first intron of mouse OCTN2 are functional, we generated three mutant constructs from the PPREint-1-8-10 construct each containing a targeted mutation in either PPRE1, PPRE8 or PPRE10. As shown in Fig. 2C, cells transiently transfected with the mutant PPREint-1-8-10 constructs harboring a mutation in PPRE8 (PPREint-1-8-10-mut8) or PPRE10 (PPREint-1-8-10-mut10) showed an about 70% and 85%, respectively, reduced luciferase activity in response to WY-14,643 and/or co-expression of mouse PPARα/RXRα compared to cells transiently transfected with the wild-type PPREint-1-8-10 construct. However, transient transfection of the mutant PPREint-1-8-10 construct harboring a mutation in PPRE1 (PPREint-1-8-10-mut1) caused a complete loss of responsiveness to WY-14,643 and/or co-expression of mouse PPARα/RXRα compared to transfection with the wild-type PPREint-1-8-10 construct.

3.4. In vitro-binding of PPARα/RXRα heterodimer to PPREint-1-8-10 of mouse OCTN2

To examine *in vitro*-binding of mouse PPARα/RXRα heterodimer to the PPREint-1-8-10, we performed gel shift assays (EMSA) using *in vitro*-translated PPARα and RXRα and DIG-labelled oligonucleotide corresponding to the PPREint-1-8-10 of mouse OCTN2. As shown in Fig. 3, a strong DNA–protein complex was formed between the specific probe of rat-ACO-PPRE and the *in vitro*-translated PPARα/RXRα heterodimer as positive control (lane 2). DNA–protein complex formation between the oligonucleotide corresponding to the PPREint-1-8-10 of mouse OCTN2 and *in vitro*-translated PPARα/RXRα heterodimer was also observed (lane 4). In addition, competition experiments using the oligonucleotide corresponding to the PPREint-1-8-10 and 5-, 50- 100-fold molar excess of unlabelled specific probe (rat-ACO-PPRE) (lanes 5–7) were performed to test the specificity of PPARα/RXRα binding. As demonstrated in Fig. 3, a weak complex was formed at 5-fold molar excess of unlabelled specific probe (lane 5), whereas this complex was absent at 50-fold (lane 6) and 100-fold (lane 7) molar excess of unlabelled specific probe being indicative of complete competition. No DNA–protein complex formation was observed between the mutant construct PPREint-1-8-10-mut harboring a mutation in PPRE1, PPRE8, and PPRE10 and *in vitro*-translated PPARα/RXRα heterodimer (lane 8). These results demonstrate that the PPARα/RXRα heterodimer binds specifically to the PPREint-1-8-10 of mouse OCTN2.

To further evaluate which putative PPRE in the identified PPREint-10-8-1 cluster in the first intron of mouse OCTN2 is functional, we performed EMSA using individual oligonucleotides corresponding to either PPREint-10, PPREint-8 or PPREint-1 as well as their mutant counterparts PPREint-10-mut, PPREint-8-mut, and PPREint-1-mut. As shown in Fig. 4, a DNA–protein complex was formed between the oligonucleotide corresponding to the PPREint-1 of mouse OCTN2 and *in vitro*-translated PPARα/RXRα heterodimer (lane 5). In addition, a DNA–protein complex was formed between the specific probe of rat-ACO-PPRE as positive control and the *in vitro*-translated PPARα/RXRα heterodimer (lane 7). However, no DNA–protein complex formation was observed between oligonucleotides corresponding to either PPREint-10 (lane 3) or PPREint-8 (lane 4) and *in vitro*-translated PPARα/RXRα heterodimer. In addition, no DNA–protein complex was formed between oligonucleotides corresponding to the mutant PPREint-10 (lane 8), mutant PPREint-8 (lane 9), and mutant PPREint-1 (lane 10). These results demonstrate that the PPARα/RXRα heterodimer binds specifically to the PPREint-1 of mouse OCTN2.

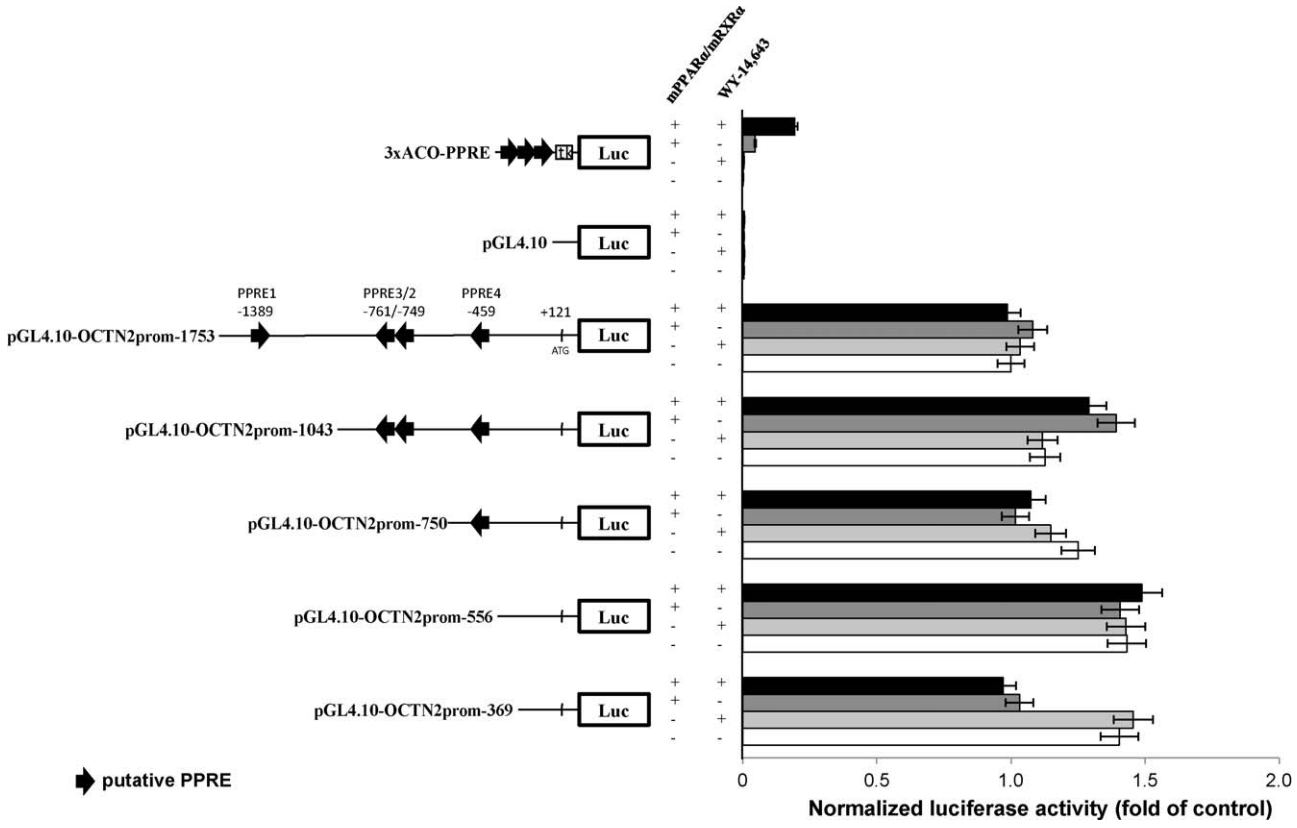

Fig. 1. Effect of exogenous mouse PPARα/RXRα and PPARα ligand WY-14,643 on transcriptional activity of OCTN2 promoter-truncation reporter constructs. HepG2 cells were transiently transfected with serial 5′-deletion OCTN2 promoter reporter constructs, and a renilla luciferase expression vector for normalization. Cells were also co-transfected with or without (empty vector) expression vectors for mouse PPARα and RXRα. After transfection, cells were stimulated or not with 50 μM of WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the OCTN2 promoter-truncation reporter vectors and the renilla luciferase expression vector determined by dual-luciferase assay. Results represent means ± SEM for one out of three independent experiments each performed in triplicate.

4. Discussion

Recent studies from different groups provided strong evidence to suggest that OCTN2 is a direct target gene of PPARα [21,26,27]. Direct PPARα target genes are characterized by the presence of one or more functional PPRE located in and/or around their promoter region [3–9]. Although *in silico*-analysis of nuclear receptor binding sites revealed several putative PPRE upstream of the transcription start site of rat OCTN2 [28,33], further studies employing promoter reporter gene and gel shift assays could not establish a major functional role of these putative PPRE [33]. This was evidenced by a low response to PPARα activation of promoter reporter vectors containing these PPRE and a weak binding of PPARα to these PPRE [33]. Since transcription factor binding sites are also frequently observed in intronic regions of genes [6–8,34], we hypothesized that the OCTN2 gene is transcriptionally induced by PPARα via one or more functional PPRE located in the first intron.

The present study shows using NUBIScan analysis that the first 2 kb of the first intron of mouse OCTN2 contain 11 putative PPRE with high similarity to the conserved consensus PPRE, which is indicative of their importance for transcriptional regulation of the OCTN2 gene. The present study, moreover, demonstrates that three of these putative PPRE, namely PPRE1, PPRE8 and PPRE10, which show a partially overlapping alignment in the first intron of mouse OCTN2, are highly responsive to both, exogenous PPARα/RXRα and PPARα agonist WY-14,643. This could be shown in reporter gene assays using a reporter vector containing an approximately 200 bp fragment of the OCTN2 first intron including PPRE1, PPRE8 and PPRE10 (PPREint-1-8-10) in front of a luciferase reporter gene. The reporter activity of this construct could be dramatically increased by co-expression of exogenous mouse PPARα/RXRα (20-fold compared to empty vector) and further markedly enhanced by the addition of WY-14,643 (5-fold compared to PPARα/RXRα co-expression). The magnitude of induction of reporter activity by WY-14,643 is consistent with the increase in OCTN2 mRNA concentration by different PPARα agonists in mouse and rat liver [21,26–29]. Together with these recent findings, the present results indicate that the intronic region containing these PPRE is probably of great importance for PPARα-dependent regulation of OCTN2. In contrast, transient transfection of other OCTN2 intron reporter vectors containing either PPRE9 and PPRE4 (PPREint-9-4), PPRE7 and PPRE6 (PPREint-7-6) or PPRE2, PPRE3, PPRE5, and PPRE11 (PPREint-2-3-5-11) did not result in any increase in reporter activity by either exogenous PPARα/RXRα or WY-14,643. This suggests that these intronic regions of OCTN2 are probably of minor importance for PPARα-dependent regulation of mouse OCTN2.

To further examine in more detail the functional role of the intronic region containing PPRE1, PPRE8, and PPRE10, we generated three different mutant PPREint-1-8-10 reporter constructs each harboring a selective mutation in either PPRE1, PPRE8 or PPRE10, and investigated their responsiveness to exogenous PPARα/RXRα and/or WY-14,643. These experiments revealed that a selective mutation in either PPRE8 or PPRE10 caused a substantial loss of the responsiveness to PPARα activation, and that a mutation in PPRE1 despite the presence of unmutated PPRE8 and PPRE10 resulted in a complete loss of responsiveness to both, exogenous PPARα/RXRα and WY-14,643. These observations suggest that PPRE1 in the first intron of OCTN2 plays a decisive role in the PPARα-dependent regulation of mouse OCTN2, whereas

PPRE8 and PPRE10 are of less importance. However, the fact that a mutation in either PPRE8 or PPRE10 also caused a significant reduction of reporter activity in response to PPARα activation (70–85% reduction compared to PPREint-1-8-10 vector) indicates that a proper function of PPRE1 is also dependent on the nucleotide sequence adjacent to PPRE1. An explanation for this could be that mutations in the nucleotide sequence surrounding PPRE1 might impair proper binding of the PPARα/RXRα complex including transcriptional co-activators to PPRE1 and, thereby, decrease transcriptional activity of this intronic region. In summary, these results suggest that the intronic region from PPRE1 to PPRE10 (positions 1850–1881 in mouse OCTN2 first intron) is of great importance for PPARα-dependent regulation of mouse OCTN2. This assumption is also supported by the observation that the PPREint-1-8-10 sequence is highly conserved between species as demonstrated by the high similarity between mouse (positions 1850–1881), rat (positions 2019–2050), and human (positions 2271–2302) and to the consensus PPRE. The OCTN2-PPREint-1 showed a complete (100%) sequence homology between mouse, rat and human. This finding, therefore, suggests that regulation of the OCTN2 gene by PPARα agonists is similar between mouse, rat and human.

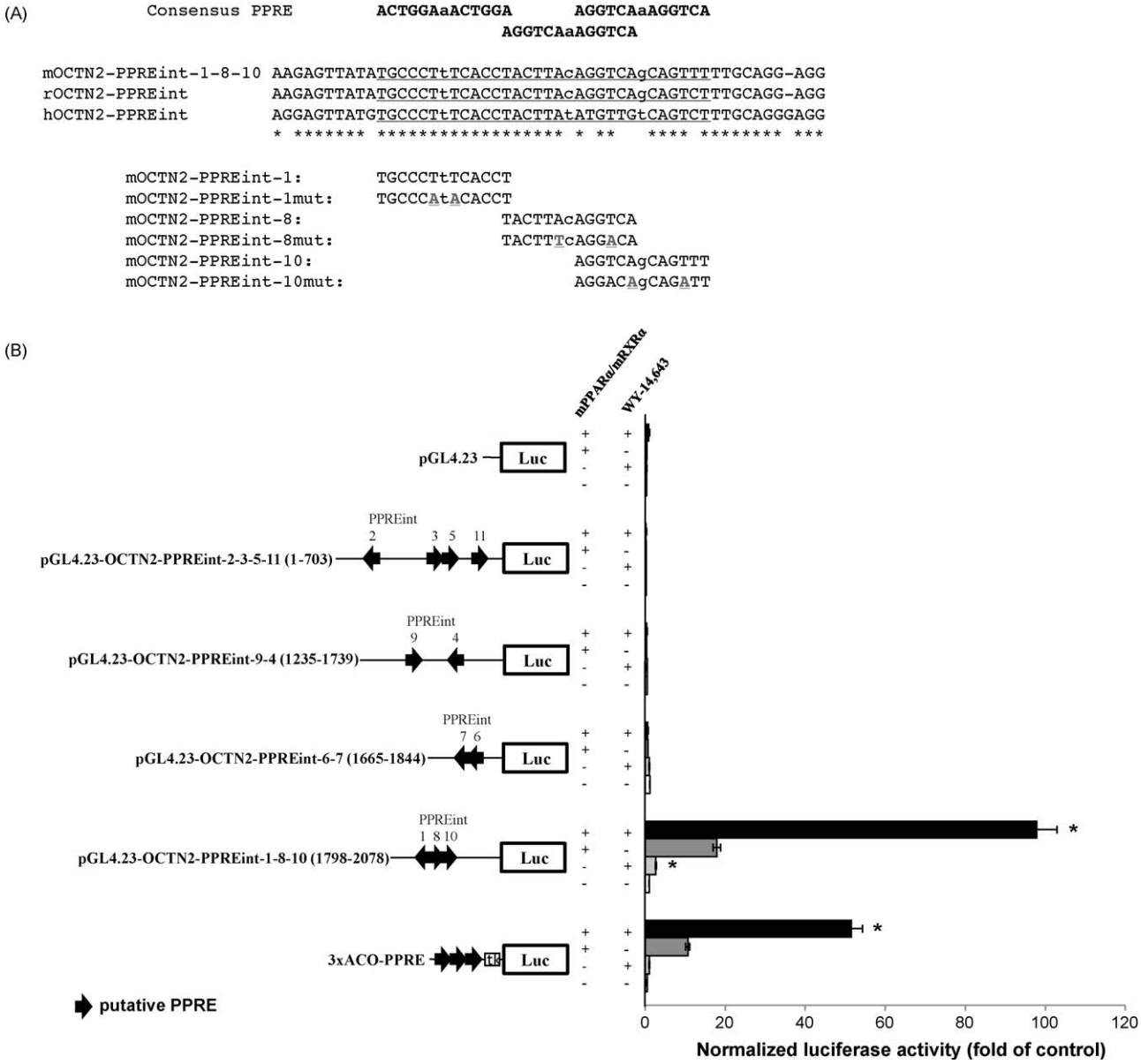

Fig. 2. Effect of exogenous mouse PPARα/RXRα and PPARα ligand WY-14,643 on transcriptional activity of OCTN2 intron reporter constructs. (A) Sequence alignment of consensus PPRE with the PPREint-1-8-10 sequence found in OCTN2 first intron of mouse (nucleotide positions from 1850 to 1881) and rat (nucleotide positions from 2019 to 2050). Matching nucleotides between mouse, rat and human are shown by asterisks. Sequence of putative PPREint-1 and PPREint-8 overlapped with one nucleotide, and putative PPREint-8 and PPREint-10 overlapped with six nucleotides. The mutated nucleotides introduced in the OCTN2 intron reporter constructs for mutation analysis are underlined. (B) HepG2 cells were transiently transfected with different OCTN2 intron reporter constructs, and a renilla luciferase expression vector for normalization. Cells were also co-transfected with or without (empty vector) expression vectors for mouse PPARα and RXRα. After transfection, cells were stimulated or not with 50 μM of WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the OCTN2 promoter-truncation reporter vectors and the renilla luciferase expression vector determined by dual-luciferase assay. Results represent means ± SEM for one out of three independent experiments each performed in triplicate. (C) HepG2 cells were transiently transfected with mutant constructs from OCTN2-PPREint-1-8-10 construct each containing a targeted mutation in either PPRE1, PPRE8 or PPRE10, and a renilla luciferase expression vector for normalization. Cells were also co-transfected with or without (empty vector) expression vectors for mouse PPARα and RXRα. After transfection, cells were stimulated or not with 50 μM of WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the OCTN2 promoter-truncation reporter vectors and the renilla luciferase expression vector determined by dual-luciferase assay. Results represent means ± SEM for one out of three independent experiments each performed in triplicate.

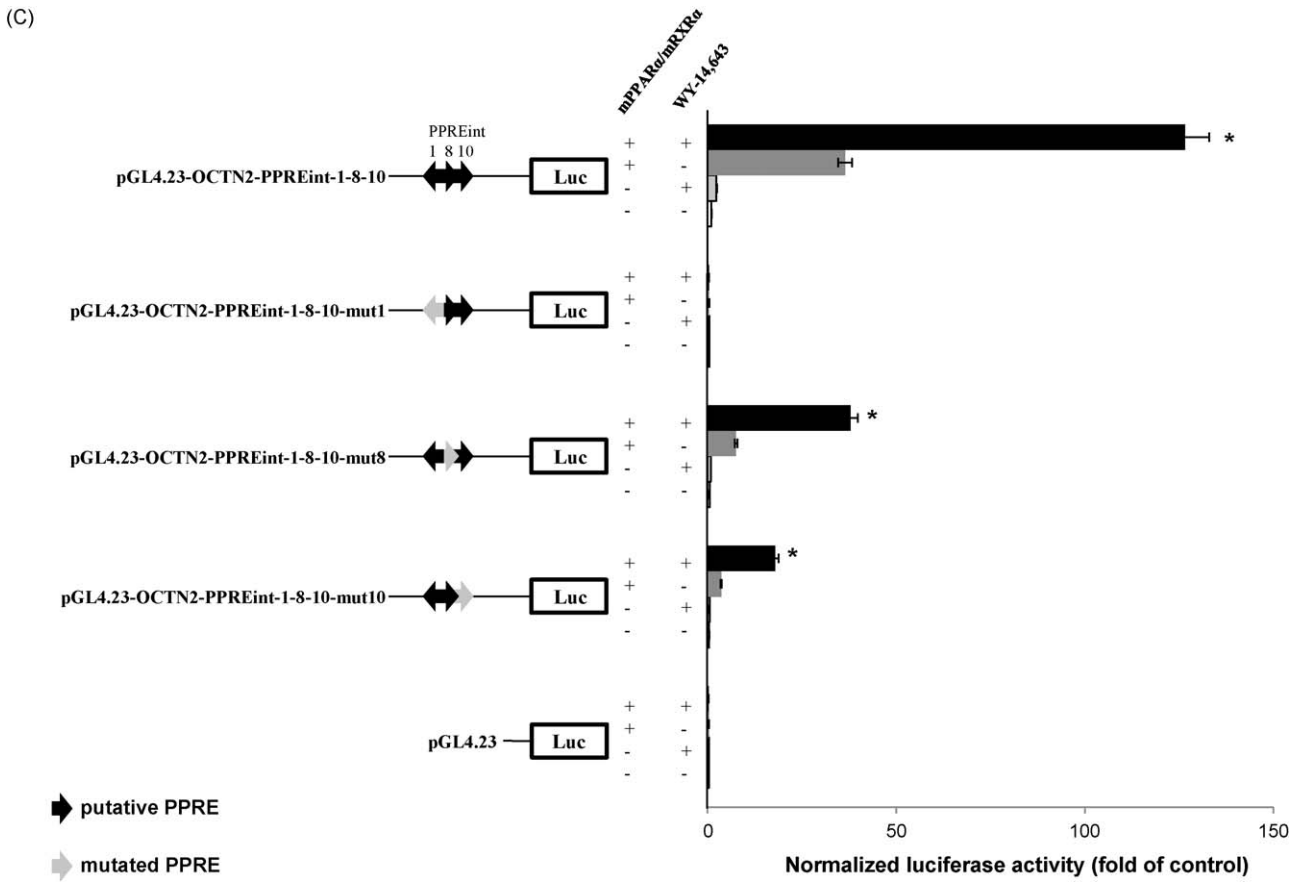

Fig. 2. (*Continued*).

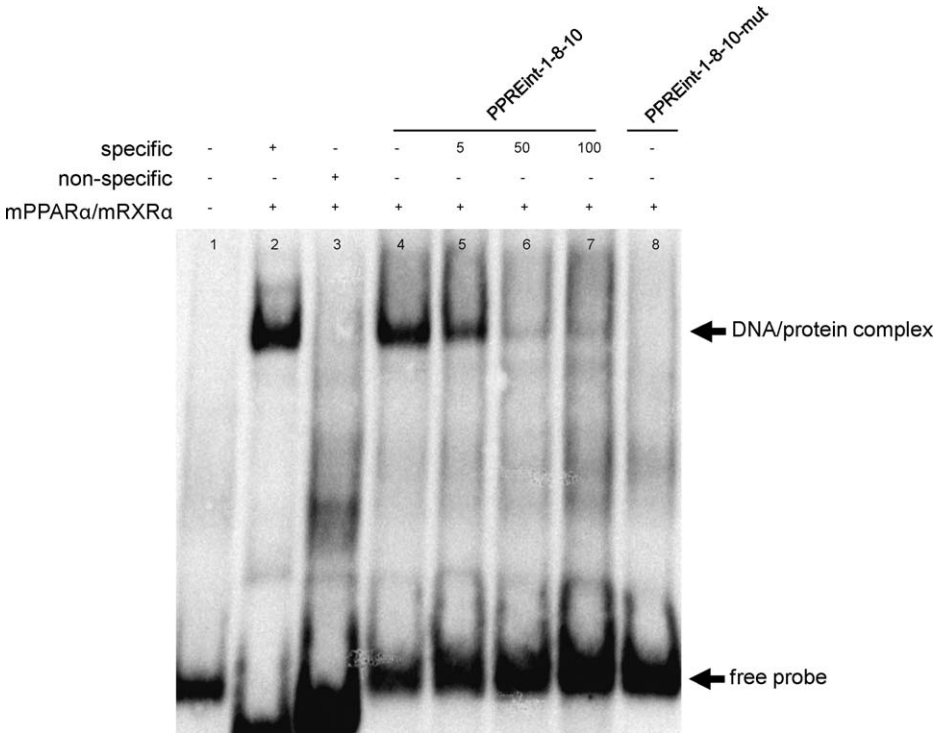

Fig. 3. Binding of *in vitro*-translated mouse PPARα/RXRα to the PPREint-1-8-10 of mouse OCTN2 intron. EMSA was performed using *in vitro*-translated mouse PPARα/RXRα and DIG-labelled oligonucleotide corresponding to either wild-type or mutated PPREint-1-8-10. Fold molar excess of unlabelled specific probe for competition (rat-ACO-PPRE) is indicated. The use of DIG-labelled specific probe (corresponding to rat-ACO-PPRE oligonucleotides) and non-specific probe (corresponding to random oligonucleotides of OCTN2 intron 1) is also indicated.

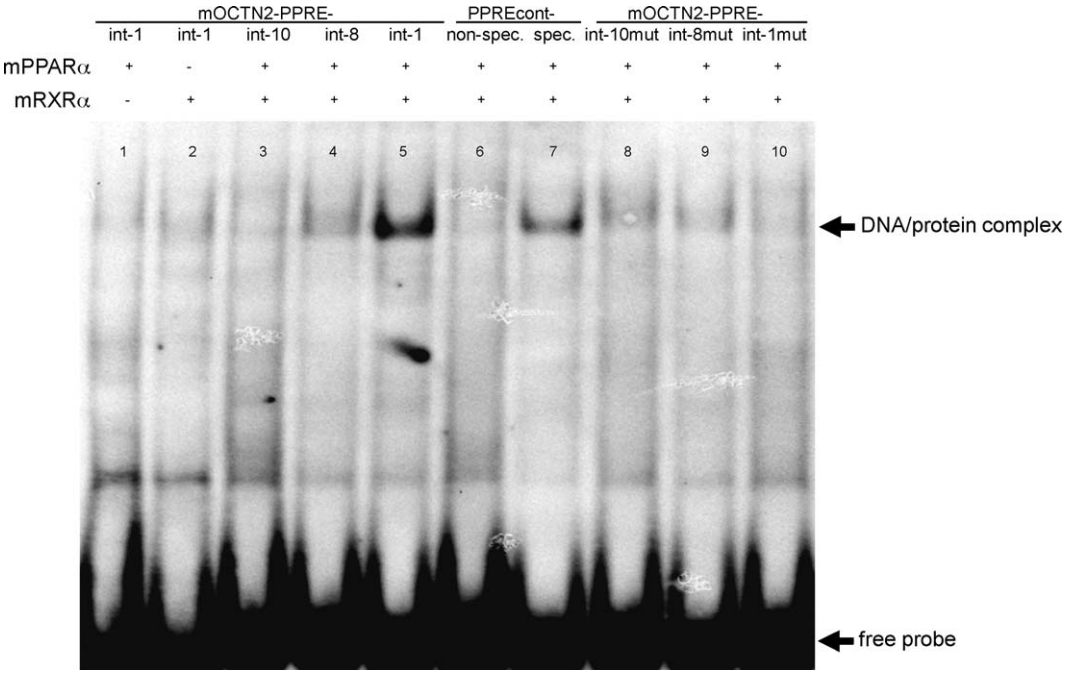

Fig. 4. Binding of *in vitro*-translated mouse PPARα/RXRα to the PPREint-1 of mouse OCTN2 intron. EMSA was performed using *in vitro*-translated mouse PPARα/RXRα and DIG-labelled oligonucleotides corresponding to either PPREint-10, PPREint-8 or PPREint-1 as well as their mutant counterparts PPREint-10-mut, PPREint-8-mut, and PPREint-1-mut. The use of DIG-labelled specific probe (corresponding to rat-ACO-PPRE oligonucleotides) and non-specific probe (corresponding to random oligonucleotides of OCTN2 intron 1) is also indicated.

To further explore the functional role of the PPREint-1-8-10 intronic sequence, we performed gel shift assays using *in vitro*-translated mouse PPARα/RXRα proteins and an oligonucleotide representing PPREint-1-8-10. In the presence of PPARα/RXRα proteins, a strong band appeared representing the DNA–PPARα/RXRα complex which disappeared in the presence of an excess of unlabelled specific oligonucleotide. No band for the DNA–PPARα/RXRα complex was observed when an oligonucleotide containing a mutated PPREint-1-8-10 sequence (PPREint-1-8-10-mut) was used. In addition, EMSA using individual oligonucleotides corresponding to either PPREint-10, PPREint-8 or PPREint-1 as well as their mutant counterparts PPREint-10-mut, PPREint-8-mut, and PPREint-1-mut revealed a DNA–protein complex formation between the oligonucleotide corresponding to the PPREint-1 of mouse OCTN2 and *in vitro*-translated PPARα/RXRα heterodimer. In contrast, no DNA–protein complex formation was observed when oligonucleotides corresponding to either wild-type or mutant PPREint-10 and PPREint-8 and mutant PPREint-1-mut were used. Although we did not perform antibody supershift assays, these results strongly suggest that the PPARα/RXRα heterodimer binds specifically to the PPREint-1 of mouse OCTN2. These findings again support our aforementioned assumption that the PPRE1 in the PPREint-1-8-10 cluster is of decisive importance for transcriptional regulation of mouse OCTN2. Moreover, these results provide further evidence to suggest that mouse OCTN2 is a direct PPARα target gene with a functional PPREint-1 in its first intron.

The present study, moreover, shows that the mouse OCTN2 promoter region like the rat OCTN2 promoter [33] contains several PPRE with high similarity to the conserved consensus PPRE (AGGTCAAAGGTCA). However, these PPRE predicted from NUBISCAN analysis are obviously not functional as demonstrated by reporter gene assays. Using serial 5′-deletion OCTN2 promoter constructs we clearly showed herein that the mouse OCTN2 promoter region is largely unresponsive to both, exogenous PPARα/RXRα and treatment with WY-14,643. These results indicate that the PPRE in the mouse OCTN2 promoter are probably not involved in the induction of the OCTN2 gene by PPARα.

We have recently postulated that up-regulation of OCTN2 by PPARα activation is a means to supply cells with sufficient carnitine required for transport of excessive amounts of fatty acids into the mitochondrion, and therefore plays an important role in the adaptive response of cells to PPARα activation [21]. Since OCTN2 is polyspecific and able to bind other monovalent cations and various drugs such as verapamil, spironolactone or mildronate [38–43], up-regulation of OCTN2 by PPARα activation in tissues due to treatment with pharmacological PPARα activators may be not only relevant with respect to carnitine homeostasis but also to tissue distribution and intestinal absorption of various other compounds. In fact, we have recently observed that treatment of rats with PPARα activator clofibrate increases intestinal absorption of dietary carnitine [29]. It is, therefore, not unlikely that fibrates and other PPARα agonists influence intestinal absorption and tissue distribution of such compounds.

In conclusion, the present study shows using reporter gene and gel shift assays that mouse OCTN2 is a direct target gene of PPARα and that transcriptional up-regulation of OCTN2 by PPARα is likely mediated via PPRE1 in its first intron. Although we could demonstrate binding of PPARα/RXRα heterodimer to this intronic PPRE1, we cannot exclude the possibility that transactivation of the OCTN2 gene in response to PPARα activation is also mediated by additional functional PPRE in other intronic regions or by binding of other transcription factors to the promoter region. For instance, Mandard et al. [8] reported regulation of the PPAR target gene glycogen synthase 2 by hepatic nuclear factor 4α (HNF4α) via a HNF4α response element in the promoter and by PPARα via a PPRE in the first intron. Since HNF4α is known to recognize DR-1 sequences like PPRE as well, it may be speculated that the "non-functional" PPRE identified in the mouse OCTN2 promoter region are probably also occupied by other transcription factors such as HNF4α. Thus, future studies have to demonstrate whether HNF4α might induce the OCTN2 gene via a DR-1 motif in its promoter region.

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