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

3

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15

16 Key words: peroxisome proliferator-activated receptor α (PPAR α), carnitine, novel organic

17 cation transporter 2 (OCTN2)

18

18 Recent studies provided strong evidence to suggest that OCTN2 is a direct target gene of
19 PPAR α . However, subsequent studies failed to demonstrate a functional peroxisome
20 proliferator response element (PPRE) in the promoter region of the OCTN2 gene. In the
21 present study we hypothesized that the OCTN2 gene is transcriptionally induced by PPAR α
22 via a functional PPRE located in the first intron. *In silico*-analysis of the first intron of mouse
23 OCTN2 revealed eleven putative PPRE with high similarity to the consensus PPRE. In
24 addition, reporter gene assays using a mouse OCTN2 intron reporter construct containing a
25 cluster of three partially overlapping PPRE (PPREint-1-8-10) revealed a marked response to
26 exogenous mouse PPAR α /RXR α and subsequent stimulation with PPAR α agonist WY-
27 14,643. Introduction of a selective mutation in either PPRE8 or PPRE10 in the PPREint-1-8-
28 10 reporter constructs caused a substantial loss of the responsiveness to PPAR α activation, but
29 a selective mutation in PPRE1 resulted in a complete loss of responsiveness to PPAR α
30 activation. Moreover, gel shift assays revealed binding of PPAR α /RXR α heterodimer to the
31 PPRE1 of mouse OCTN2 first intron. In conclusion, the present study shows that mouse
32 OCTN2 is a direct target gene of PPAR α and that transcriptional up-regulation of OCTN2 by
33 PPAR α is likely mediated via PPRE1 in its first intron.

34

34 INTRODUCTION

35 Peroxisome proliferator-activated receptor α (PPAR α) is a ligand-activated transcription
36 factor that acts as an important regulator of lipid metabolism and energy homeostasis [1].
37 PPAR α is abundantly expressed in tissues with high rates of fatty acid oxidation such as liver,
38 heart muscle, skeletal muscle, and kidney [2]. Transcriptional regulation of genes by PPAR α
39 is mediated by binding of activated PPAR/retinoid X receptor (RXR) heterodimers to specific
40 DNA sequences, called peroxisome proliferator response elements (PPRE), present in and
41 around the promoter region of target genes [3-9], thereby stimulating the expression of those
42 genes. Proteins encoded by these genes are involved in all aspects of fatty acid catabolism
43 including cellular fatty acid uptake, intracellular fatty acid transport, fatty acid transport
44 through the mitochondrial membrane, mitochondrial and peroxisomal fatty acid oxidation,
45 ketogenesis as well as gluconeogenesis [2, 10]. PPAR α can be activated by both endogenous
46 and synthetic ligands. Endogenous ligands of PPAR α are fatty acids and their derivatives
47 (eicosanoids) [11, 12]. Endogenous ligand-activation of PPAR α is observed during fasting
48 [10, 13], since free nonesterified fatty acids are released from adipose tissue and taken up into
49 tissues at increased levels during this state. Consequently, in the liver, where PPAR α is most
50 abundant, β -oxidation, ketogenesis as well as gluconeogenesis are dramatically increased as a
51 consequence of the increased expression of PPAR α target genes [2, 10]. In addition to
52 endogenous ligands, PPAR α is also activated by a heterogenous group of synthetic
53 compounds including WY-14,643 and the fibrate class of lipid lowering drugs (clofibrate,
54 fenofibrate, bezafibrate, and gemfibrozil) [11, 12].

55 Many years ago it has been shown that starvation or treatment of rats with clofibrate
56 increases the hepatic concentration of carnitine [14-17], which is an essential metabolite that
57 is required for the β -oxidation of long-chain fatty acids in the mitochondrial matrix [18-20].
58 Hence, all tissues that use fatty acids as a fuel source require carnitine for normal function.
59 The reason for the fasting- and clofibrate-induced increase in hepatic carnitine concentrations

60 became clear until recently we observed a marked, about 8-fold increase in the hepatic mRNA
61 content of novel organic cation transporter 2 (OCTN2) in the liver of rats treated with the
62 PPAR α agonist clofibrate [21]. OCTN2 and other OCTN isoforms (OCTN1 and OCTN3) are
63 polyspecific transporters mediating the transport of several cations and l-carnitine [22, 23].
64 Due to its high binding affinity for carnitine and its wide expression, OCTN2 is the
65 physiologically most important carnitine transporter, operating for the reabsorption of
66 carnitine from the urine as well as playing a major role in tissue distribution, and intracellular
67 carnitine homeostasis [24, 25]. Subsequent studies in PPAR α knockout mice clearly revealed
68 that transcriptional up-regulation of hepatic OCTN2 by fasting or treatment with PPAR α
69 agonist WY-14,643 is dependent on PPAR α [26, 27]. In addition, studies in rats and pigs
70 showed that OCTN2 is induced by fasting or clofibrate also in several other tissues with
71 abundant PPAR α expression including kidney, skeletal muscle, heart, and small intestine [28-
72 32]. Moreover, *in silico*-analysis of the promoter region of rat OCTN2 revealed several
73 putative PPRE upstream of the transcription start site [28]. Collectively, these findings
74 provided strong evidence to suggest that OCTN2 is a direct PPAR α target gene. Nevertheless,
75 a more recent study employing reporter gene and gel shift assays revealed only a weak
76 response of rat OCTN2 promoter gene constructs to fenofibrate or exogenous PPAR α and a
77 weak binding of PPAR α to a proximal PPRE of the rat OCTN2 promoter, respectively [33].
78 Based on these results it has been concluded that the contribution of this proximal PPRE to
79 the OCTN2 promoter activity is low and that a more functional PPRE might be located in
80 other regulatory regions of the OCTN2 gene [33]. Since regulatory elements are frequently
81 observed in intronic regions [7, 8, 34] we hypothesized that the OCTN2 gene is
82 transcriptionally induced by PPAR α via one or more functional PPRE located in intronic
83 regions of the OCTN2 gene. In this study, we therefore performed *in silico*-analyses, reporter
84 gene experiments and gel shift assays to investigate whether functional PPRE exist in intronic

85 regions of the mouse OCTN2 gene, and to get more insights into molecular mechanism
86 underlying PPAR α -dependent up-regulation of the mouse OCTN2 gene.

87

88 **MATERIALS AND METHODS**

89

90 **Chemicals**

91 WY-14,643 was purchased from Sigma-Aldrich (Steinheim, Germany). RPMI1640
92 GlutaMax-1 medium, fetal calf serum (FCS) and gentamycin were from Invitrogen (Karsruhe,
93 Germany).

94

95 **Cell culture**

96 The human hepatoma cell line HepG2 (DSMZ, Braunschweig, Germany), which is commonly
97 used for transient transfection assays [35], was used in the present study. HepG2 cells were
98 cultured in RPMI1640 GlutaMax-1 medium supplemented with 10% FCS and 0.05 mg/ml
99 gentamycin. Cells were maintained at 37°C in a humidified atmosphere of 95 % air and 5 %
100 CO₂. Medium was changed every 2 days. After reaching a confluence of 70-80%, the cells
101 were seeded in 96-well culture plate at a density of 4 - 5 x 10⁴/well. The cells were used for
102 transfection 24 h after reaching a confluence of 70 %.

103

104 ***In silico*-analysis of mouse OCTN2 promoter and first intron**

105 Using cDNA and genomic sequences from NCBI Genbank (Accession number BC031118
106 and CT571271) an approximately 2 kb fragment of mouse OCTN2 promoter from -1632 to
107 +121 relative to transcription start site, and a 2 kb fragment of mouse OCTN2 first intron
108 were scanned and analyzed for putative PPRE using NUBIScan (nuclear receptor binding site
109 Scanner [36]).

110

111 Generation of mouse OCTN2 promoter reporter gene constructs

112 Five mouse OCTN2 promoter-truncation constructs were designed. The 1,753 bp promoter
113 fragment pGL4.10-PPREprom-1753 (from -1632 to +121 relative to transcription start site)
114 containing four identified putative PPRE was PCR amplified from mouse BAC clone RP23-
115 303F24 (imaGene, Berlin, Germany). The generated PCR fragment with XhoI and HindIII
116 restriction sites introduced at the 5' and 3' ends was subcloned into the XhoI and HindIII
117 digested pGL4.10 [luc2] vector (Promega, Mannheim, Germany) upstream of the luciferase
118 reporter gene. The OCTN2 promoter-truncation constructs pGL4.10-PPREprom-1043 (from -
119 922 to +121) containing three putative PPRE, pGL4.10-PPREprom-750 (from -629 to +121)
120 containing one putative PPRE, and pGL4.10-PPREprom-556 (from -435 to +121) containing
121 no PPRE but a TATA box and three Sp1 sites, and pGL4.10-PPREprom-369 (from -248 to
122 +121) containing no PPRE but a TATA box and one Sp1 site were PCR amplified from
123 parental clone pGL4.10-PPREprom-1753 by using different 5'-primers flanking the putative
124 PPRE and a common 3'-primer. The primer sequences and PCR product sizes are shown in
125 Table 1. The generated PCR products containing two adapters of XhoI and HindIII site at the
126 end were subcloned into the XhoI and HindIII digested pGL4.10 [luc2] vector upstream of the
127 luciferase reporter gene. The cloned DNA fragments were sequenced to confirm the integrity
128 of the constructs.

129

130 Generation of mouse OCTN2 first intron reporter gene constructs

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

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

140

141 **Site-directed mutagenesis**

142 Three mutation constructs of the OCTN2-PPREint-1-8-10 construct were prepared by
143 selectively introducing a mutation in either PPRE1, PPRE8 or PPRE10 of the OCTN2 first
144 intron with Site-Directed Mutagenesis kit (Stratagene Europe, Amsterdam, Netherlands) using
145 the following oligonucleotides: PPREint-10mut (forward: 5'-
146 GCCTCCTGCAAAATCTGCAGACCTGTAAGTAGGTG-3', reverse: 5'-
147 CACCTACTTACAGGTCTGCAGATTTTGCAGGAGGC-3'), PPREint-8mut (forward: 5'-
148 GCAAAAAGTCTGCTGTCCTGAAAGTAGGTGAAAGGGC-3', reverse: 5'-
149 GCCCTTTCACCTACTTTCAGGACAGCAGTTTTTGC-3'), PPREint-1mut (forward: 5'-
150 GACCTGTAAGTAGGTGTATGGGCATATAACTCTTA-3', reverse: 5'-
151 TAAGAGTTATATGCCATACACCTACTTACAGGTC-3'). The mutant constructs were
152 controlled for the presence of the intended mutation and the absence of any unexpected
153 mutations by DNS sequencing.

154

155 **Transient transfection**

156 HepG2 cells were transiently transfected with the generated reporter gene constructs and co-
157 transfected with either mouse PPAR α expression plasmid (pCMX-mPPAR α) and mouse
158 RXR α expression plasmid (pCMX-mRXR α) (both, generous gifts from R.M. Evans, Salk
159 Institute for Biological Studies, San Diego, CA, USA) or empty vector (pCMX) using
160 FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the
161 manufacturer's protocol. Cells were also co-transfected with pGL4.74 Renilla luciferase
162 (Rluc) (encoding the renilla luciferase reporter gene; Promega), which was used as an internal

163 control reporter vector to normalize for differences in transfection efficiency. A 3X ACO-
 164 PPRE vector (containing three copies of consensus PPRE from the ACO promoter in front of
 165 a luciferase reporter gene; a generous gift from Dr. Sander Kersten, Nutrigenomics
 166 Consortium, Top Institute (TI) Food and Nutrition, Wageningen, Netherlands) and pGL4.10
 167 (or pGL4.23) vector were used as positive and negative control vectors, respectively.
 168 Following transfection, cells were treated with either 50 μ M WY-14,643 to achieve activation
 169 of PPAR α or vehicle only (DMSO = control) for 24 h. Afterwards, cells were washed with
 170 phosphate-buffered saline and lysed with passive lysis buffer (Promega). Luciferase activities
 171 were determined with the Dual-Luciferase Reporter Assay System from Promega according to
 172 the manufacturer's instructions using a Mithras LB940 luminometer (Berthold Technologies,
 173 Bad Wildbad, Germany) as described recently in more detail [37].

174

175 **Electrophoretic mobility shift assay (EMSA)**

176 The mouse PPAR α and mouse RXR α proteins were generated from the expression vectors by
 177 *in vitro* transcription/translation using TNT[®] Quick Coupled Transcription/Translation Kit
 178 (Promega) according to the manufacturer's protocol. The following oligonucleotides were
 179 annealed with annealing buffer (10 mM Tris, 1 mM EDTA, 0.1 mM NaCl; pH 8.0): OCTN2-
 180 PPREint-1-8-10 (forward: 5'-TCGTAAGAGTTATATGCCCTTTCACCTACTTAC
 181 AGGTCAGCAGTTTTTGCAGGAGG-3', reverse: 5'-CTTGCCTCCTGCAAAAAGTCTGCTG
 182 ACCTGTAAGTAGGTGAAAGGGCATATAACTCTT-3'), for mutation OCTN2-PPREint-
 183 1-8-10mut (forward: 5'-TCGTAAGAGTTATATGCCCATACACCTACTTTCAGGACGC
 184 AGATTTTGCAGGAGG-3', reverse: 5'-CTTGCCTCCTGCAAAATCTGCTGCTCCTGAAA
 185 GTAGGTGTATGGGCATATAACTCTT-3'), PPREcont-spec (rat-ACO-PPRE) as specific
 186 control and for competition (forward: 5'-TTCCCGAACGTGACCTTTGTCCTGGTCCCCTT
 187 TGAT C-3', reverse: 5'-AAAGGGGACCAGGACAAAGGTCACGTTCCGGGAAGATC-3'),
 188 PPREcont-non-spec as non-specific control (forward: 5'-GATCGTGACTCTTGTGGGGAT

189 ACTCTCTGACTCTA-3', reverse: 5'- AGCTTAGAGTCAGAGAGTATCCCCACAAGAG
190 TCAC-3'). For OCTN2-PPREint-10 (forward: 5'-GACTGCCTCCTGCAAAAACTGCTGA
191 CCTGTAAGTAG-3', reverse: 5'-AGCTCTACTTACAGGTCAGCAGTTTTTGCAGGAGG
192 C-3'), for mutation OCTN2-PPREint-10mut (forward: 5'-GACTGCCTCCTGCAAAATCTG
193 CAGACCTGTAAGTAG-3', reverse: 5'-AGCTCTACTTACAGGTCTGCAGATTTTGCAG
194 GAGGC-3'). For OCTN2-PPREint-8 (forward: 5'-GACTAAAACTGCTGACCTGTAAGT
195 AGGTGAAAGGG-3', reverse: 5'-AGCTCCCTTTCACCTACTTACAGGTCAGCAGTTTT
196 T-3'), for mutation OCTN2-PPREint-8mut (forward: 5'-GACTAAAACTGCTGTTCCTGAA
197 AGTAGGTGAAAGGG-3', reverse: 5'-AGCTCCCTTTCACCTACTTTTCAGGACAGCAGT
198 TTTT-3'). For OCTN2-PPREint-1 (forward: 5'-GACTCTGTAAGTAGGTGAAAGGGCAT
199 ATAACTCTTA-3', reverse: 5'-AGCTTAAGAGTTATATGCCCTTTCACCTACTTACAG-
200 3'), for mutation OCTN2-PPREint-1mut (forward: 5'-GACTCTGTAAGTAGGTGTATGGG
201 CATATAACTCTTA-3', reverse: 5'-AGCTTAAGAGTTATATGCCCATACACCTACTTA
202 CAG-3'). After annealing 100 ng of double-stranded DNA-probes were labelled with 0.05
203 mM DIG-ddUTP in 1X labelling buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25
204 ng/ml bovine serum albumin; pH 6.6), 5 mM CoCl₂, 20 U/μl Terminal transferase (Roche)
205 and incubated for 15 min at 37°C. Then 2 μl of each *in vitro* translated PPARα and RXRα
206 proteins were incubated with 4 ng DIG-labelled probes and 5-, 50- and 100-fold molar excess
207 of unlabelled specific probes for competition in 1 μg Poly d(I-C) and EMSA binding buffer
208 (10 mM Tris-HCl, 120 mM KCl, 0.5 mM EDTA, 0.1 % Triton-X-100, 12.5 % glycerol, 0.2
209 mM DTT) for 30 min at RT. The protein-DNA complexes were subjected to electrophoresis
210 on 6 % native polyacrylamid gels, and transferred to a positive charged nylon membrane. The
211 DIG-labelled DNA was detected by chemiluminescence using Anti-Digoxigenin-AP
212 Conjugate and CSPD (both from Roche) according to the manufacturer's protocol (Roche),
213 and a Bio-Imaging system (Biostep, Jahnsdorf, Germany).

214

215 **Statistical analysis**

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

218

219 **RESULTS**

220

221 ***In silico*-analysis of mouse OCTN2 for the existence of putative PPRE**

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

230

231 **Transcriptional activity of putative PPRE in the proximal promoter of mouse** 232 **OCTN2**

233 To evaluate whether the putative PPRE identified in the mouse OCTN2 promoter region are
234 responsible for PPAR α -mediated transactivation of OCTN2, we generated a series of plasmid
235 constructs containing 5'-deleted fragments of mouse OCTN2 promoter upstream of a firefly
236 luciferase reporter gene. These promoter reporter constructs were transiently transfected into
237 HepG2 cells with co-transfection of either pCMX-mPPAR α and pCMX-mRXR α or empty
238 vector (pCMX). As shown in Figure 1, we did not observe an increase of luciferase activity
239 by WY-14,643 and/or co-expression of mouse PPAR α and RXR α in cells transfected with

240 either of these OCTN2 promoter reporter constructs indicating that the putative PPRE in the
241 promoter region are probably not functional.

242

243 **Transcriptional activity of putative PPRE in the first intron of mouse OCTN2**

244 Next, we evaluated whether the putative PPRE identified in the first intron of mouse OCTN2
245 are responsible for PPAR α -mediated transactivation of OCTN2. As shown in Figure 2A and
246 2B, some of the eleven putative PPRE identified in the first intron of mouse OCTN2
247 overlapped each other: PPREint-1, PPREint-8 and PPREint-10 overlapped with each other,
248 PPREint-6 overlapped with PPREint-7, and PPREint-5 overlapped with PPREint-3. PPRE2,
249 PPRE4, PPRE9, and PPRE11 did not overlap with the other PPRE. Therefore, we generated
250 different OCTN2 intron reporter gene constructs each containing a cluster of these partially
251 overlapping PPRE, and transiently transfected these OCTN2 intron reporter constructs into
252 HepG2 cells with co-transfection of either pCMX-mPPAR α and pCMX-mRXR α or empty
253 vector (pCMX). As shown in Figure 2B, the luciferase activity did not increase in response to
254 WY-14,643 and/or co-expression of mouse PPAR α /RXR α in cells transiently transfected with
255 the reporter constructs PPREint-6-7, PPREint-9-4 and PPREint-2-3-5-11. However, in cells
256 transiently transfected with the reporter construct PPREint-1-8-10 luciferase activity increased
257 approximately 20-fold by co-expression of mouse PPAR α /RXR α and about 100-fold by co-
258 expression of mouse PPAR α /RXR α and subsequent stimulation with WY-14,643 when
259 compared to cells treated without WY-14,643 and without co-expression of mouse
260 PPAR α /RXR α ($P < 0.05$). These findings indicated that PPRE1, PPRE8, and/or PPRE10 in
261 the first intron at positions 1850 to 1869 are probably functional, and responsible for PPAR α -
262 dependent regulation of mouse OCTN2.

263 To further explore which of PPRE1, PPRE8, and PPRE10 in the first intron of mouse
264 OCTN2 are functional, we generated three mutant constructs from the PPREint-1-8-10
265 construct each containing a targeted mutation in either PPRE1, PPRE8 or PPRE10. As shown

266 in Figure 2C, cells transiently transfected with the mutant PPREint-1-8-10 constructs
267 harboring a mutation in PPRE8 (PPREint-1-8-10-mut8) or PPRE10 (PPREint-1-8-10-mut10)
268 showed an about 70 and 85 %, respectively, reduced luciferase activity in response to WY-
269 14,643 and/or co-expression of mouse PPAR α /RXR α compared to cells transiently
270 transfected with the wild-type PPREint-1-8-10 construct. However, transient transfection of
271 the mutant PPREint-1-8-10 construct harboring a mutation in PPRE1 (PPREint-1-8-10-mut1)
272 caused a complete loss of responsiveness to WY-14,643 and/or co-expression of mouse
273 PPAR α /RXR α compared to transfection with the wild-type PPREint-1-8-10 construct.

274

275 ***In vitro*-binding of PPAR α /RXR α heterodimer to PPREint-1-8-10 of mouse**
276 **OCTN2**

277 To examine *in vitro*-binding of mouse PPAR α /RXR α heterodimer to the PPREint-1-8-10, we
278 performed gel shift assays (EMSA) using *in vitro*-translated PPAR α and RXR α and DIG-
279 labelled oligonucleotide corresponding to the PPREint-1-8-10 of mouse OCTN2. As shown in
280 Figure 3, a strong DNA-protein complex was formed between the specific probe of rat ACO-
281 PPRE and the *in vitro*-translated PPAR α /RXR α heterodimer as positive control (lane 2).
282 DNA-protein complex formation between the oligonucleotide corresponding to the PPREint-
283 1-8-10 of mouse OCTN2 and *in vitro*-translated PPAR α /RXR α heterodimer was also
284 observed (lane 4). In addition, competition experiments using the oligonucleotide
285 corresponding to the PPREint-1-8-10 and 5-, 50- 100-fold molar excess of unlabelled specific
286 probe (rat ACO-PPRE) (lane 5, 6, and 7) were performed to test the specificity of
287 PPAR α /RXR α binding. As demonstrated in Figure 3A, a weak complex was formed at 5-fold
288 molar excess of unlabelled specific probe (lane 5), whereas this complex was absent at 50-
289 fold (lane 6) and 100-fold (lane 7) molar excess of unlabelled specific probe being indicative
290 of complete competition. No DNA-protein complex formation was observed between the
291 mutant construct PPREint1-8-10-mut harboring a mutation in PPRE1, PPRE8, and PPRE10

292 and *in vitro*-translated PPAR α /RXR α heterodimer (lane 8). These results demonstrate that the
293 PPAR α /RXR α heterodimer binds specifically to the PPREint-1-8-10 of mouse OCTN2.

294 To further evaluate which putative PPRE in the identified PPREint-10-8-1 cluster in
295 the first intron of mouse OCTN2 is functional, we performed EMSA using individual
296 oligonucleotides corresponding to either PPREint-10, PPREint-8 or PPREint-1 as well as their
297 mutant counterparts PPREint-10mut, PPREint-8mut, and PPREint-1mut. As shown in Figure
298 4, a DNA-protein complex was formed between the oligonucleotide corresponding to the
299 PPREint-1 of mouse OCTN2 and *in vitro*-translated PPAR α /RXR α heterodimer (lane 5). In
300 addition, a DNA-protein complex was formed between the specific probe of rat ACO-PPRE
301 as positive control and the *in vitro*-translated PPAR α /RXR α heterodimer (lane 7). However,
302 no DNA-protein complex formation was observed between oligonucleotides corresponding to
303 either PPREint-10 (lane 3) or PPREint-8 (lane 4) and *in vitro*-translated PPAR α /RXR α
304 heterodimer. In addition, no DNA-protein complex was formed between oligonucleotides
305 corresponding to the mutant PPREint-10 (lane 8), mutant PPREint-8 (lane 9), and mutant
306 PPREint-1 (lane 10). These results demonstrate that the PPAR α /RXR α heterodimer binds
307 specifically to the PPREint-1 of mouse OCTN2.

308

309 DISCUSSION

310 Recent studies from different groups provided strong evidence to suggest that OCTN2 is a
311 direct target gene of PPAR α [21, 26, 27]. Direct PPAR α target genes are characterized by the
312 presence of one or more functional PPRE located in and/or around their promoter region [3-
313 9]. Although *in silico*-analysis of nuclear receptor binding sites revealed several putative
314 PPRE upstream of the transcription start site of rat OCTN2 [28, 33], further studies
315 employing promoter reporter gene and gel shift assays could not establish a major functional
316 role of these putative PPRE [33]. This was evidenced by a low response to PPAR α activation
317 of promoter reporter vectors containing these PPRE and a weak binding of PPAR α to these

318 PPRE [33]. Since transcription factor binding sites are also frequently observed in intronic
319 regions of genes [6-8, 34], we hypothesized that the OCTN2 gene is transcriptionally induced
320 by PPAR α via one or more functional PPRE located in the first intron.

321 The present study shows using NUBIScan analysis that the first 2 kb of the first intron
322 of mouse OCTN2 contain eleven putative PPRE with high similarity to the conserved
323 consensus PPRE, which is indicative of their importance for transcriptional regulation of the
324 OCTN2 gene. The present study, moreover, demonstrates that three of these putative PPRE,
325 namely PPRE1, PPRE8 and PPRE10, which show a partially overlapping alignment in the
326 first intron of mouse OCTN2, are highly responsive to both, exogenous PPAR α /RXR α and
327 PPAR α agonist WY-14,643. This could be shown in reporter gene assays using a reporter
328 vector containing an approximately 200 bp fragment of the OCTN2 first intron including
329 PPRE1, PPRE8 and PPRE10 (PPREint-1-8-10) in front of a luciferase reporter gene. The
330 reporter activity of this construct could be dramatically increased by co-expression of
331 exogenous mouse PPAR α /RXR α (20-fold compared to empty vector) and further markedly
332 enhanced by the addition of WY-14,643 (5-fold compared to PPAR α /RXR α co-expression).
333 The magnitude of induction of reporter activity by WY-14,643 is consistent with the increase
334 in OCTN2 mRNA concentration by different PPAR α agonists in mouse and rat liver [21, 26-
335 29]. Together with these recent findings, the present results indicate that the intronic region
336 containing these PPRE is probably of great importance for PPAR α -dependent regulation of
337 OCTN2. In contrast, transient transfection of other OCTN2 intron reporter vectors containing
338 either PPRE9 and PPRE4 (PPREint-9-4), PPRE7 and PPRE6 (PPREint-7-6) or PPRE2,
339 PPRE3, PPRE5, and PPRE11 (PPREint-2-3-5-11) did not result in any increase in reporter
340 activity by either exogenous PPAR α /RXR α or WY-14,643. This suggests that these intronic
341 regions of OCTN2 are probably of minor importance for PPAR α -dependent regulation of
342 mouse OCTN2.

343 To further examine in more detail the functional role of the intronic region containing
344 PPRE1, PPRE8, and PPRE10, we generated three different mutant PPREint-1-8-10 reporter
345 constructs each harboring a selective mutation in either PPRE1, PPRE8 or PPRE10, and
346 investigated their responsiveness to exogenous PPAR α /RXR α and/or WY-14,643. These
347 experiments revealed that a selective mutation in either PPRE8 or PPRE10 caused a
348 substantial loss of the responsiveness to PPAR α activation, but that a mutation in PPRE1
349 despite the presence of unmutated PPRE8 and PPRE10 resulted in a complete loss of
350 responsiveness to both, exogenous PPAR α /RXR α and WY-14,643. These observations
351 suggest that PPRE1 in the first intron of OCTN2 plays a decisive role in the PPAR α -
352 dependent regulation of mouse OCTN2, whereas PPRE8 and PPRE10 are of less importance.
353 However, the fact that a mutation in either PPRE8 or PPRE10 also caused a significant
354 reduction of reporter activity in response to PPAR α activation (70 to 85 % reduction
355 compared to PPREint-1-8-10 vector) indicates that a proper function of PPRE1 is also
356 dependent on the nucleotide sequence adjacent to PPRE1. An explanation for this could be
357 that mutations in the nucleotide sequence surrounding PPRE1 might impair proper binding of
358 the PPAR α /RXR α complex including transcriptional co-activators to PPRE1 and, thereby,
359 decrease transcriptional activity of this intronic region. In summary, these results suggest that
360 the intronic region from PPRE1 to PPRE10 (positions 1850 to 1881 in mouse OCTN2 first
361 intron) is of great importance for PPAR α -dependent regulation of mouse OCTN2. This
362 assumption is also supported by the observation that the PPREint-1-8-10 sequence is highly
363 conserved between species as demonstrated by the high similarity between mouse (positions
364 1850 to 1881), rat (positions 2019 to 2050), and human (positions 2271 to 2302) and to the
365 consensus PPRE. The OCTN2 PPREint-1 showed a complete (100%) sequence homology
366 between mouse, rat and human. This finding, therefore, suggests that regulation of the
367 OCTN2 gene by PPAR α agonists is similar between mouse, rat and human.

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

387 The present study, moreover, shows that the mouse OCTN2 promoter region like the
388 rat OCTN2 promoter [33] contains several PPRE with high similarity to the conserved
389 consensus PPRE (AGGTCAAAGGTCA). However, these PPRE predicted from NUBIScan
390 analysis are obviously not functional as demonstrated by reporter gene assays. Using serial 5'-
391 deletion OCTN2 promoter constructs we clearly showed herein that the mouse OCTN2
392 promoter region is largely unresponsive to both, exogenous PPAR α /RXR α and treatment with

393 WY-14,643. These results indicate that the PPRE in the mouse OCTN2 promoter are probably
394 not involved in the induction of the OCTN2 gene by PPAR α .

395 We have recently postulated that up-regulation of OCTN2 by PPAR α activation is a
396 means to supply cells with sufficient carnitine required for transport of excessive amounts of
397 fatty acids into the mitochondrion, and therefore plays an important role in the adaptive
398 response of cells to PPAR α activation [21]. Since OCTN2 is polyspecific and able to bind
399 other monovalent cations and various drugs such as verapamil, spironolactone or mildronate
400 [38-43], up-regulation of OCTN2 by PPAR α activation in tissues due to treatment with
401 pharmacological PPAR α activators may be not only relevant with respect to carnitine
402 homeostasis but also to tissue distribution and intestinal absorption of various other
403 compounds. In fact, we have recently observed that treatment of rats with PPAR α activator
404 clofibrate increases intestinal absorption of dietary carnitine [29]. It is, therefore, not unlikely
405 that fibrates and other PPAR α agonists influence intestinal absorption and tissue distribution
406 of such compounds.

407 In conclusion, the present study shows using reporter gene and gel shift assays that
408 mouse OCTN2 is a direct target gene of PPAR α and that transcriptional up-regulation of
409 OCTN2 by PPAR α is likely mediated via PPRE1 in its first intron. Although we could
410 demonstrate binding of PPAR α /RXR α heterodimer to this intronic PPRE1, we cannot exclude
411 the possibility that transactivation of the OCTN2 gene in response to PPAR α activation is also
412 mediated by additional functional PPRE in other intronic regions or by binding of other
413 transcription factors to the promoter region. For instance, Mandard et al. [8] reported
414 regulation of the PPAR target gene glycogen synthase 2 by hepatic nuclear factor 4 α (HNF4 α)
415 via a HNF4 α response element in the promoter and by PPAR α via a PPRE in the first intron.
416 Since HNF4 α is known to recognize DR-1 sequences like PPRE as well, it may be speculated
417 that the “non-functional” PPRE identified in the mouse OCTN2 promoter region are probably
418 also occupied by other transcription factors such as HNF4 α . Thus, future studies have to

419 demonstrate whether HNF4 α might induce the OCTN2 gene via a DR-1 motif in its promoter
420 region.

421

422 REFERENCES

- 423 1 Desvergne, B. and Wahli, W. (1999) Peroxisome proliferator-activated receptors:
424 Nuclear control of metabolism. *Endocr. Rev.* **20**, 649-688
- 425 2 Mandard, S., Müller, M. and Kersten, S. (2004) Peroxisome proliferator receptor α
426 target genes. *Cell. Mol. Life Sci.* **61**, 393-416
- 427 3 Schoonjans, K., Martin, G., Staels, B. and Auwerx, J. (1997) Peroxisome proliferator-
428 activated receptors, orphans with ligands and functions. *Curr. Opin. Lipidol.* **8**, 159-
429 166
- 430 4 Tan, N.S., Michalik, L., Desvergne, B. and Wahli, W. (2005) Multiple expression
431 control mechanisms of peroxisome proliferator-activated receptors and their target
432 genes. *J. Steroid Biochem. Mol. Biol.* **93**, 99-105
- 433 5 Qi, C., Zhu, Y. and Reddy, J.K. (2000) Peroxisome proliferator-activated receptors,
434 coactivators, and downstream targets. *Cell Biochem. Biophys.* **32**, 187-204
- 435 6 Hansmannel, F., Clémencet, M.C., Le Jossic-Corcos, C., Osumi, T., Latruffe, N. and
436 Nicolas-Francés, V. (2003) Functional characterization of a peroxisome proliferator
437 response-element located in the intron 3 of rat peroxisomal thiolase B gene. *Biochem.*
438 *Biophys. Res. Commun.* **311**, 149-155
- 439 7 Helledie, T., Grøntved, L., Jensen, S.S., Kiilerich, P., Rietveld, L., Albrektsen, T.,
440 Boysen, M.S., Nøhr, J., Larsen, L.K., Fleckner, J. et al. (2002) The gene encoding the
441 Acyl-CoA-binding protein is activated by peroxisome proliferator-activated receptor
442 gamma through an intronic response element functionally conserved between humans
443 and rodents. *J. Biol. Chem.* **277**, 26821-26830

- 444 8 Mandard, S., Stienstra, R., Escher, P., Tan, N.S., Kim, I., Gonzalez, F.J., Wahli, W.,
445 Desvergne, B., Müller, M. and Kersten, S. (2007) Glycogen synthase 2 is a novel
446 target gene of peroxisome proliferator-activated receptors. *Cell. Mol. Life Sci.* **64**,
447 1145-1157
- 448 9 Gutgesell, A., Wen, G., König, B., Koch, A., Spielmann, J., Stangl, G.I., Eder, K. and
449 Ringseis, R. (2009) Mouse carnitine-acylcarnitine translocase (CACT) is
450 transcriptionally regulated by PPAR α and PPAR δ in liver cells. *Biochim. Biophys.*
451 *Acta* (in press)
- 452 10 Kersten, S., Seydoux, J., Peters, J.M., Gonzalez, F.J., Desvergne, B. and Wahli, W.
453 (1999) Peroxisome proliferator-activated receptor α mediates the adaptive response to
454 fasting. *J. Clin. Invest.* **103**, 1489-1498
- 455 11 Forman, B.M., Chen, J. and Evans, R.M. (1997) Hypolipidemic drugs,
456 polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-
457 activated receptors α and δ . *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4312-4317
- 458 12 Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M.G. and
459 Wahli, W. (1997) Fatty acids, eicosanoids, and hypolipidemic agents identified as
460 ligands of peroxisome proliferator-activated receptors by coactivator-dependent
461 receptor ligand assay. *Mol. Endocrinol.* **11**, 779-791
- 462 13 Leone, T.C., Weinheimer, C.J. and Kelly, D.P. (1999) A critical role for the
463 peroxisome proliferator-activated receptor α (PPAR α) in the cellular fasting response:
464 the PPAR α -null mouse as a model of fatty acid oxidation disorders. *Proc. Natl. Acad.*
465 *Sci. U. S. A.* **96**, 7473-7478
- 466 14 McGarry, J.D., Robles-Valdes, C. and Foster, D.W. (1975) Role of carnitine in hepatic
467 ketogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **72**, 4385-4388
- 468 15 Brass, E.P. and Hoppel CL. (1978) Carnitine metabolism in the fasting rat. *J. Biol.*
469 *Chem.* **253**, 2688-2693

- 470 16 Paul, H.S. and Adibi, S.A. (1979) Paradoxical effects of clofibrate on liver and muscle
471 metabolism in rats. Induction of myotonia and alteration of fatty acid and glucose
472 oxidation. *J. Clin. Invest.* **64**, 405-412
- 473 17 Paul, H.S., Gleditsch, C.E. and Adibi, S.A. (1986) Mechanism of increased hepatic
474 concentration of carnitine by clofibrate. *Am. J. Physiol.* **251**, E311-E315
- 475 18 McGarry, J.D. and Brown, N.F. (1997) The mitochondrial carnitine
476 palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.*
477 **244**, 1-14
- 478 19 Brass, E.P. (2002) Pivalate-generating prodrugs and carnitine homeostasis in man.
479 *Pharmacol. Rev.* **54**, 589-598
- 480 20 Steiber, A., Kerner, J. and Hoppel, C.L. (2004) Carnitine: a nutritional, biosynthetic,
481 and functional perspective. *Mol. Asp. Med.* **25**, 455-473
- 482 21 Luci, S., Geissler, S., König, B., Koch, A., Stangl, G.I., Hirche, F. and Eder, K. (2006)
483 PPAR α agonists up-regulate organic cation transporters in rat liver cells. *Biochem.*
484 *Biophys. Res. Commun.* **350**, 704-708
- 485 22 Ohashi, R., Tamai, I., Yabuuchi, H., Nezu, J.I., Oku, A., Sai, Y., Shimane, M. and
486 Tsuji, A. (1999) Na⁺-dependent carnitine transport by organic cation transporter
487 (OCTN2): its pharmacological and toxicological relevance. *J. Pharmacol. Exp. Ther.*
488 **291**, 778-784
- 489 23 Ohashi, R., Tamai, I., Nezu, J.I., Nikaido, H., Hashimoto, N., Oku, A., Sai, Y.,
490 Shimane, M. and Tsuji, A. (2001) Molecular and physiological evidence for
491 multifunctionality of carnitine/organic cation transporter OCTN2. *Mol. Pharmacol.* **59**,
492 358-366
- 493 24 Tamai, I., Ohashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y. and
494 Tsuji, A. (1998) Molecular and functional identification of sodium iondependent, high
495 affinity human carnitine transporter OCTN2. *J. Biol. Chem.* **273**, 20378-20382

- 496 25 Tamai, I., Ohashi, R., Nezu, J.I., Sai, Y., Kobayashi, D., Oku, A., Shimane, M. and
497 Tsuji, A. (2000) Molecular and functional characterization of organic cation/carnitine
498 transporter family in mice. *J. Biol. Chem.* **275**, 40064-40072
- 499 26 van Vlies, N., Ferdinandusse, S., Turkenburg, M., Wanders, R.J. and Vaz, F.M. (2007)
500 PPAR α -activation results in enhanced carnitine biosynthesis and OCTN2-mediated
501 hepatic carnitine accumulation. *Biochim. Biophys. Acta* **1767**, 1134-1142
- 502 27 Koch, A., König, B., Stangl, G.I. and Eder, K. (2008) PPAR α mediates transcriptional
503 upregulation of novel organic cation transporters-2 and -3 and enzymes involved in
504 hepatic carnitine synthesis. *Exp. Biol. Med. (Maywood)* **233**, 356-365
- 505 28 Ringseis, R., Pösel, S., Hirche, F. and Eder, K. (2007) Treatment with
506 pharmacological peroxisome proliferator-activated receptor alpha agonist clofibrate
507 causes upregulation of organic cation transporter 2 in liver and small intestine of rats.
508 *Pharmacol. Res.* **56**, 175-183
- 509 29 Ringseis, R., Lüdi, S., Hirche, F. and Eder K. (2008) Treatment with pharmacological
510 peroxisome proliferator-activated receptor alpha agonist clofibrate increases intestinal
511 carnitine absorption in rats. *Pharmacol. Res.* **58**, 58-64
- 512 30 Ringseis, R., Luci, S., Spielmann, J., Kluge, H., Fischer, M., Geissler, S., Wen, G.,
513 Hirche, F. and Eder, K. (2008) Clofibrate treatment up-regulates novel organic cation
514 transporter (OCTN)-2 in tissues of pigs as a model of non-proliferating species. *Eur. J.*
515 *Pharmacol.* **583**, 11-17
- 516 31 Ringseis, R., Wege, N., Wen, G., Rauer, C., Hirche, F., Kluge, H. and Eder K. (2008)
517 Carnitine synthesis and uptake into cells are stimulated by fasting in pigs as a model of
518 nonproliferating species. *J. Nutr. Biochem.* [Epub ahead of print]
- 519 32 Luci, S., Hirche, F. and Eder K. (2008) Fasting and caloric restriction increases
520 mRNA concentrations of novel organic cation transporter-2 and carnitine
521 concentrations in rat tissues. *Ann. Nutr. Metab.* **52**, 58-67

- 522 33 Maeda, T., Wakasawa, T., Funabashi, M., Fukushi, A., Fujita, M., Motojima, K. and
523 Tamai, I. (2008) Regulation of Octn2 transporter (SLC22A5) by peroxisome
524 proliferator activated receptor α . *Biol. Pharm. Bull.* **31**, 1230-1236
- 525 34 Schupp, M., Lefterova, M.I., Janke, J., Leitner, K., Cristancho, A.G., Mullican, S.E.,
526 Qatanani, M., Szwegold, N., Steger, D.J., Curtin, J.C. et al. (2009) Retinol saturase
527 promotes adipogenesis and is downregulated in obesity. *Proc. Natl. Acad. Sci. U. S. A.*
528 **106**, 1105-1110.
- 529 35 Kast-Woelbern, H.R., Dana, S.L., Cesario, R.M., Sun, L., de Grandpre, L.Y., Brooks,
530 M.E., Osburn, D.L., Reifel-Miller, A., Klausning, K. and Leibowitz, M.D. (2004)
531 Rosiglitazone induction of Insig-1 in white adipose tissue reveals a novel interplay of
532 peroxisome proliferator-activated receptor gamma and sterol regulatory element-
533 binding protein in the regulation of adipogenesis. *J. Biol. Chem.* **279**, 23908-23915
- 534 36 Podvinec, M., Kaufmann, M.R., Handschin, C. and Meyer, U.A. (2002) NUBIScan,
535 an in silico approach for prediction of nuclear receptor response elements. *Mol.*
536 *Endocrinol.* **16**, 1269-1279
- 537 37 Ringseis, R., König, B., Leuner, B., Schubert, S., Nass, N., Stangl, G. and Eder, K.
538 (2006) LDL receptor gene transcription is selectively induced by t10c12-CLA but not
539 by c9t11-CLA in the human hepatoma cell line HepG2. *Biochim. Biophys. Acta* **1761**,
540 1235-1243
- 541 38 Wu, X., Huang, W., Prasad, P.D., Seth, P., Rajan, D.P., Leibach, F.H., Chen, J.,
542 Conway, S.J. and Ganapathy, V. (1999) Functional characteristics and tissue
543 distribution pattern of organic cation transporter 2 (OCTN2), an organic
544 cation/carnitine transporter. *J. Pharmacol. Exp. Ther.* **290**, 1482-1492
- 545 39 Koepsell, H. and Endou, H. (2004) The SLC22 drug transporter family. *Pflugers Arch.*
546 **447**, 666-676

- 547 40 Lahjouji, K., Elimrani, I., Lafond, J., Leduc, L., Qureshi, I.A. and Mitchell, G.A.
548 (2004) L-Carnitine transport in human placental brush-border membranes is mediated
549 by the sodium-dependent organic cation transporter OCTN2. *Am. J. Physiol. Cell*
550 *Physiol.* **287**, C263-C269
- 551 41 Grube, M., Meyer zu Schwabedissen, H.E., Prager, D., Haney, J., Möritz, K.U.,
552 Meissner, K., Roskopf, D., Eckel, L., Böhm, M., Jedlitschky, G. and Kroemer, H.K.
553 (2006) Uptake of cardiovascular drugs into the human heart: expression, regulation,
554 and function of the carnitine transporter OCTN2 (SLC22A5). *Circulation* **113**, 1114-
555 1122
- 556 42 Hirano, T., Yasuda, S., Osaka, Y., Kobayashi, M., Itagaki, S. and Iseki, K. (2006)
557 Mechanism of the inhibitory effect of zwitterionic drugs (levofloxacin and
558 grepafloxacin) on carnitine transporter (OCTN2) in Caco-2 cells. *Biochim. Biophys.*
559 *Acta* **1758**, 1743-1750
- 560 43 Kato, Y., Sugiura, M., Sugiura, T., Wakayama, T., Kubo, Y., Kobayashi, D., Sai, Y.,
561 Tamai, I., Iseki, S. and Tsuji, A. (2006) Organic cation/carnitine transporter OCTN2
562 (Slc22a5) is responsible for carnitine transport across apical membranes of small
563 intestinal epithelial cells in mouse. *Mol. Pharmacol.* **70**, 829-837
564

564 **Table 1** Oligonucleotides used for PCR amplification of promoter region and first
 565 intron of mouse OCTN2

Oligonucleotide name	Oligonucleotide sequence (5' to 3')	PCR product size (bp)
PPREprom-F	ATAAAGCTTGCCGTCCTCAGCCTCCCAC	
PPREprom-369R	ATACTCGAGGTTCTGAAGCCCGGTTTCAGCC	369
PPREprom-556R	ATACTCGAGCTGCTCCAGGAAGTGTCTG	556
PPREprom-750R	ATACTCGAGCTGTCGTTGTCAATGAGACTC	750
PPREprom-1043R	ATACTCGAGCACCTTTCTCATCACCAGTCT	1043
PPREprom-1753R	ATACTCGAGCAGCATTCTCTCCTGGCTT	1753
PPREint-1810F	ATAGGTACCCAATGGATCTGGATGCTATTG	
PPREint-1810R	ATAAAGCTTGCTGACTAGCTGCAGTTGTC	280
PPREint-67F	ATAGGTACCCTTCACTGTCGTCTTGCTCG	
PPREint-67R	ATAAAGCTTCTCTTACGAAACTGCTAAGATA	179
PPREint-94F	ATAGGTACCCTGGTCAGTGATGGAGCACA	
PPREint-94R	ATAAAGCTTTTGCTGATCAGCCAGCCTCT	504
PPREint-23511F	ATAGGTACCGTAGGTGACAGCCCCCT	
PPREint-23511R	ATAAAGCTTCCAGCAGAGTGACTGAGAAAT	703

566

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

PPRE denomination	Position	Sequence
Consensus PPRE	-	<u>AGGTCA</u> A <u>AGGTCA</u>
<i>Putative PPRE in promoter region of mouse OCTN2</i>		
PPRE1 [#]	-1389 to -1401 (+)*	AGGGAA a GGGTGA
PPRE2	-749 to -761 (-)	AAGGCA t AGGGCA
PPRE3	-761 to -773 (-)	AGCTCA c AGGCCC
PPRE4	-459 to -471 (-)	AGGTCA a AGCTAC
<i>Putative PPRE in intron 1 of mouse OCTN2</i>		
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 GGTAAG
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

569 [#] PPRE are numbered by ascending p value as obtained from *in silico*-analysis for putative
 570 PPRE of promoter region and first intron of mouse OCTN2 using NUBIScan software.

571 * The direction of the PPRE is indicated in brackets with “+” or “-“.

572

572 **LEGENDS TO FIGURES**

573

574 **Figure 1 Effect of exogenous mouse PPAR α /RXR α and PPAR α ligand WY-14,643**
575 **on transcriptional activity of OCTN2 promoter-truncation reporter**
576 **constructs**

577 HepG2 cells were transiently transfected with serial 5'-deletion OCTN2 promoter reporter
578 constructs, and a renilla luciferase expression vector for normalization. Cells were also co-
579 transfected with or without (empty vector) expression vectors for mouse PPAR α and RXR α .
580 After transfection, cells were stimulated or not with 50 μ M of WY-14,643 for 24 h.
581 Afterwards, cells were lysed, and luciferase activities of the OCTN2 promoter-truncation
582 reporter vectors and the renilla luciferase expression vector determined by dual luciferase
583 assay. Results represent means \pm SEM for one out of three independent experiments each
584 performed in triplicate.

585

586 **Figure 2 Effect of exogenous mouse PPAR α /RXR α and PPAR α ligand WY-14,643**
587 **on transcriptional activity of OCTN2 intron reporter constructs**

588 (A) Sequence alignment of consensus PPRE with the PPREint-1-8-10 sequence found in
589 OCTN2 first intron of mouse (nucleotide positions from 1850 to 1881) and rat (nucleotide
590 positions from 2019 to 2050). Matching nucleotides between mouse, rat and human are shown
591 by asterisks. Sequence of putative PPREint-1 and PPREint-8 overlapped with one nucleotide,
592 and putative PPREint-8 and PPREint-10 overlapped with six nucleotides. The mutated
593 nucleotides introduced in the OCTN2 intron reporter constructs for mutation analysis are
594 underlined. (B) HepG2 cells were transiently transfected with different OCTN2 intron
595 reporter constructs, and a renilla luciferase expression vector for normalization. Cells were
596 also co-transfected with or without (empty vector) expression vectors for mouse PPAR α and
597 RXR α . After transfection, cells were stimulated or not with 50 μ M of WY-14,643 for 24 h.

598 Afterwards, cells were lysed, and luciferase activities of the OCTN2 promoter-truncation
599 reporter vectors and the renilla luciferase expression vector determined by dual luciferase
600 assay. Results represent means \pm SEM for one out of three independent experiments each
601 performed in triplicate. (C) HepG2 cells were transiently transfected with mutant constructs
602 from OCTN2-PPREint-1-8-10 construct each containing a targeted mutation in either PPRE1,
603 PPRE8 or PPRE10, and a renilla luciferase expression vector for normalization. Cells were
604 also co-transfected with or without (empty vector) expression vectors for mouse PPAR α and
605 RXR α . After transfection, cells were stimulated or not with 50 μ M of WY-14,643 for 24 h.
606 Afterwards, cells were lysed, and luciferase activities of the OCTN2 promoter-truncation
607 reporter vectors and the renilla luciferase expression vector determined by dual luciferase
608 assay. Results represent means \pm SEM for one out of three independent experiments each
609 performed in triplicate.

610

611 **Figure 3 Binding of *in vitro*-translated mouse PPAR α /RXR α to the PPREint-1-8-10**
612 **of mouse OCTN2 intron**

613 EMSA was performed using *in vitro*-translated mouse PPAR α /RXR α and DIG-labelled
614 oligonucleotide corresponding to either wild-type or mutated PPREint-1-8-10. Fold molar
615 excess of unlabelled specific probe for competition (rat-ACO-PPRE) is indicated. The use of
616 DIG-labelled specific probe (corresponding to rat-ACO-PPRE oligonucleotides) and non-
617 specific probe (corresponding to random oligonucleotides of OCTN2 intron 1) is also
618 indicated.

619

620 **Figure 4 Binding of *in vitro*-translated mouse PPAR α /RXR α to the PPREint-1 of**
621 **mouse OCTN2 intron**

622 EMSA was performed using *in vitro*-translated mouse PPAR α /RXR α and DIG-labelled
623 oligonucleotides corresponding to either PPREint-10, PPREint-8 or PPREint-1 as well as their

624 mutant counterparts PPREint-10mut, PPREint-8mut, and PPREint-1mut. The use of DIG-
625 labelled specific probe (corresponding to rat-ACO-PPRE oligonucleotides) and non-specific
626 probe (corresponding to random oligonucleotides of OCTN2 intron 1) is also indicated.

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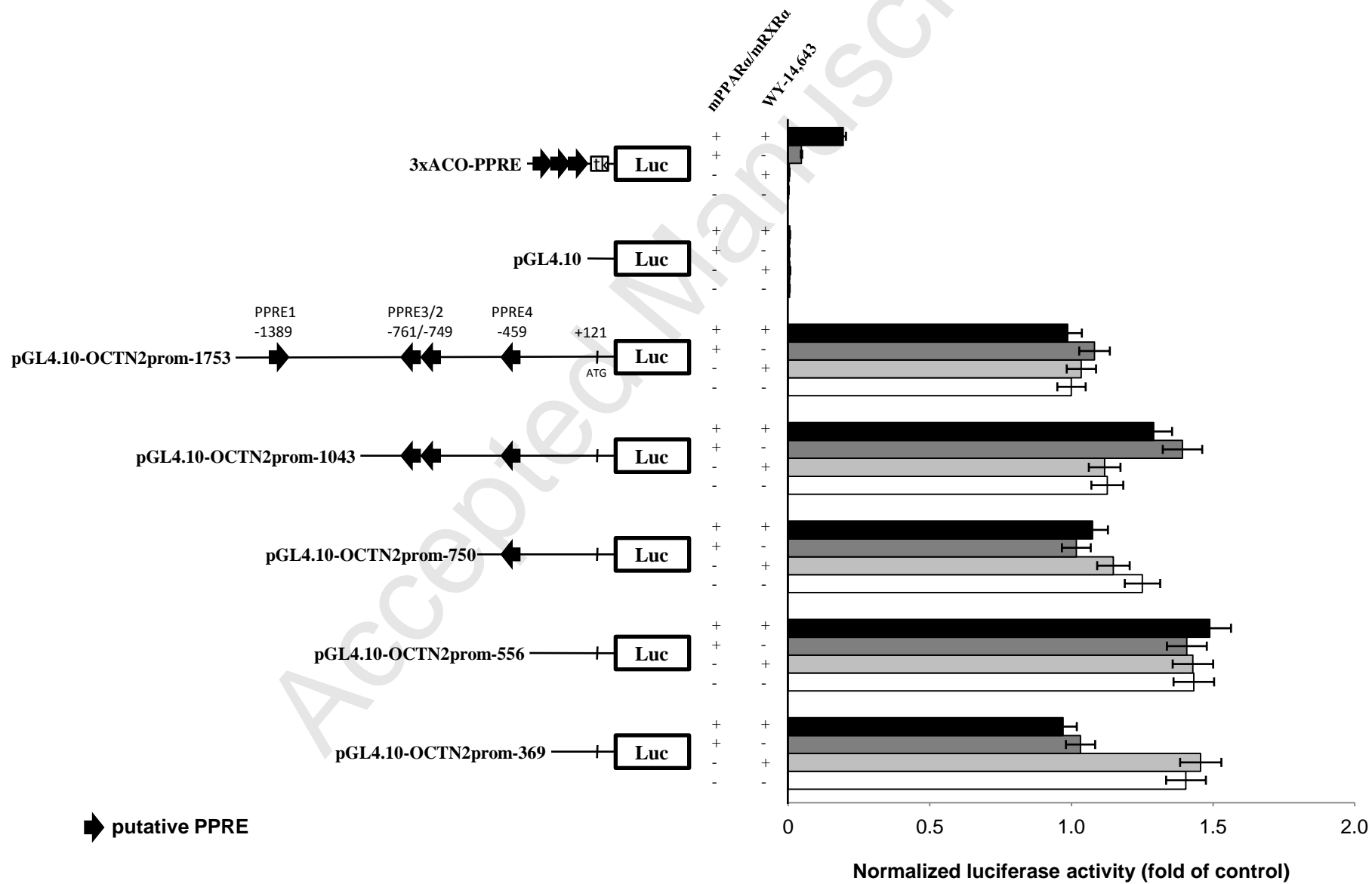


Figure 1

```

Consensus PPRE           ACTGGAaACTGGA           AGGTCAaAGGTCA
                             AGGTCAaAGGTCA

mOCTN2-PPREint-1-8-10  AAGAGTTATATGCCCTtTCACCTACTTAcAGGTCAgCAGTTTTTGCAGG-AGG
rOCTN2-PPREint         AAGAGTTATATGCCCTtTCACCTACTTAcAGGTCAgCAGTCTTTGCAGG-AGG
hOCTN2-PPREint        AGGAGTTATATGCCCTtTCACCTACTTAaATGTTGtCAGTCTTTGCAGGGAGG
* ***** ***** * **      **** ***** **

mOCTN2-PPREint-1:      TGCCCTtTCACCT
mOCTN2-PPREint-1mut:   TGCCCAAtACACCT
mOCTN2-PPREint-8:     TACTTAcAGGTCA
mOCTN2-PPREint-8mut:  TACTTTcAGGACA
mOCTN2-PPREint-10:    AGGTCAgCAGTTT
mOCTN2-PPREint-10mut: AGGACAgCAGATT
    
```

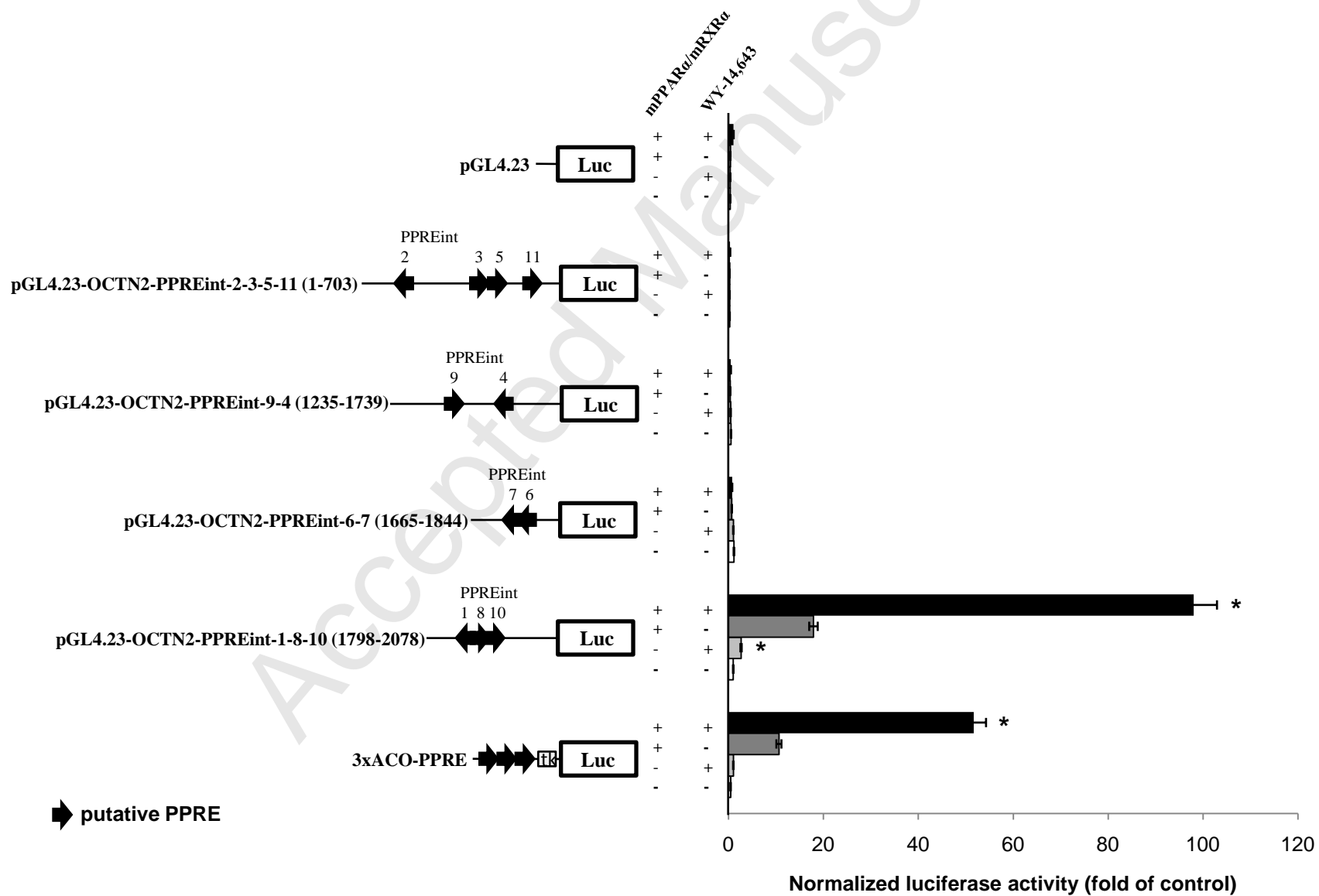



Figure 2B

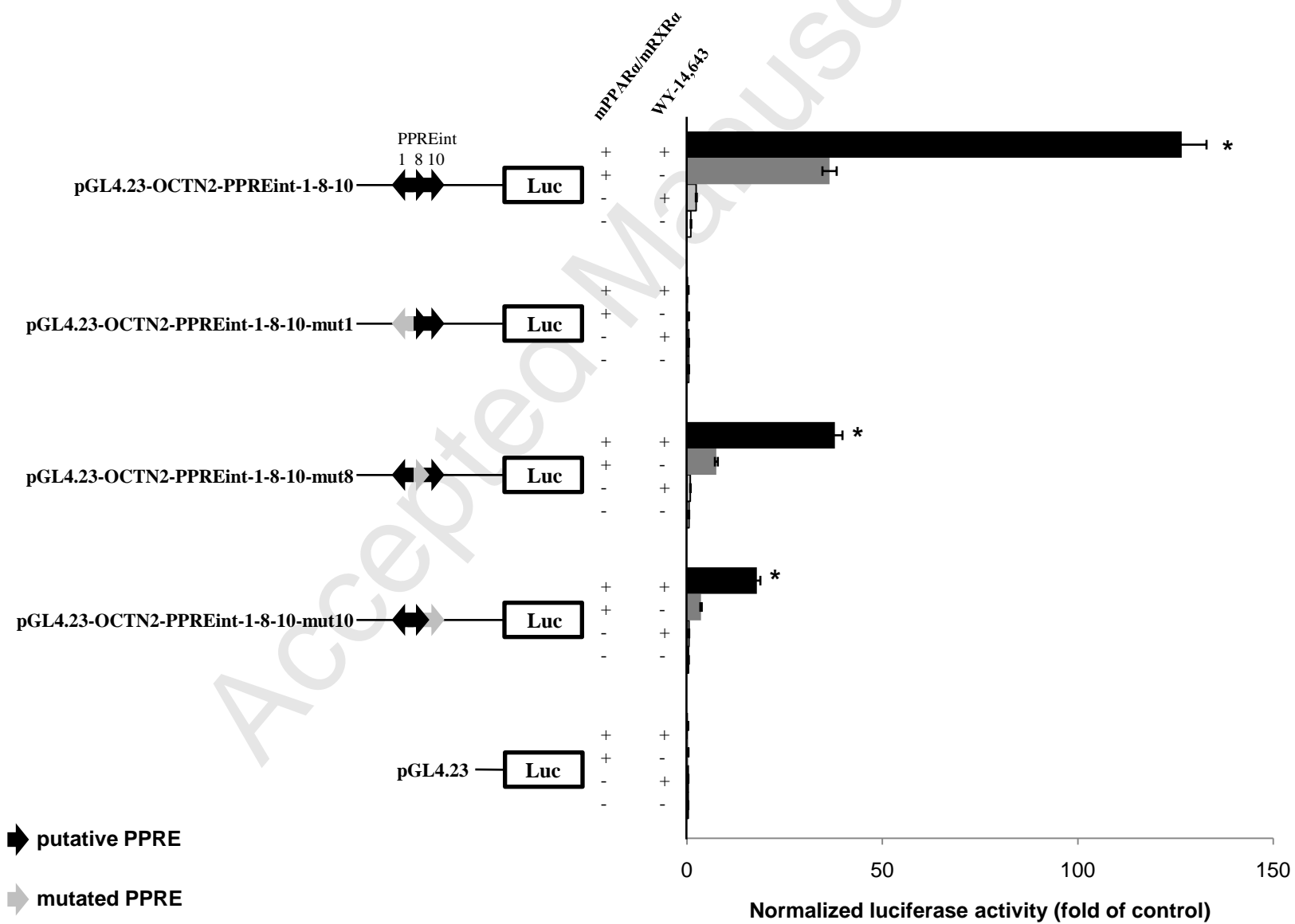
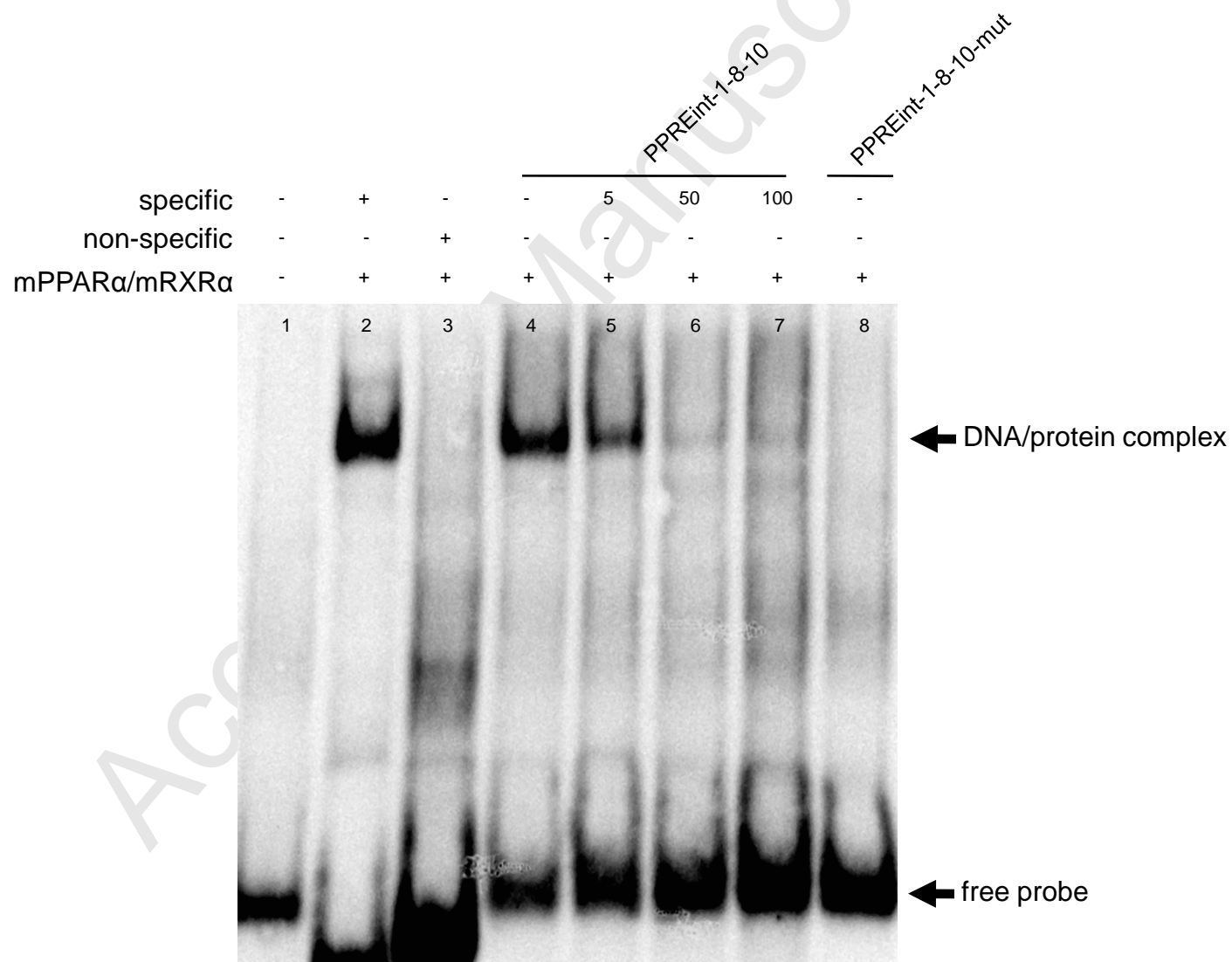
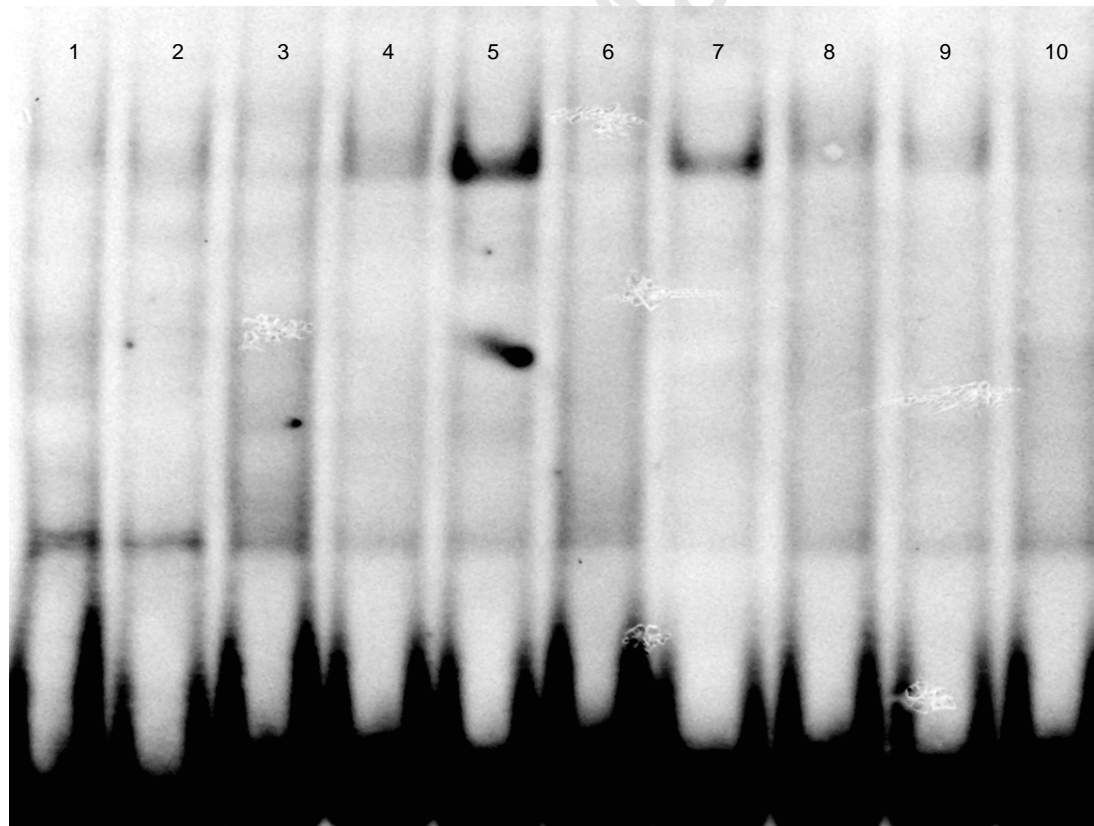


Figure 2C



	mOCTN2-PPRE-					PPREcont-		mOCTN2-PPRE-		
	int-1	int-1	int-10	int-8	int-1	non-spec.	spec.	int-10mut	int-8mut	int-1mut
mPPAR α	+	-	+	+	+	+	+	+	+	+
mRXR α	-	+	+	+	+	+	+	+	+	+



← DNA/protein complex

← free probe

