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Functional characterization of a peroxisome proliferator response-element located in the intron 3 of rat peroxisomal thiolase B gene☆

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Abstract

Expression of the rat peroxisomal 3-ketoacyl-CoA thiolase gene B is induced by peroxisome proliferators. Although a sequence element like a peroxisome proliferator-activated receptor (PPAR)-binding site is located in the promoter region of this gene, we previously found that this element is competent for the activation by hepatocyte nuclear factor-4, but not functional with PPARα. We describe here a new peroxisome proliferator-response element located in the intron 3 (+1422/+1434) that binds in vitro the PPARα/retinoid X receptor α heterodimer and confers the induction by PPARα in transfection assays. We propose a model of regulation of the rat thiolase B gene involving those elements in the promoter and intron 3.

Keywords: Peroxisome; Fatty acid β oxidation; Thiolase; Peroxisome proliferator activated receptor α

The enzymes required for β-oxidation of fatty acyl-CoA are present both in peroxisomes and in mitochondria. The feature of liver peroxisomes is their inducibility. It has been well established that the administration of hypolipidemic compounds such as clofibrate or plasticizers including di(2-ethylhexyl)phthalate to the rats leads to an increase in the volume density of peroxisomes in liver cells. These proliferators simultaneously induce the expression of the three genes coding for the enzymes involved in the peroxisomal β-oxidation of long chain fatty acids, i.e., acyl-CoA oxidase (AOX), enoyl-CoA hydratase-hydroxyacyl-CoA dehydrogenase (bifunctional/multifunctional-enzyme) (BFE) and 3-ketoacyl-CoA thiolase. Similar results are observed when rat hepatocytes are cultured with the proliferators [1].

The last step of peroxisomal β-oxidation of long chain fatty acids in rat peroxisomes depends at least on two distinct 3-keto-acyl CoA thiolases, A and B. These enzymes have the same specificities regarding ketoacyl-CoA substrates [2], but they are differently regulated. Thiolase A is constitutively expressed whereas the expression of thiolase B (TB) is induced by peroxisome proliferators [3]. The expression of TB gene depends on the nuclear receptor PPARα because it is not induced by fenofibrate in PPARα knockout mice [4].

Like other inducible genes involved in fatty acid β-oxidation, the rat TB (rTB) gene has a putative peroxisome proliferator response element (PPRE) upstream of the basal promoter, at the positions −681/−669 [5]. It was previously shown that this PPRE-like sequence is able to bind the PPARα–RXRα heterodimer in vitro [5,6], but it does not confer induction by PPARα to

☆ *Abbreviations:* BFE, bifunctional enzyme; AOX, acyl-CoA oxidase; HNF-4, hepatocyte nuclear factor 4; luc, luciferase; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator-response element; RXRα, retinoid X receptor α; TB, peroxisomal 3-ketoacyl-CoA thiolase B.

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a reporter gene [6]. Thus, we concluded that this PPRE, named here PPRE1, was non-functional for thiolase B gene activation by PPARα [6] but we demonstrated that this element is competent for HNF4-dependent activation. Here we set the evidence of a new PPRE, named PPRE2, located between nucleotides +1422 and +1434 in intron 3 of the rat TB (rTB) gene.

Peroxisomal β-oxidation of long chain fatty acids takes place mainly in the liver, and the thiolase genes are expressed at high levels in this organ compared to others [7, Chevillard et al., personal communication for the mouse]. HNF-4 is one of the nuclear receptors highly expressed in the liver and is known to be implicated in the hepatic specific gene expression and liver differentiation [8,9]. Moreover, studies with HNF-4 knockout mice showed that HNF-4 is involved in the control of the lipid homeostasis [10]. Otherwise HNF-4 and PPARα binding sequences are closely related and competition or cooperation between PPARα and HNF-4 was soon described for the PPREs of acyl-CoA oxidase and multifunctional enzyme genes [11]. In the present work, being given the important function of HNF-4 in liver specific gene expression, we also looked for HNF-4 activation in comparison with PPARα activation on the newly identified PPRE in the intron 3 of the rTB gene.

Materials and methods

Plasmid construction. A rat genomic fragment, BE1, was obtained by subcloning in *Eco*RI site of pBluescript a 10 kb *Eco*RI fragment of λPTB1 clone [3], extending from position −8331 to position +1909 located in intron 3 of the TB gene (the transcription initiation site was numbered +1). The sequencing has been achieved by Genome Express (Meylan, France). The sequence analysis was performed essentially using the algorithms contained in Infobiogen site (http://www.infobiogen.fr).

pGluc is a luciferase expression vector containing the β-globin promoter upstream of the luciferase coding sequence [12]. Previously and in the present work, this plasmid was used for generating constructs allowing the study of isolated response elements The pairs of annealed oligonucleotides with *Bam*HI and *Hin*dIII ends reported in Table 1 were ligated with *Hin*dIII/*Bam*HI double-digested pGluc upstream of the β-globin promoter to provide the plasmids pGluc BFE (−2954/−2918) [12] (named BFE in the present work), pGluc TB PPRE1 (−688/−664) [6] (named PPRE1), AOX-PPRE-Luc (named AOX) [13], pGluc TB PPRE2 (+1413/+1438) (named PPRE2) (this work), and pGluc TB mPPRE2 (+1413/+1438) (named mPPRE2).

The 500 bp intron 3 contructs reported in Fig. 4 were obtained by PCR on rat genomic DNA using the following primers. A *Hin*dIII site (AAGCTT) was added to the 5′ end of the forward primers, while a *Bam*HI site (GGATCC) to the 5′ end of the reverse primers. To obtain the plasmid containing PPRE2 (Fig. 4, 500I3PPRE2), we used anoligonucleotide ACAAGCTT^{+966} ACTTAGAGGGAGTGT GTTG C^{+985} as the forward primer and ACGGATCC^{+1444} CAG TTTGCTATGACTTTTGA^{+1425} as the reverse primer. To delete PPRE2 (Fig. 4, plasmid 500I3ΔPPRE2) ACGGATCC^{+1421} ACAC AGCAGAAAGAACCGAG^{+1402} was used as the reverse primer while the forward primer was the same as below. The PCR-amplified fragments were digested with *Hin*dIII and *Bam*HI, and subcloned in the *Hin*dIII/*Bam*HI digested pGLuc, upstream of the β-globin promoter.

pSG5mPPARα and pSG5mRXRα, gifts from Dr. Green (Zeneca Macclesfield, UK), contain, respectively, the mouse PPARα and RXRα cDNAs downstream of the SV40 promoter of pSG5. The pMT7-rHNF4, a gift from Dr. Sladek (University of California), contains the rat HNF4 cDNA downstream of the adenovirus promoter of pMT7 vector. pCMVβ vector (Clontech), a β-galactosidase reporter plasmid, was co-transfected for the correction of transfection efficiency.

Table 1
Sequence of different oligonucleotides containing PPREs used in this study

Rat gene	Oligonucleotide	Plasmid
BFE PPRE	AGCT^{-2954}**TTCCT**T**TGACCT**AT**TGAACT**A**TTACCT**ACATTTGAGG^{-2918} **AAGGA**A**ACTGGA**TA**ACTTGA**T**AATGGA**TGTAAACTCC**CTAG** DR1 DR1 DR2	pGluc BFE(-2954/-2918)
AOX PPRE	AGCT^{-575}TGAACG**TGACCT**T**TGTCCT**GGTCCCCTTTTG^{-545} ACTTGC**ACTGGA**A**ACAGGA**CCAGGGGAAAAC**CTAG** DR1	AOX-PPRE-Luc
TB PPRE (-681/-669) (PPRE1)	AGCT^{-688}CTCTCAG**AGACCT**T**TGAACC**ACTTC^{-664} GAGAGTC**TCTGGA**A**ACTTGG**TGAAG**CTAG** DR1	pGlucTB (-688/-664) named PPRE1 in this work
TB PPRE (+1422/+1434) (PPRE2)	AGCT^{+1412}CTGCTGTGT**AGGTCA**A**AAGTCA**TAGC^{+1438} GACGACACA**TCCAGT**T**TTCAGT**ATCG**CTAG** DR1 AGCT^{+1412}CTGCTGTGTGCGGCCGCTAGC^{+1438} GACGACACACGCCGGCGATCG**CTAG**	pGlucTB(+1413/+1438) named PPRE 2 pGluc TB mPPRE2 (+1413/+1438) named mPPRE2

PPREs are indicated in bold and the direct repeat is underlined. The nucleotide positions are given taking as +1 the trancription initiation site. The *Hin*dIII and *Bam*HI extentions used for cloning are given in white letters.

Transfection assays. COS-7 (a monkey kidney-derived cell line) cells were seeded in 24-well plates at 5×10^4 cells per well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 20 mM Hepes, and 10% fetal calf serum. After an overnight culture, the cells were washed with a serum free medium (optiMEM) and transfected with a mixture of 1 μg of plasmid DNA (purified with a Qiagen column) containing 30 ng pCMVβ, 250 ng of a luciferase reporter plasmid, and different amounts of the nuclear factor's expression vectors with 2 μg of liposome Exgen (Euromedex) in 300 μl optiMEM. After 5 h, the transfection medium was replaced by 1 ml of the complete medium with or without 10^{-5} M Wy14,643 (Alexis Biochemicals), a peroxisome proliferator. pSG5 was used to adjust the amount of DNA to be transfected to 1 μg. HNF-4 cDNA is cloned in pMT7 vector. We always compared transfection with HNF-4 to control experiments containing the same molar quantity of pMT7.

After 48 h, the cells were washed twice with PBS, harvested in 100 μl of Reporter Lysis Buffer (Promega), and centrifuged after three freeze–thaw cycles. The cleared cytosol extract (10 μl) was added to 50 μl of luciferase assay reagent (Promega) and the light emission was measured for 10 s in TLX1 luminometer. β-Galactosidase activity was assayed using 2 μl of lysate mixed with 200 μl of 0.1 M phosphate buffer, pH 7.4, 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, and 1 mM chlorophenolred-β-D-galactopyranoside.

Transfection assays were repeated four times for each condition, and the mean and SD of corrected luciferase activity were calculated. Statistical Student's *t* tests were performed, between the data obtained with and without the nuclear receptor expression vectors (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

Electrophoretic mobility shift assay (EMSA). The oligonucleotide PPRE2 (Table 1) was used as a probe. The annealed oligonucleotide was labeled with ^{32}P using Klenow DNA polymerase and [α-^{32}P]dCTP. In vitro translation of proteins was performed with the TNT coupled reticulocyte lysate system (Promega) using pSG5mPPARα, pSG5mRXRα, and pMT7-rHNF4 as DNA templates. For each plasmid, a 50 μl translation mixture was prepared according to the manufacturer's protocol. To produce proteins with unlabeled methionine (20 pmol), 40 μl of the translation mixture was used. To the remaining 10 μl of the translation mixture, 4 μCi of the [^{35}S]methionine was added to compare the translation efficiency between different plasmids. Total [^{35}S]methionine incorporation into a TCA-insoluble fraction was measured on 8 μl aliquots. On the basis of these results and according to the relative number of methionines in the different proteins, equivalent amounts of the non-radiolabeled proteins (obtained in the 40 μl assay) were used in electrophoretic mobility shift assay while keeping the same lysate volume between the different assays by adding unprogrammed reticulocyte lysate.

A typical DNA-binding assay contained in 10 mM Tris–HCl, pH 7.1, 1 mM EDTA, 80 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 5% glycerol, 10 mM β-mercaptoethanol, 0.5 mg/ml BSA, and 1.5 μg poly(dIdC), in a total volume of 20 μl. Binding reaction was started by the addition of radiolabeled oligonucleotides (100,000 cpm) and proceeded on ice for 30 min. The reaction mixtures were loaded on a 5% polyacrylamide gel equilibrated in 0.5× TBE. Finally, the gels were dried and autoradiographed.

Results

rTB gene sequencing

We sequenced intron 3 of the rTB gene from nucleotide +1012 to +1909, as counted from the transcription start site (Accession No. in EMBL AJ438953). PPREs were searched for, using 5′ TGACCT N TGNCCT 3′, or the complementary sequence, as the consensus sequence proposed by Juge Aubry et al. [14], allowing 1, 2 or 3 nucleotide-mismatches. In this work, we focused on PPRE2 located in the intron 3 of the rTB gene between positions +1422 and +1434 (Table 1).

Interaction of PPRE2 with in vitro translated PPARα, RXRα, and HNF-4

Binding studies on PPRE2 were performed using in vitro translated PPARα, RXRα, and HNF-4. PPRE2 interacted with the heterodimer PPARα/RXRα (Fig. 1, lane 3) but not with either one, separately (Fig. 1, lanes 1 and 2). PPRE2 also interacted with HNF-4 as shown in lane 4 (Fig. 1). These data suggest that PPRE2 interacts, at least in vitro, with both the PPARα/RXRα heterodimer and HNF-4.

Activation of PPRE-driven reporter genes by PPARα in transfection assays

We first examined if PPARα could activate a PPRE2-driven reporter luciferase gene (Table 1, plasmid pGlucTB(+1413/+1438), named PPRE2). In comparison, we also used two other PPRE-driven reporter genes: PPRE1 (Table 1, plasmid pGlucTB(−688/−664), named PPRE1) that we previously showed to be non-responsive to the activation by PPARα [6], and PPRE AOX (plasmid AOX-PPRE-Luc named AOX) as a well-known PPARα-stimulated responsive element.

COS-7 cells were transfected by the different PPRE plasmids in the presence of increasing amounts of the PPARα expression vector. After transfection, the cells were treated or not with Wy14,643, a strong peroxisome proliferator (Fig. 2). PPARα activated the expression of PPRE2 and AOX, but not PPRE1. The strongest

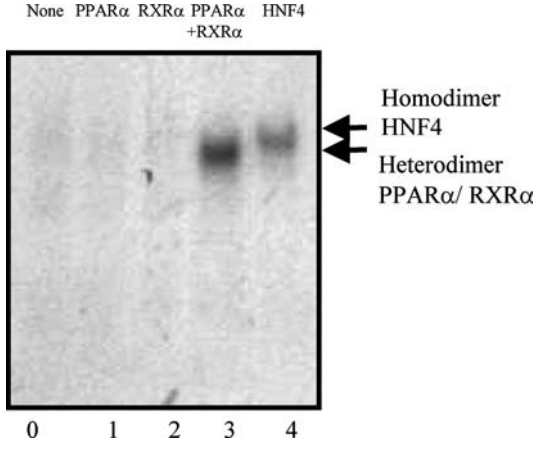

Fig. 1. EMSA of in vitro translated nuclear factors with the PPRE2 probe. Labeled PPRE2 double-strand oligonucleotide was incubated with unprogrammed reticulocyte lysate (lane 0) or programmed reticulocyte lysate containing the same amounts (based on the TCA precipitable radioactivity in a pilot assay) of PPARα (lane 1), RXRα (lane 2), PPARα + RXRα (lane 3), and HNF4 (lane 4).

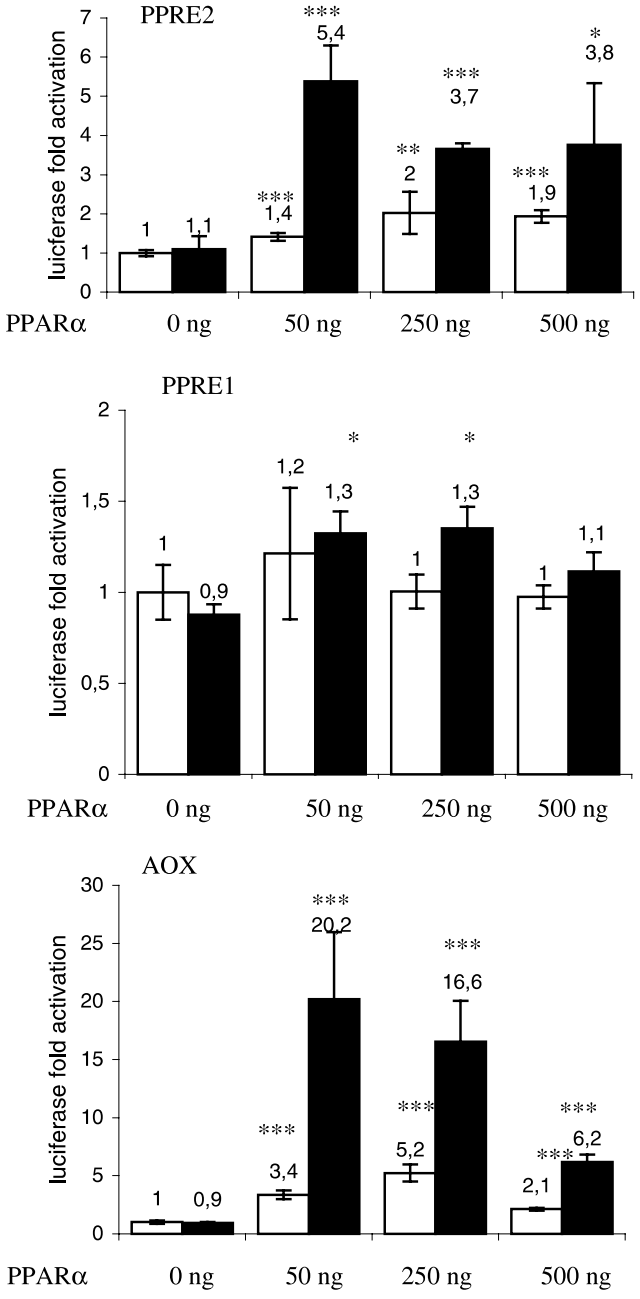

Fig. 2. Effects of increasing amounts of pSG5 PPARα on the PPRE-driven-luciferase activities with or without peroxisome proliferator treatment. PPRE2, PPRE1, and AOX PPRE-driven luciferase reporter vectors (250 ng) were transfected to COS-7 cells together with increasing amounts of pSG5PPARα, as indicated below the bars. After transfection, cells were cultured with (black bars) or without (white bars) Wy14,643 (10 μM). Relative luciferase activities are given, taking as 1 the activity obtained for each PPRE-driven reporter gene in the absence of PPARα and the treatment with Wy14,643. Data represent means ± SD of quadruplicate samples. Differences statistically significant are shown (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

activation on PPRE2 and AOX was obtained with the smallest amount of PPARα expression vector (50 ng) upon Wy14,643 (10 μM) treatment (activation of 5.4- and 20.2-fold, respectively), and higher vector dose rather suppressed the activation, particularly in the presence of Wy14,643. This effect of the amount of expression vector was reproduced in several batches of fetal calf serum and with all cell preparations. Using an even lower amount of PPARα expression vector (10 ng), we found that 50 ng of the vector was optimal for the transactivation by PPARα of the PPRE-driven reporter genes in our experimental system (data not shown). By comparing the responsiveness of different PPREs to PPARα (Fig. 2), we conclude that PPRE2 confers activation of a reporter gene by PPARα, though at a lower level than AOX PPRE. However, under any transfection conditions, PPRE1 did not allow activation of the reporter gene, confirming the previous conclusion that PPRE1 is not by itself a PPARα-response element [6].

In Fig. 3, we compared the peroxisome proliferator-dependent activation by PPARα, on different PPREs so far identified in the three genes for the peroxisomal β-oxidation enzymes. As previously reported by several authors, the complex PPRE of the BFE (bifunctional enzyme or MFP1) gene is a stronger PPRE than that of AOX, which in turn is stronger than PPRE2 found in the rTB gene. At the same time, we examined a mutated PPRE2 (mPPRE2) in which the PPRE2 sequence was replaced by a *Not*I site. As expected, this substitution totally abolished the PPARα-dependent activation.

PPARα-dependent activation of the intron 3-driven reporter gene in transfection assays

The above results suggest that PPRE2 is a PPARα-response element of the rTB gene. To verify this, we studied whether PPRE2 conferred the activation by PPARα, when surrounded by the rTB intron 3 sequences (Fig. 4, 500I3PPRE2). More than twofold activation by PPARα was found with the 500 bp intron 3 fragment containing PPRE2, whereas deletion of the PPRE2 abolished the activation by PPARα (Fig. 4, 500I3ΔPPRE2). This result shows that PPRE2 is functional even if surrounded by the intron 3 sequence, though the level of activation is less marked than that obtained with PPRE2 alone.

Activation of PPRE-driven reporter genes by HNF-4 in transfection assays

As HNF-4 binds to PPRE2 in vitro, we examined whether HNF-4 activated the PPRE2-driven reporter gene in transfection assays. We found a twofold activation by HNF-4 on the PPRE1-driven reporter gene as previously reported [6], but not on the gene driven by PPRE2 (Fig. 5A). This experiment was repeated using different amounts of the HNF-4 expression vector (between 50 and 250 ng), but we did not find stimulation of the PPRE2-driven reporter expression under any experimental conditions (data not shown).

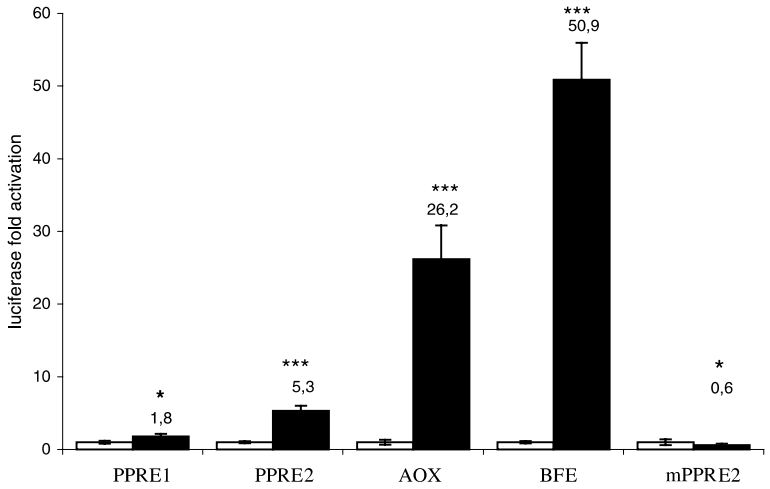

Fig. 3. Comparison of the activation by PPARα of different PPRE-driven luciferase reporter genes. Luciferase reporter vectors driven by PPRE2, PPRE1, AOX, BFE-PPREs, and mPPRE2 (250 ng) were transfected to COS-7 cells with pSG5 PPARα (50 ng) and Wy14,643 (10 μM) treatment (black bars) or without pSG5 PPARα and Wy14,643 (white bars). Relative luciferase activities are given, taking as 1 the activity obtained for each PPRE-driven reporter gene in the absence of PPARα and treatment. Data represent means ± SD of quadruplicate samples. Differences statistically significant are shown (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

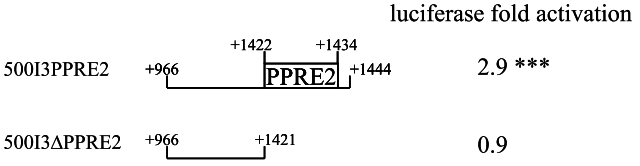

Fig. 4. Effect of the surrounding intron 3 sequences on the PPRE2-targeted activation by PPARα in transfection assays. The 500 bp I3 construct (with PPRE2 (500I3PPRE2) or without PPRE2 (500I3ΔPPRE2) was obtained by PCR amplification as described in Materials and methods, and inserted upstream of the β-globin promoter of the pGluc vector. Equal amounts of the two reporter genes (250 ng) were transfected to COS-7 cells with or without pSG5PPARα (50 ng) and Wy14,643 (10 μM) treatment. The luciferase fold-activation represents for each construct the ratio of the luciferase activity obtained in the presence of PPARα and Wy14,643 treatment to that obtained without PPARα and Wy14,643 treatment. Data represent means of quadruplicate. Differences statistically significant are shown (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

We looked for the effect of HNF-4 on the PPARα-dependent activation of the PPRE2-driven reporter gene (Fig. 5B). Increasing amounts of the HNF-4 expression vector were transfected with the PPRE2-driven reporter gene in the presence of 50 ng of the PPARα expression vector. The PPARα-dependent activation diminished with higher amounts of HNF-4 vector. This result shows a competition between HNF-4 and PPARα on PPRE2, as described for the PPRE of the AOX gene [11,15].

Discussion

Until now, the only proposed PPRE of the peroxisomal 3-ketoacyl-CoA thiolase gene was the one located between nucleotides −681 and −669 of the rTB gene [5]. Our previous work showed that this PPRE (PPRE1) would be non-functional for the induction of rTB gene by peroxisome proliferators, but rather a responsive element for the liver factor HNF-4 [6]. In addition, we showed that a fragment of 2.7 kb of the rTB gene promoter did not allow the activation by PPARα [6]. These observations suggest that a genuine PPRE of the rTB gene is outside the proximal promoter region. By sequencing the rTB gene, we found a sequence closely related to the PPRE consensus (named PPRE2) in intron 3. PPRE2 interacted in vitro with the PPARα/RXRα heterodimer and HNF-4, and allowed an activation of a reporter gene by PPARα. On the other hand, HNF-4 did not activate the PPRE2-driven reporter gene but probably could bind to PPRE2 also in vivo, because HNF-4 diminished the activating effect of PPARα on PPRE2 in transfection assays.

Several genes were reported to contain more than one PPRE-like motifs, e.g., malic enzyme [16], PEPCK [17], AOX [18], and aP2 [19] genes. For malic enzyme [20], the proximal motif only seemed functional. For rat AOX and aP2 genes, gel shift assays showed that one of the two PPRE-like motifs was far more effective than the other for PPAR binding [14]. In the case of PEPCK gene, the two motifs were equivalent for PPAR binding, but in vivo analysis showed that they could have different roles for PEPCK gene regulation [21]. The last situation seems to best fit the case of rTB gene. The rTB gene indeed contains at least two putative PPREs (PPRE1 and PPRE2). PPRE1 was probably a regulatory element recognized by HNF-4, whereas PPRE2 would preferentially be activated by PPARα. An important question is how the regulatory mechanisms involving these sequence elements are interrelated.

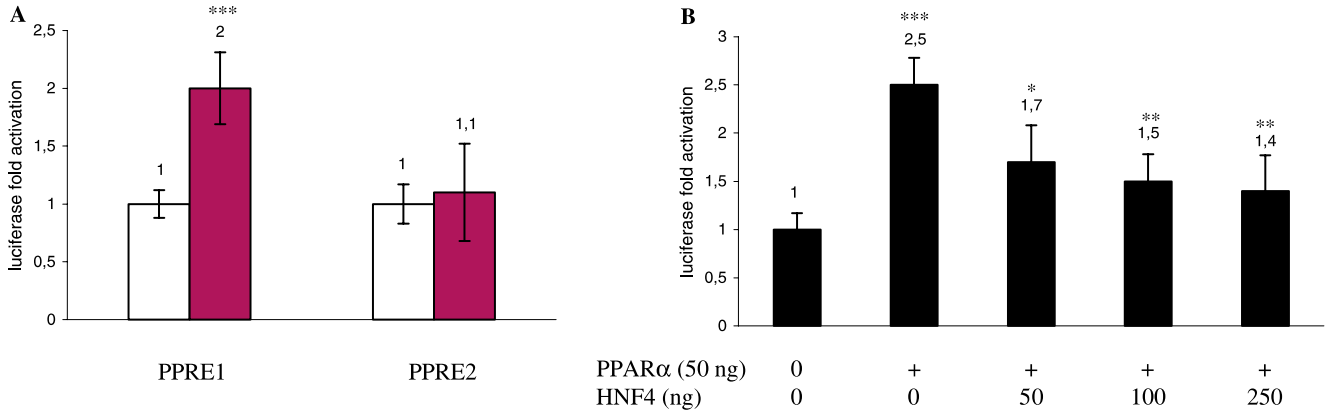

Fig. 5. Effect of the HNF-4 vector on various PPRE-driven luciferase reporter genes (A) and competition between HNF-4 and PPARα on the PPRE2-driven reporter gene (B). (A) PPRE2- and PPRE1-driven reporter plasmids (250 ng) were transfected to COS-7 cells with pMT7-rHNF-4 vector (100 ng) (black bars).In the control experiments without HNF-4 (white bars), pMT7 of the same molar quantity was used for the transfection assay. (B) PPRE2-driven reporter plasmids (250 ng) were transfected to COS-7 cells with or without pSG5PPARα (50 ng), and with increasing amounts of pMT7-rHNF4 as indicated below the bars. The molar quantity of pMT7 more pMT7rHNF-4 keeps constant between assays. The cells were incubated with Wy14,643 (10 μM) after transfection. Relative luciferase activities are given, taking as 1 the activity obtained for each PPRE-driven reporter gene in the absence of any nuclear receptor expression vector. Data represent means ± SD of quadruplicate samples. Differences statistically significant are shown (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

The relationship between HNF-4 and PPARα regulations seems to be complex. HNF-4 is indispensable to the hepatic gene expression [22]. At the same time, PPARα and HNF-4 recognize very similar sequences [23] with some nucleotide differences. The functional relationship between PPARα and HNF-4 on PPREs may depend on the sequences. For example, in the case of AOX gene, there is a competition between HNF-4 and PPARα on the PPRE [11,15], whereas there is a cooperation between these two nuclear receptors on the BFE PPRE [11]. Mutual regulation between PPARα and HNF-4 was also described on genes not involved in the peroxisomal β-oxidation, which was probably due to either the competition for binding to the regulatory element [24] or a suppressive effect of PPARα on the expression of HNF-4 gene [25]. On the other hand, at least in humans, HNF-4 may modulate PPARα gene expression [26].

Recently, a functional PPRE for PPARγ was localized in the intron 1 of the acyl-CoA-binding protein gene [27]. Chromatin immunoprecipitation has shown an in vivo interaction between this element and the PPARγ/RXR heterodimer in the adipocytes. How an element in the intron could interfere with the rate of the transcription remains to be established. It has been suggested that an intron would affect the elongation step of transcription [28], or at the step of initiation of transcription by an interaction of an element in the intron and the promoter, the sequence in between looping out [29,30].

This work described for the first time a PPRE localized in the intron of a gene of the peroxisomal β oxidation, the interactions between the promoter and the intron 3 of the rTB gene remaining to be studied.

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