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Identification of a Novel Peroxisome Proliferator-Activated Receptor (PPAR) γ Promoter in Man and Transactivation by the Nuclear Receptor RORα1

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PPARγ has been extensively studied for the past decade mainly due to its central role in promoting and maintaining the adipocyte phenotype. To date, three PPARγ isoforms have been described in man. Here we show the presence of a fourth PPARγ promoter with its cognate mRNA initiating at exon 1, as evidenced by primer extension analysis. The presence of a putative responsive element (RORE) for RORα, a representative of the ROR/RZR orphan receptor superfamily, in the novel promoter was investigated. By gelshift experiments and site-directed mutagenesis we show that this RORE specifically binds the RORα1 isoform. We further demonstrate that overexpression of RORα1, but not the RORα2 and RORα3 isoforms, induced a 40-fold increase in promoter activity in transient transfection assays in various cell lines. Considering the strong transcriptional activation it is likely that RORα1 forms a part of the multifactorial regulatory mechanisms that control expression of the human PPARγ gene.

Key Words: **nuclear receptor; promoter; gene regulation; proliferation; differentiation; primer extension; electroforetic mobility gel shift assay; transfection.**

The gamma subtype of the peroxisome proliferator-activated receptors (PPARγ) has attracted considerable attention since it was demonstrated to be the key regulator of adipocyte differentiation (1, 2). Upon ligand-binding PPAR heterodimerise with the retinoid X receptor (RXR) and regulate the transcription of genes, preferentially those involved in -lipid and -lipoprotein metabolism, through binding to specific response elements, or PPREs, in the target gene promoter (3). Identification of the anti-diabetic drugs thiazolidinediones as synthetic ligands for PPARγ (4, 5) has linked its function to metabolic disorders such as obesity and noninsulin-dependent diabetes mellitus (NIDDM). A potential role for PPARγ as modulator of inflammatory response and in the development of chronic inflammatory diseases have been described (6). Furthermore, the capability of PPARγ in promoting macrophage differentiation (7) has suggested a regulatory role of the receptor in the progression of atherosclerosis (8, 9).

Due to different promoter usage and alternative splicing the human PPARγ gene generates three PPARγ mRNA, -γ1, -γ2 (10), and -γ3 (11). Although PPAR -γ1 and -γ3 mRNA give rise to the same protein, the transcripts show differential tissue distribution in man (10, 11). Whereas PPARγ1 mRNA is relatively ubiquitously expressed (10, 12, 13), PPARγ3 mRNA is restricted to adipose tissue, colon epithelium, and macrophages (11, 14). PPARγ2 expression is confined to adipose tissue and its protein contains an additional 30 amino acid N-terminal sequence compared to the -γ1 and -γ3 isoforms (10, 15). Until now, few studies have addressed the molecular mechanisms underlying the regulation of PPAR gene expression. It has been shown that PPARγ expression is under transcriptional control by the sterol regulatory element-binding protein (SREBP/ADD-1) and CCAAT-enhancer-binding protein (C/EBP) family of transcription factors (16) which are important players in the regulation of adipocyte differentiation.

The retinoic acid receptor-related orphan receptor (RORα, also termed RZR) gene gives raise to four isoforms, -α1, -α2, -α3, and RZRα, by different promoter usage and alternative splicing (17–20). The isoforms differ in their N-terminal domains and display distinct DNA-binding and transactivation properties (17). RORα binds as monomers to similar response elements (REs), that consist of a single core motif AGGTCA preceded by a 6-bp A + T-rich region (17). RORα is widely expressed as manifested by its mRNA in adipose tissue, brain, liver, spleen, lung, heart, muscle, ovary, and peripheral blood leukocytes (17, 18, 20–22).

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The exact function of RORα has not been elucidated but its protein has been shown to play an important role in cerebellar development, as demonstrated with its mutation in naturally occurring staggerer mice (23), as well as in targeted knockout experiments (24). The homozygous staggerer (sg/sg) mutant mouse shows cerebellar ataxia and neurodegeneration (25, 26) and exhibits immune abnormalities such as hyperproduction of inflammatory cytokines (27). In addition, these mice are more susceptible to develope atherosclerosis (28).

Several target genes for RORα1 have been identified and characterised based on the presence of putative response elements in their promoter (29–34). Among these are the mouse apolipoprotein AI (32), as well as both human and mouse apolipoprotein CIII (33) which are positively regulated by RORα1, suggesting a role of this orphan receptor as a regulator of lipid and lipoprotein metabolism. The importance of RORα in cardiovascular and metabolic diseases is supported by the observation that staggerer mice maintained on a high fat atherogenic diet develop a severe hypoalphalipoproteinemia and atherosclerosis (28). Additionally, a role for RORα has been proposed in adipocyte differentiation (35), bone metabolism (36), and in modulating the immune response (29, 34, 37).

Here we report the presence of a new PPARγ mRNA in man, PPARγ4, with a transcription initiation site localised at the boundary of exon 1. Our data suggest that the human PPARγ gene, through the newly identified PPARγ4 promoter, is a novel target for the RORα1 receptor.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotide sequences[2] used in this study listed from 5′ to 3′ are:

(a) LF18-gct gat ccc aaa gtt ggt ggg cca gaa (as) exon 1*;
(b) LF120-ctc aat tga tga cct aat gta gaa gtt aat (s)-intron 1*;
(c) LF121-aat aac ttc tac att agg tca tca att gag (as)-intron 1*;
(d) LF123-ctc aat tga aga tct aat gta gaa gtt aat-(s)-intron 1*;
(e) LF124-att aac ttc tac att aga tct tca att gag-(as)-intron 1*;
(f) LF127-ctg gga taa cag gtg tga gcc act (s)-intron 1;
(g) LF128-ctg aaa gga aaa ata gac tag ctg (as)-intron 1;
(h) 613-gat ctt gac cta cat tct aag ctg (s);
(i) 614-gat cca gct tag aat gta ggt caa (as).

Plasmids. pCMX-RORα1, pCMX-RORα2, and pCMX-RORα3 was obtained from V. Giguere. pGL3γ1p3000 and pGL3γ2p1000 are previously described (10) and pGL3-γ3p800 (11).

Cell cultures. Standard cell culture conditions were used to maintain 3T3-L1 (obtained from ATCC), CaCO2 (ATCC), HepG2 (ATCC), and COS-1 (ATCC).

Cloning and construction of recombinant plasmids. The PAC clone p-8856 (Fajas *et al.*, 1997), containing the full-length of the human PPARγ gene, was sequenced with the oligonucleotide *a* using Big Dye Terminator (Perkin Elmer). 433 bp in front of exon 1 was recovered and amplified by PCR using the oligonucleotides *f* and *g*. The PCR fragment was treated with *Pfu polymerase* and subsequently inserted into the *Eco*RV site of pBluescript SK+ (Stratagene, La Jolla, CA). After digestion with *Sac*I and *Xho*I the insert was subcloned into pGL3 (Promega, Madison, WI), creating the reporter vector pGL3-γp4.

Site-directed mutagenesis. Site-directed mutagenesis of the RORE site in the hPPARγ4 promoter was performed by PCR amplification of pGL3-γp4 using high fidelity PCR enzyme according to the manufacturer (Boehringer) with the oligonucleotide pair *d*/*e*. This changed the two bases underlined in the sequence from 5′-TGA TGA CCT AAT-3′ to 5′-TGA AGA TCT-3′ to generate the plasmid pGL3-γp4$_{mut}$. Ten cycles of amplification were performed at 94°C for 15 s, 60°C for 30 s, and 68°C for 3 min, followed by 20 cycles of amplification at 94°C for 15 s, 60°C for 30 s, and 68°C for 4 min. The resulting PCR fragment was digested with 20 units *Dpn*I in order to destroy the methylated, parental strands, and transformed in XL-1 cells. Four out of 15 clones were containing the mutated site as verified by sequencing.

Primer extension. Total cellular RNA was prepared as described previously (38). For primer extension, 10 pmol (50 ng) of the oligonucleotide *a* was labelled, using T4-polynucleotide kinase (T4-PNK) (Amersham, Courtaboeuf, France) and ^{32}P-γATP to a specific activity of 500,000 CPM, and subsequently purified from excess nucleotides by a Nucleotide removal kit (Qiagen). Primer extension analysis was performed using 10 μg of total RNA from human adipose tissue and approximately 5-ng probe, labelled to a specific activity of 50,000 cpm, according to a standard protocol utilising 50 U of AMV reverse transcriptase (Life Technologies, Paisley, UK). A sequencing reaction was used as molecular mass standard to map the 5′ end of the extension products. The products were separated at an 5% urea/acrylamidgel.

Electrophoretic mobility gel shift assays (EMSA). The pCMX-based RORα1 expression vector was *in vitro* transcribed and translated using TNT-T7 Quick coupled transcription/translation kit as directed by the manufacturer (Promega). Double-stranded oligonucleotides serving as probes for EMSA were labeled with T4-PNK and ^{32}P-γATP and unincorporated nucleotides were subsequently removed using a Nucleotide removal kit (Qiagen). Two microliters of programmed reticulocyte lysate was incubated for 15 min on ice in a total volume of 20 μl with 1 mg poly(dI:dC) and 1 mg herring sperm DNA in binding buffer (10 mM Tris–HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40, and 1 mM DTT). Approximately 0.15 pmol of probe was added to the preincubated protein reaction-mix and incubation continued for 20 min at room temperature. For competition experiments, increasing amounts of cold double-stranded oligonucleotide (50-, 100-, and 200-fold molar excess) was included just before adding labelled oligonucleotide. DNA–protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25 × TBE buffer at 4°C (39). The following oligonucleotides were used as probes: RORE$_{wt}$: *b* and *c*, RORE$_{conc}$: *h* and *i*, and RORE$_{mut}$: *d* and *e*.

Transfections and transient expression assay. Human colon carcinoma cells ($CaCO_2$), mouse preadipocytes (3T3-L1), monkey kidney fibroblasts (COS-1), and human hepatocytes (HepG2) were transiently transfected by an electroporation procedure using 10 μg of reporter vector and 4 μg of RORα1 expression vector (gift from Dr. V. Giguere) or empty pCMX plasmid vector. A RSV-driven β-galactosidase expression vector (2 μg) was included as a control for transfection efficiency. For transfections performed by electroporation, approximately 4×10^6 cells were transfected for each condition and plated into 12-well plates. Medium was changed after 2-h incubation and incubation continued for at least 24 h. Luciferase and β-galactosidase assays were performed as described previously (40). Each transfection was performed at least three times.

RESULTS

Identification and Cloning of the PPARγ4 Promoter: Determination of the Transcription Initiation Site

The sequence of the exon–intron boundaries of the human PPARγ gene have previously been described

[2] * corresponds to hPPARγ sequence; RORE is underlined.

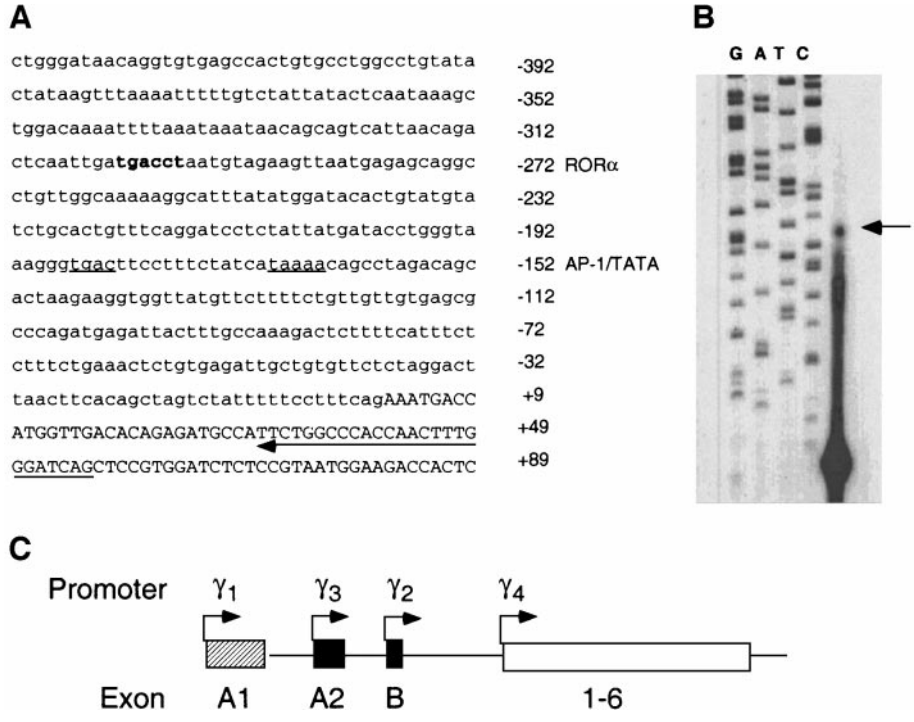

FIG. 1. Determination of the transcription initiation site of PPARγ4 mRNA and sequence of the 5′ proximal region. (A) Sequence showing part of the genomic sequence of hPPARγ (Accession No. AY043357). Small letters correspond to intron B and capital letters to exon 1. Sequence motifs are underlined (TATA box, AP1) and RORE is indicated in bold. The oligonucleotides *a* used for primer extension is indicated by an arrow. (B) Primer extension of human white adipose tissue RNA with the PPARγ specific primer *a* was performed as described. The major extension product is indicated by an arrow. Size standards, on the left, consist of a sequencing reaction. (C) A scheme of the genomic structure of the 5′ end of the human PPARγ gene. Exons 1–6 are shared by all four isoforms. PPARγ1 contains in addition the untranslated exons A1 and A2, PPARγ2 contains exon B, which is translated, PPARγ3 contains only the untranslated exon A2 and PPARγ4 initiate at exon 1.

(10). A more detailed analysis of the 5′ sequence boundaries of exon 1 indicated the presence of some potential regulatory elements such as a TATA-like sequence, AP-1 site, and a RORE, suggesting the presence of a promoter upstream of exon 1 in the human PPARγ gene (Fig. 1A).

In order to define the 5′ end of the putative PPARγ4 transcript we performed primer extension analysis of total RNA from human adipose tissue. An antisense oligonucleotide (*a*) localised in exon 1 was used as primer (Fig. 1A). One major extension product was detected, localised 29 nucleotides upstream from the primer, and revealed the existence of a mRNA extending up to exon 1 (Fig. 1B). These data indicate the presence of a novel transcription initiation site giving rise to a new PPARγ mRNA, which we designated PPARγ4.

We next isolated, by PCR, a 433-bp genomic fragment corresponding to the region 5′ to exon 1 (intron B) of the human PPARγ gene. This putative promoter fragment was inserted into the pGL3-basic reporter vector (Promega) to generate the construct pGL3-γp4. To evaluate the promoter activity of pGL3-γp4, the construct was transfected into 3T3-L1, HepG2, COS-1, and $CaCO_2$ cells. Promoter activity was monitored by evaluation of the luciferase activity. Relative to the promoterless parent vector, the PPARγ4 promoter construct stimulated luciferase expression by a 3- to 10-fold (data not shown), and thus supported the evidence that this was a functional promoter.

In summary, the structure of the human PPARγ gene corresponds to the scheme shown in Fig. 1C.

RORα1 Binds to and Transactivates the hPPARγ4 Promoter

Sequence analysis of the intronic sequence upstream of exon 1 (intron B) of the human PPARγ gene revealed the presence of a putative-binding site for the RORα transcription factor which we defined as RORE (Fig. 2A). In order to demonstrate direct binding of RORα to this site, EMSA analysis was performed using double-stranded oligonucleotides (*b*/*c*) corresponding to the PPARγ RORE ($RORE_{wt}$) as a probe. As shown in Fig. 2B *in vitro* translated RORα1, but not RORα2 or RORα3, binds to the hPPARγ RORE. Spesificity of the binding was demonstrated by competition assays using increasing amounts of a cold double-stranded oligonucleotide containing the hPPARγ-RORE wild-type ($RORE_{wt}$), mutated ($RORE_{mut}$), or consensus ($RORE_{conc}$). Binding of RORα1 to the hPPARγ RORE-element was competed by both the cold wild-type $RORE_{wt}$ and the

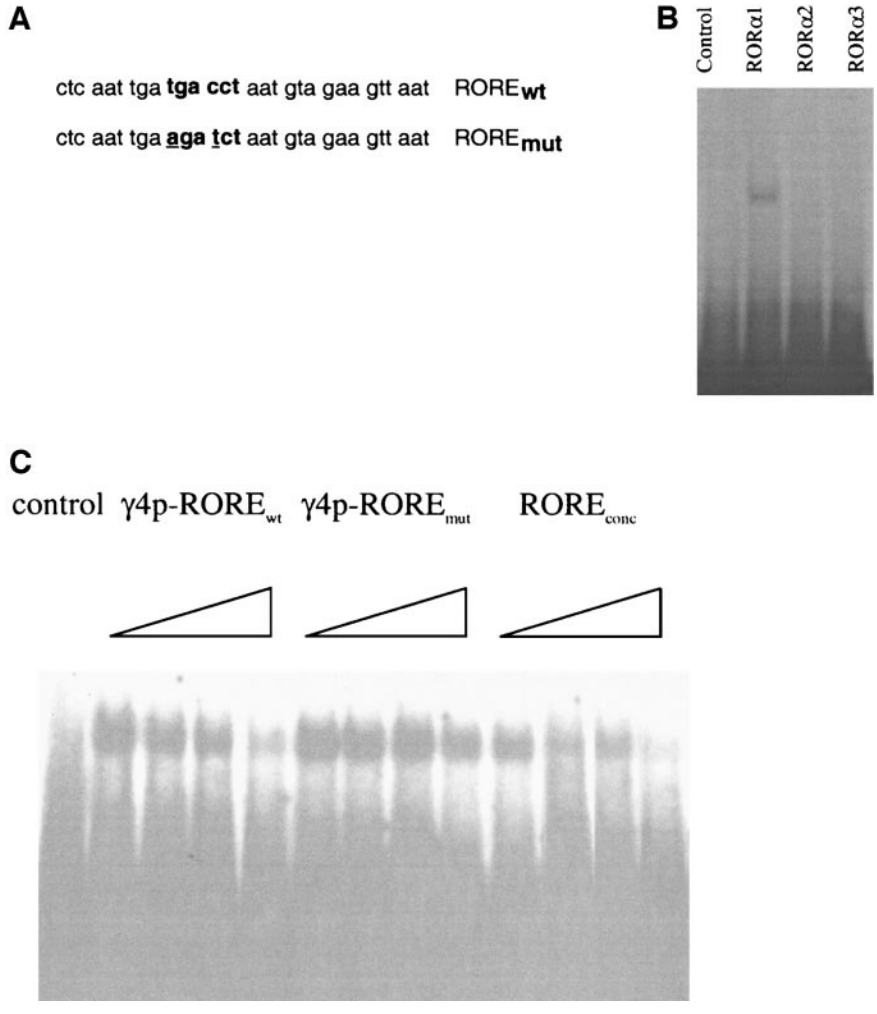

FIG. 2. RORα1 binds to the concensus site in the hPPARγ4 promoter. (A) Scheme depicting the wild-type (wt) and the mutated (mt) oligonucleotides used as probes in electrophoretic mobility shift assay (EMSA). The RORE site (AGGTCA) is indicated in bold. (B) EMSA of hPPARγ-RORE$_{wt}$ in presence of *in vitro* transcribed/translated RORα1, RORα2, RORα3, or unprogrammed reticulolysate. (C) EMSA of hPPARγ-RORE$_{wt}$ in presence of *in vitro* transcribed/translated RORα1, RORα2, RORα3, or unprogrammed reticulolysate. Competition experiments were performed with unlabeled hPPARγ-RORE$_{wt}$, hPPARγ-RORE$_{mut}$, and RORE$_{conc}$ (50-, 100-, and 200-fold molar excess of oligonucleotides).

consensus RORE$_{conc}$ oligonucleotides whereas the mutated RORE$_{mut}$ was unable to compete for binding of RORα1 (Fig. 2C).

Next, in order to assess the capability of RORα1 to mediate transactivation from the hPPARγ4 promoter through the RORE, cotransfection experiments were performed using expression vectors coding for either RORα1, RORα2, or RORα3 together with the pGL3-γp4 luciferase reporter construct (Fig. 2A). A 44-fold increase in the luciferase activity of the pGL3-γp4 reporter construct was observed when COS-1 cells were cotransfected with the RORα1 expression vector (Fig. 3A). In contrast, no change in the luciferase activity was observed when either RORα2 or RORα3 were cotransfected (Fig. 3A). These results suggest that RORα1 transactivates the expression of the hPPARγ4 mRNA through binding to the responsive element located at the PPARγ4 promoter. In addition, the observed transcription driven from the PPARγ4 promoter seemed to be spesific for the RORα1 isoform since neither ROR-α2 or -α3 are able to stimulate the PPARγ4 promoter. Furthermore, expression of PPARγ is regulated by RORα1 through the newly identified PPARγ4 promoter and not through the -γ1, -γ2, or -γ3 promoters of PPAR as demonstrated by cotransfection experiments using the promoter-constructs pGL3-γ1p3000, pGL3-γ2p1000, or pGL3-γ3p800 (Fig. 3B).

Finally, to unequivocally demonstrate that it was through binding to RORE that RORα1 stimulated the activity of the pGL3-γp4 reporter construct, we substituted two bases in PPARγ RORE (Fig. 2A) to generate the mutated reporter plasmid pGL3-γp4$_{mut}$. Cotransfected RORα1 was unable to stimulate the mutated reporter construct in COS-1 cells whereas in the same

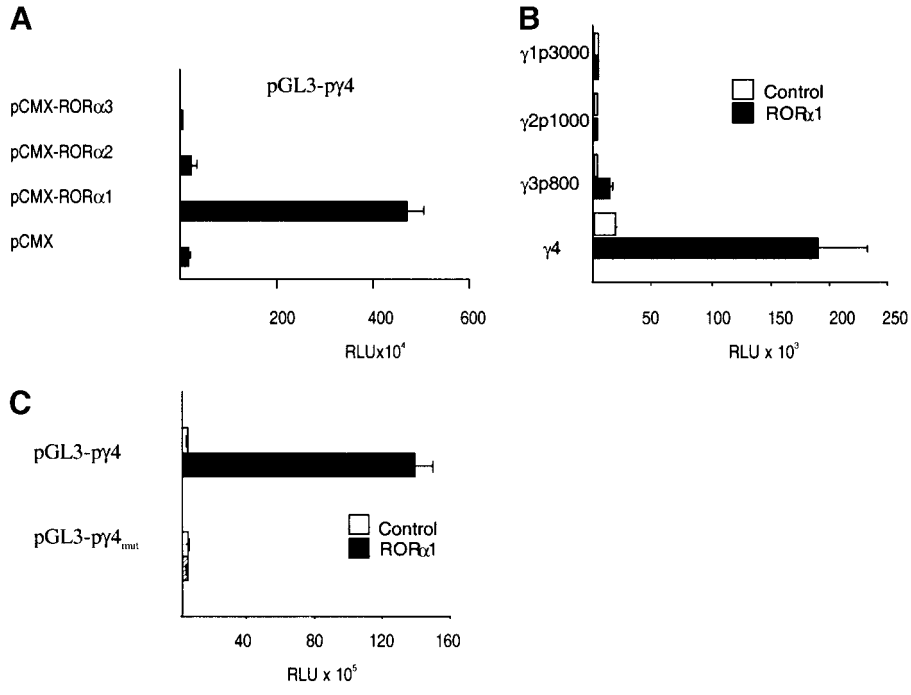

FIG. 3. The promoter of hPPARγ4 contains a functional RORE. (A) COS-1 cells transfected with the reporter plasmid pGL3-γp4 containing the RORE and the expression vector pCMX-RORα1, -RORα2, -RORα3, or empty plasmid vector pCMX. Luciferase activity was determined and normalized to RSV-β-galactosidase activity. (B) Human intestinal carcinomas cells $CaCO_2$ were transfected with reporter plasmid containing the sequence of four PPARγ isoforms, pGL3-γ1p3000, pGL3-γ2p1000, pGL3-γ3p800, or pGL3-γ4p, respectively, in the presence of the expression construct pCMX-RORα1 or empty plasmid vector pCMX. Luciferase activity was determined and normalized to RSV-β-galactosidase activity. (C) Human intestinal carcinomas cells $CaCO_2$ and COS-1 cells were transfected with reporter plasmid, pGL3-γp4 or pGL3-γp4$_{mut}$, in the presence of the expression construct pCMX-RORα1 or empty plasmid vector pCMX. Luciferase activity was determined and normalized to RSV-β-galactosidase activity.

experiment, the wild-type reporter construct (pGL3-γp4) was induced by a 155-fold (Fig. 3C).

DISCUSSION

While PPARγ activity has been extensively studied, little is known about the regulation of PPARγ gene expression. The identification of transcription factors responsible for such regulation may help to further understand the complex functional role of PPARγ in human physiology. Sequence analysis of the genomic sequence upstream of exon 1 (intron B) of the human PPARγ gene revealed the existence of some putative regulatory elements (AP-1, RORE, TATA), suggesting the presence of a new promoter. We have demonstrated by primer extension analysis and promoter activity assays that a novel PPARγ mRNA, PPARγ4, is transcribed from the newly isolated -γ4 promoter in the human PPARγ gene. Expression analysis to detect the novel -γ4 mRNA is, however, not possible since the -γ4 sequence is common for all four PPARγ mRNAs. Although the -γ1, -γ3-, and -γ4 mRNA of hPPARγ, give rise to the same protein, the presence of different promoters is thought to allow a more fine tuning in the control of gene expression.

As such, we report here the presence of a consensus site for RORα (RORE) in the PPARγ4 promoter and that this RORE specifically binds the RORα1 isoform, and not -α2 or -α3. This is consistent with the observation of Giguère and colleagues (17) that RORα1 and RORα2 have different preferences for sequences 5′-flanking to the core-binding motif. Transfection studies demonstrated that RORα1 could, in contrast to RORα2 and RORα3, activate transcription from the reporter construct containing the hPPARγ4 promoter, resulting in a 44–155-fold induction of transcription. Whereas RORα1 induced transactivation from the hPPARγ4 promoter construct, no induction was detected from the promoters of PPAR-γ1, -γ2, or -γ3, consistent with the absence of RORE in these promoters.

The high level of transactivation mediated by RORα1 in the absence of ligand is similar to observations in other studies (19, 41, 42). It has thus been speculated whether this receptor actually requires a ligand for its proper function or whether it is activated by interaction with a endogenous ligand present in the cell system used. Another possible explanation could involve regulation of its activity by phosphorylation (43). More recently, it has been reported that RORα is a Ca^{2+}-responsive transcription factor that can be ac-

tivated by Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) (44). So far, known activators of RORα are the endogenous pineal gland hormone melatonin (18, 43) and the synthetic antiarthritic thiazolidinedione CGP52608 (37, 43). CPG52608 represents a new class of thiazolidinediones with high potency in inhibiting inflammation (37). In addition, both melatonin and CGP52608 exert similar inhibitory effects on the proliferation of neoplastic cells in mouse colonic adenocarcinoma, human prostata cancer, and human breast carcinoma (45–47).

Crosstalk between different nuclear signalling pathways has been demonstrated for various member of the nuclear receptor superfamily (2, 22, 48). Here we provide evidence for an interaction between the RORα and PPAR signalling pathway in which PPARγ is a direct target for RORα1. The *in vivo* role for such an interaction is supported by the similar role of the two receptors as modulators in atherosclerosis and inflammation where they mediate anti-atherogenic and anti-inflammatory effects (6, 27–29, 34). Both receptors also modulate cell proliferation in that synthetic RORα- and PPARγ agonists show similar effects in exerting antiproliferative activity on tumour cells (6, 47, 49–51).

Although the role of PPARγ in atheroscleoris, inflammation, and cancer seems contradictory and rather complex (6), the knowledge of factors that govern PPARγ gene expression might be of crucial importance to understand the interaction with other signalling pathways and the *in vivo* role of the receptor. Our data indicate a regulatory role of RORα on human PPARγ expression that might explain some of the similar effects of both receptors in human physiology.

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REFERENCES

1. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) mPPARg2: Tissue-specific regulator of an adipocyte enhancer. *Genes & Dev.* **8,** 1224–1234.
2. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPARg2, a lipid-activated transcription factor. *Cell* **79,** 1147–1156.
3. Schoonjans, K., Staels, B., and Auwerx, J. (1996) The peroxisome proliferator-activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochim. Biophys. Acta* **1302**(2), 93–109.
4. Berger, J., Bailey, P., Biswas, C., Cullinan, C. A., Doebber, T. W., Hayes, N. S., Saperstein, R., Smith, R. G., Leibowitz, M. D. (1996) Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-g: Binding and activation correlate with antidiabetic actions in db/db mice. *Endocrinology* **137,** 4189–4195.
5. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor g (PPARg). *J. Biol. Chem.* **270,** 12953–12956.
6. Debril, M. B., Renaud, J. P., Fajas, L., and Auwerx, J. (2001) The pleiotropic functions of peroxisome proliferator-activated receptor gamma. *J. Mol. Med.* **79**(1), 30–47.
7. Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A., and Evans, R. M. (1998) PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* **93,** 241–252.
8. Marx, N., Schonbeck, U., Lazar, M. A., Libby, P., and Plutzky, J. (1998) Peroxisome proliferator-activated receptor gamma activators inhibit gene expression and migration in human vascular smooth muscle cells. *Circ. Res.* **83,** 1097–1103.
9. Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H., and Evans, R. M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **93,** 229–240.
10. Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, C., Lefebvre, A. M., Saladin, R., Najib, Laville, J. M., Fruchart, J. C., Deeb, S., Vidal-Puig, A., Flier, J., Briggs, M. R., Staels, B., Vidal, H., and Auwerx, J. (1997) Organization, promoter analysis, and expression of the human PPARg gene. *J. Biol. Chem.* **272,** 18779–18789.
11. Fajas, L., Fruchart, J. C., and Auwerx, J. (1998) PPARgamma3 mRNA: A distinct PPARgamma mRNA subtype transcribed from an independent promoter. *FEBS Lett.* **438,** 55–60.
12. Elbrecht, A., Chen, Y., Cullinan, C. A., Hayes, N., Leibowitz, M. D., Moller, D. E., and Berger, J. (1996) Molecular cloning, expression and characterization of human peroxisome proliferator activated receptors gamma 1 and gamma 2. *Biochem. Biophys. Res. Comm.* **224,** 431–437.
13. Mukherjee, R., Jow, L., Croston, G. E., and Paterniti, J. R., Jr. (1997) Identification, characterization, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARgamma2 versus PPARgamma1 and activation with retinoid X receptor agonists and antagonists. *J. Biol. Chem.* **272,** 8071–8076.
14. Ricote, M., Huang, J., Fajas, L., Li, A., Welch, J., Najib, J., Witztum, J. L., Auwerx, J., Palinski, W., and Glass, C. K. (1998) Expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA* **95**(13), 7614–7619.
15. Auboeuf, D., Rieusset, J., Fajas, L., Vallier, P., Frering, V., Riou, J. P., Staels, B., Auwerx, J., Laville, M., and Vidal, H. (1997) Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: No alteration in adipose tissue of obese and NIDDM patients. *Diabetes* **46**(8), 1319–1327.
16. Fajas, L., Schoonjans, K., Gelman, L., Kim, J. B., Najib, J., Martin, G., Fruchart, J. C., Briggs, M., Spiegelman, B. M., and Auwerx, J. (1999) Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: Implications for adipocyte differentiation and metabolism. *Mol. Cell. Biol.* **8,** 5495–5503.
17. Giguère, V., Tini, M., Flock, G., Ong, E. S., Evans, R. M., and Otulakowski, G. (1994) Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan nuclear receptors. *Genes Dev.* **8,** 538–553.

18. Becker-Andrè, M., Andrè, E., and Delamarter, J. F. (1993) Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. *Biochem. Biophys. Res. Comm.* **194,** 1371–1379.
19. Carlberg, C., Hooft van Huijsduijnen, R., Staple, J. K., DeLamarter, J. F., and Becker-Andre, M. (1994) RZRs, a new family of retinoid-related orphan receptors that function as both monomers and homodimers. *Mol. Endocrinol.* **6,** 757–770.
20. Hirose, T., Smith, R. J., and Jetten, A. M. (1994) ROR gamma: The third member of ROR/RZR orphan receptor subfamily that is highly expressed in skeletal muscle. *Biochem. Biophys. Res. Commun.* **205,** 1976–1983.
21. Giguère, V., McBroom, L. D. B., and Flock, G. (1995) Determinants of target gene specificity for RORα: Monomeric DNA-binding by an orphan nuclear receptor. *Mol. Cell. Biol.* **15,** 2517–2526.
22. Hiroshi, A., Dawson, M. I., and Jetten, A. M. (1996) Suppression by retinoids of the induction of the CCAATT/enhancer-binding protein α and the nuclear receptors PPARγ and RORγ during adipocyte differentiation of 3T3-L1 cells. *Mol. Cell. Differ.* **4,** 365–381.
23. Hamilton, B. A., Frankel, W. N., Kerrebrock, A. W., Hawkins, T. L., FitzHugh, W., Kusumi, K., Russell, L. B., Mueller, K. L., van Berkel, V., Birren, B. W., Kruigiyak, L., and Lander, E. S. (1996) Disruption of the nuclear hormone receptor RORalpha in staggerer mice. *Nature* **379,** 736–739.
24. Dussault, I., Fawcett, D., Matthyssen, A., Bader, J. A., and Giguere, V. (1998) Orphan nuclear receptor ROR alpha-deficient mice display the cerebellar defects of staggerer. *Mech. Dev.* **70,** 147–153.
25. Sidman, R. L., Lane, P. V., and Divkie, M. M. (1962) Staggerer, a new mutation in the mouse affecting the cerebellum. *Science* **136,** 610–612.
26. Herrup, K., and Mullen, R. J. (1979) Staggerer chimeras: Intrinsic nature of Purkinje cell defects and implications for normal cerebellar development. *Brain Res.* **178,** 443–457.
27. Trenkner, E., and Hoffmann, M. K. (1986) Defective development of the thymus and immunological abnormalities in the neurological mouse mutation staggerer. *J. Neurosci.* **6,** 1733–1737.
28. Mamontova, A., Seguret-Mace, S., Esposito, B., Chaniale, C., Bouly, M., Delhaye-Bouchaud, N., Luc, G., Staels, B., Duverger, N., Mariani, J., and Tedgui, A. (1998) Severe atherosclerosis and hypoalphalipoproteinemia in the staggerer mouse, a mutant of the nuclear receptor RORalpha. *Circulation* **98,** 2738–2743.
29. Steinhilber, D., Brungs, M., Werz, O., Wiesenberg, C. D., Kahlen, J-P., Nayer, S., Schräder, M., and Carlberg, C. (1995) The nuclear receptor for melatonin represses 5-lipooxygenase gene expression in human B lymphocytes. *J. Biol. Chem.* **270,** 7037–7040.
30. Schraeder, M., Danielsson, C., Wiesenberg, I., and Carlberg, C. (1996) Identification of natural monomeric response elements of the nuclear receptor RZR/ROR: Interference with COUP-TF. *J. Biol. Chem.* **271,** 19732–19736.
31. Chu, K., and Zing, H. H. (1999) Activation of the mouse oxytocin promoter by the orphan receptor RORalpha. *J. Mol. Endocrinol.* **23,** 337–346.
32. Vu-Dac, N., Gervois, P., Grötzinger, T., De Vois, P., Schoonjans, K., Fruchart, J-C., Auwerx, J., Mariani, J., Tedgui, A., and Staels, B. (1997) Transcriptional regulation of apolipoprotein A-I gene expression by the nuclear receptor RORα. *J. Biol. Chem.* **272,** 22401–22404.
33. Raspé, E., Duez, H., Gervois, P., Fievet, C., Fruchart, J. C., Besnard, S., Mariani, J., Tedgui, A., and Staels, B. (2001) Transcriptional regulation of apolipoprotein C-III gene expression by the orphan nuclear receptor RORalpha. *J. Biol. Chem.* **276**(4), 2865–2871.
34. Delerive, P., Monte, D., Dubois, G., Trottein, F., Fruchart-Najib, J., Mariani, J., Fruchart, J. C., and Staels, B. (2001) The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. *EMBO* **2**(1), 42–48.
35. Austin, S., Medveddev, A., Yan, Z-H., Adachi, H., Hirose, T., and Jetten, A. M. (1998) Induction of the nuclear orphan receptor RORγ during adipocyte differentiation of D1 and 3T3-L1 cells. *Cell Growth Differ.* **9,** 267–276.
36. Meyer, T., Kneissel, M., Mariani, J., and Fournier, B. (2000) In vitro and in vivo evidence for orphan nuclear receptor RORalpha function in bone metabolism. *Proc. Natl. Acad. Sci. USA* **97**(16), 9197–9202.
37. Missbach, M., Jagher, J., Sigg, I., Nayeri, S., Carlberg, C., and Wiesenberg, I. (1996) Thiazolidine Diones specific ligands of the nuclear receptor retinoid Z receptor/retinoid acid receptor-related orphan receptor α with potent antiarthritic activity. *J. Biol. Chem.* **271,** 13515–13522.
38. Saladin, R., De Vos, P., Guerre-Millo, M., Leturque, A., Girard, J., Staels, B., and Auwerx, J. (1995) Transient increase in obese gene expression after food intake or insulin administration. *Nature* **377,** 527–529.
39. Fried, M. G., and Crothers, D. M. (1983) CAP and RNA polymerase interactions with the lac promoter: Binding stoichiometry and long range effects. *Nucleic Acids Res.* **11,** 141–158.
40. Schoonjans, K., Watanabe, M., Suzuki, H., Mahfoudi, A., Krey, G., Wahli, W., Grimaldi, P., Staels, B., Yamamoto, T., and Auwerx, J. (1995) Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator-response element in the C promoter. *J. Biol. Chem.* **270,** 19269–19276.
41. Harding, H. P., Atkins, G. B., Jaffe, A. B., Seo, W. J., and Lazar, M. A. (1997) Transcriptional activation and repression by RORalpha, an orphan nuclear receptor required for cerebellar development. *Mol. Endocrinol.* **11**(11), 1737–1746.
42. Atkins, G. B., Hu, X., Guenther, M. G., Rachez, C., Freedman, L. P., and Lazar, M. A. (1999) Coactivators for the orphan nuclear receptor RORalpha. *Mol. Endocrinol.* **13**(9), 1550–1557.
43. Wiesenberg, I., Missbach, M., Kahlen, J-P., Schräder, M., and Carlberg, C. (1995) Transcriptional activation of the nuclear receptor RZRα by the pineal gland hormone melatonin and identification of CGP 52608 as a synthetic ligand. *Nuc. Acids Res.* **23,** 327–333.
44. Kane, C. D., and Means, A. R. (2000) Activation of orphan receptor-mediated transcription by Ca(2+)/calmodulin-dependent protein kinase IV. *EMBO J.* **19**(4), 691–701.
45. Blask, D. E., and Hill, S. M. (1986) Effects of melatonin on cancer: Studies on MCF-7 human breast cancer cells in culture. *J. Neural. Transm.* Suppl **21,** 433–449.
46. Hill, S. M., and Blask, D. E. (1988) Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. *Cancer Res.* **48**(21), 6121–6126.
47. Karasek, M., and Pawlikowski, M. (1999) Antiproliferative effects of melatonin and CGP 52608. *Biol. Signals Recept.* **8**(1–2), 75–78.
48. Winrow, C. J., Capone, J. P., and Rachubinski, R. A. (1998) Cross-talk between orphan nuclear hormone receptor RZRalpha and peroxisome proliferator-activated receptor alpha in regulation of the peroxisomal hydratase-dehydrogenase proliferator-activated receptor alpha in regulation of the peroxisomal hydratase-dehydrogenase gene. *J. Biol. Chem.* **273,** 31442–31448.

49. Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S., and Spiegelman, B. M. (1998) Terminal differentiation of human breast cancer through PPAR gamma. *Mol. Cell.* **1,** 465–470.

50. Elstner, E., Muller, C., Koshizuka, K., Williamson, E. A., Park, D., Asou, H., Shintaku, P., Said, J. W., Heber, D., and Koeffler, H. P. (1998) Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proc. Natl. Acad. Sci. USA* **95**(15), 8806–8811.

51. Brockman, J. A., Gupta, R. A., and Dubois, R. N. (1998) Activation of PPARgamma leads to inhibition of anchorage-independent growth of human colorectal cancer cells. *Gastroenterology* **115**(5), 1283–1285.