Vol. 192, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1993 Pages 37-45

PPAR-RXR HETERODIMER ACTIVATES A PEROXISOME PROLIFERATOR RESPONSE ELEMENT UPSTREAM OF THE BIFUNCTIONAL ENZYME GENE

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Received February 19, 1993

A DNA sequence that confers a response to a class of rodent hepatocarcinogens termed peroxisome proliferators has been identified 2947bp upstream of the rat peroxisomal bifunctional enzyme gene. Two members of the steroid hormone receptor family, termed the peroxisome proliferator activated receptor (PPARα) and the retinoid X receptor (RXRα), co-operate to bind specifically to this sequence. Importantly, this response element (PPRE) is similar to that identified upstream of other peroxisome proliferator responsive genes such as those encoding acyl CoA oxidase and cytochrome P450 IVA6. These data therefore provide further evidence that PPARα plays an important role in mediating the action of peroxisome proliferators.

Several closely related members of the steroid hormone receptor superfamily have been identified that are activated by the peroxisome proliferator class of rodent hepatocarcinogens and are therefore termed peroxisome proliferator activated receptors (PPARα, β, γ, refs. 1-3 and hNucI, ref. 4). Peroxisome proliferators encompass a wide range of chemicals including hypolipidemic drugs, plasticisers and herbicides (5-8). In rats and mice, these chemicals regulate the transcription of several genes encoding enzymes important for lipid metabolism including acyl CoA oxidase and the bifunctional enzyme that are part of the peroxisomal fatty acid β-oxidation pathway (9,10). Intriguingly, a variety of fatty acids activate PPAR and it has therefore been

0006-291X/93 $4.00

suggested that the physiological role of PPAR may be to regulate lipid homeostasis (3,11,12). A peroxisome proliferator response element (PPRE) has been identified upstream of the acyl CoA oxidase gene (13,14). This PPRE comprises an almost perfect direct repeat of the sequence TGACCT spaced by 1 base pair and binds PPAR (14). It was therefore of interest to determine whether other peroxisome proliferator-responsive genes were also regulated by PPAR. We show here, that the 5' flanking region of the rat peroxisomal bifunctional enzyme gene also contains a PPRE that binds PPAR supporting the suggestion that PPAR mediates the pleiotypic response to peroxisome proliferators.

Materials and Methods

Plasmid Constructions

Upstream sequences (-1105/+20) of the bifunctional enzyme gene (15) were amplified from rat liver genomic DNA using the polymerase chain reaction (PCR). This DNA fragment was used to screen a rat liver genomic library (Stratagene) using standard hybridisation conditions. Clone 22, that extends 3.4Kb upstream from the transcription start site, was used as a template (100ng) in a PCR reaction. After 10 rounds of amplification a fragment containing 3.4Kb of the upstream sequence was made blunt ended with Klenow DNA polymerase and inserted into the unique SmaI site of the pGL2 luciferase vector (Promega) to create the reporter plasmid BFE(-3400/+20)Luc. Oligonucleotides containing an XhoI site were used to PCR amplify the -3040/+20 region and this was inserted into the unique XhoI site of pGL2 to create the reporter plasmid BFE(-3040/+20)Luc. pG.Luc was created from pGL2 by excision of the rabbit β-globin promoter from pG.CAT (1) using the restriction enzymes HindIII and BglII and inserted into a modified polylinker, containing unique HindIII and BglII sites, upstream of the luciferase gene of pGL2. The BFE(-3040/-2751)G.Luc and BFE(-3040/-2845)G.Luc reporter plasmids were created by PCR amplification of the bifunctional enzyme upstream sequence using oligonucleotide primers with a HindIII site (5') and a BamHI site (3') and the fragments inserted upstream of the globin promoter in pG.Luc. BFE(-2952/-2918)G.Luc was created by ligating synthetic oligonucleotides (Top strand; 5' AGCTTTCCTTTGACCTATTGAACTATTACCTACATTTGAGG 3') between the HindIII and BamHI sites of pG.Luc.

Transfection Assays

Hepa1c1c7 cells were seeded into 9cm petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM glutamine and 0.1mg/ml gentamycin. When the cells were 80% confluent they were washed in serum-free medium and transfected by mixing 10μg of plasmid DNA (Qiagen column purified) with 100μg of an equal mixture of N-[1-(2,3 dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine in 5ml serum-free and phenol red-free medium which was applied to the cells (16). Each plate was transfected with 1μg of luciferase reporter plasmid, 1μg of expression vector (pSG5-mPPARα, pSG5-mRXRα), 3μg of the β-galactosidase internal control plasmid pCH110 (Pharmacia) and made up to 10μg with pBluescript (Stratagene) as carrier. After 5hr, 5ml phenol red-free DMEM containing 20% dextran coated charcoal treated

FCS was added together with either vehicle alone (0.1% DMSO) or an activator e.g. Wy-14,643 (Chemsyn science laboratories, Lenexa, Kansas) or 9-*cis* retinoic acid (Hoffmann-La Roche) prepared as 1000X stocks in DMSO. After 20hr, the medium and activators were replenished and after a total of 48hr the cells harvested in 500μl of luciferase cell lysis reagent (Promega). The cleared cytosol extract (50μl) was added to 50μl of luciferase assay reagent (Promega) and the emission of light measured in an automated Berthold plate luminometer. β-galactosidase assays were performed by incubating 100μl of cytosol extract in a final volume of 1ml containing 0.28mg ONPG in 50mM phosphate buffer pH7.0, 10mM KCl, 1mM $MgCl_2$ for 1hr at 37°C. The reaction was stopped by the addition of 400μl of 1M Na_2CO_3 and the absorbance measured at 420nM. The luminescence values were normalised using the β-galactosidase readings.

DNA Binding Assay

Oligonucleotides containing the sequences upstream of the bifunctional enzyme between -2952 and -2918 were annealed and labelled with ^{32}P using Klenow DNA polymerase and [^{32}P] dCTP. Receptor proteins were synthesised from the pSG5 expression vectors using the TNT rabbit reticulocyte *in vitro* translation system (Promega). A typical DNA binding assay contained approx. 75 fmoles of PPARα and/or RXRα in 10mM Hepes pH7.9, 50mM KCl, 1mM DTT, 2.5mM $MgCl_2$, 5μg poly(dIdC) and 10% glycerol in 20μl final volume. After 15min on ice, radiolabelled oligonucleotide (45,000cpm, 25fmol) and, where appropriate, unlabelled competitor oligonucleotide were added and incubation continued at 20°C for 12min. Reactions were loaded onto a pre-run (30min) 5% polyacrylamide gel equilibrated in 0.5X TBE and electrophoresed at 25mA at 4°C for 1hr without recirculation. Finally, gels were soaked for 15min in 5% (v/v) glycerol, dried and autoradiographed. The sequence of the acyl CoA oxidase oligonucleotide used as a competitor is 5' GATCCTTCCCGAACGTGACCTTTGTCCTG-GTCCCCTTTTGCTC 3' and that of the unrelated competitor oligonucleotide 5' GAT-CTCCAGGCTCTTCTCACGCAACTCCGGG 3'.

Results

A rat genomic library was screened with a fragment (-1105/+20) containing sequences upstream of the rat bifunctional enzyme (BFE) gene. Nine positive clones were characterised by restriction enzyme digestion and compared with the sequence of the rat gene (15). Clone 22, that extended 3.4Kb upstream from the transcription start site, was taken for further analysis. The upstream sequence (-3400/+20) of the bifunctional enzyme gene contained in clone 22 was amplified by PCR and cloned upstream of the luciferase gene to create the reporter plasmid BFE(-3400/+20)Luc. This reporter was transfected into the mouse liver cell line, Hepa1, in the absence or presence of the mouse PPARα expression vector, pSG5-mPPARα, and the peroxisome proliferator Wy-14,643. A small (1.3 fold) induction in luciferase activity was observed in the presence of PPARα and Wy-14,643 indicating the presence of a weak PPRE in the 5' regulatory sequence of the bifunctional enzyme gene (Fig.1). Deletion of 360bp from

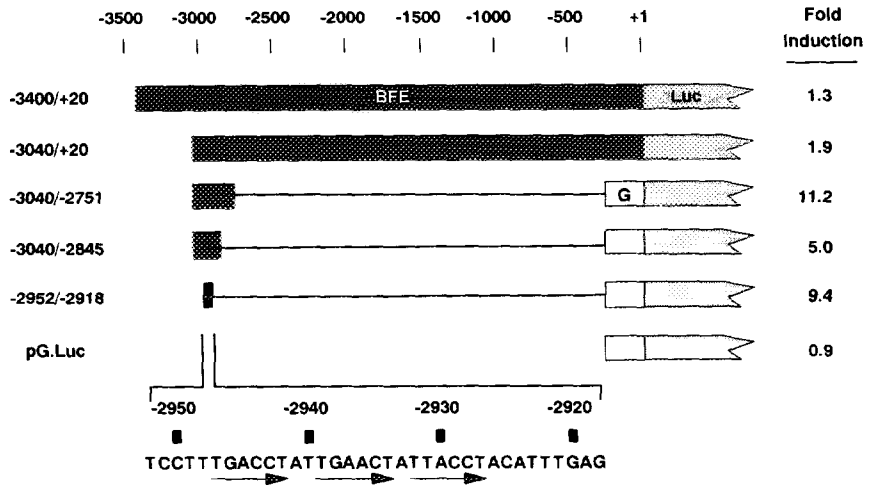

Fig.1. Localisation of a PPRE in the upstream sequence of the bifunctional enzyme gene. Reporter plasmids were constructed with the upstream sequence of the bifunctional enzyme gene inserted either upstream of the rabbit β-globin promoter (G) or directly upstream of the luciferase coding sequence (Luc). The values on the left indicate the region of the bifunctional enzyme upstream sequence used in each construct. The sequence (-2952/-2918) containing the bifunctional enzyme PPRE is shown with the TGACCT-like motifs underlined. Reporter plasmids (1μg) were transfected into Hepa1 cells in the absence or presence of pSG5-mPPARα (1μg) and Wy-14,643 (10^{-5}M). The values on the right indicate the fold stimulation in luciferase activity comparing transfections in the absence and presence of PPARα and Wy-14,643. Each experiment was performed in triplicate.

the 5' end of the bifunctional enzyme upstream sequence, to give the reporter plasmid BFE(-3040/+20)Luc, increased the response to 1.9 fold (Fig.1). Sequences were also removed from the 3' end of the bifunctional enzyme fragment and the remainder of the fragment placed upstream of the rabbit β-globin (G) gene to create the reporter plasmids BFE(-3040/-2751)G.Luc and BFE(-3040/-2845)G.Luc. The induction of luciferase activity in the presence of PPARα and Wy-14,643 was much greater with these reporters being 11.2 fold and 5.0 fold respectively (Fig.1). The greater stimulation of these reporter plasmids could be due to several factors including the closer proximity of the PPRE to the transcription start site, the use of the β-globin rather than the bifunctional enzyme transcription initiation sequences or the removal of putative inhibitory sequences between -2751 and +20. Because the region between -3040 and -2845 contains a sequence that is very similar to the acyl CoA oxidase PPRE (14) an oligonucleotide containing this putative PPRE was inserted upstream of the β-globin promoter to create the reporter plasmid BFE(-2952/2918)G.Luc. Transfection

experiments indicated that luciferase activity was induced 9.4 fold in the presence of PPARα and Wy-14,643 whereas no effect was seen using the parent vector pG.Luc that lacks the bifunctional enzyme sequences (Fig. 1 and Fig.2, compare lanes 7 and 8). Furthermore, induction was dependent upon the presence of PPARα since no induction was seen with Wy-14,643 alone (Fig.2, lane 4). Note that a small induction was observed with PPARα in the absence of Wy-14,643 (Fig.2, lane 6). This is similar to the results obtained using a reporter plasmid containing the acyl CoA oxidase PPRE (14) and may be due to the presence of endogenous ligands such as fatty acids (3-12). Together, these data demonstrate that the -2952/-2918 region of the bifunctional enzyme upstream sequence contains a functional PPRE.

We next examined whether PPARα binds to the bifunctional enzyme PPRE. Mouse PPARα and RXRα were synthesised *in vitro*, incubated with the oligonucleotide containing the bifunctional enzyme PPRE and the protein-DNA complexes resolved on a polyacrylamide gel. PPARα and RXRα alone were unable to bind to the PPRE (Fig.3,

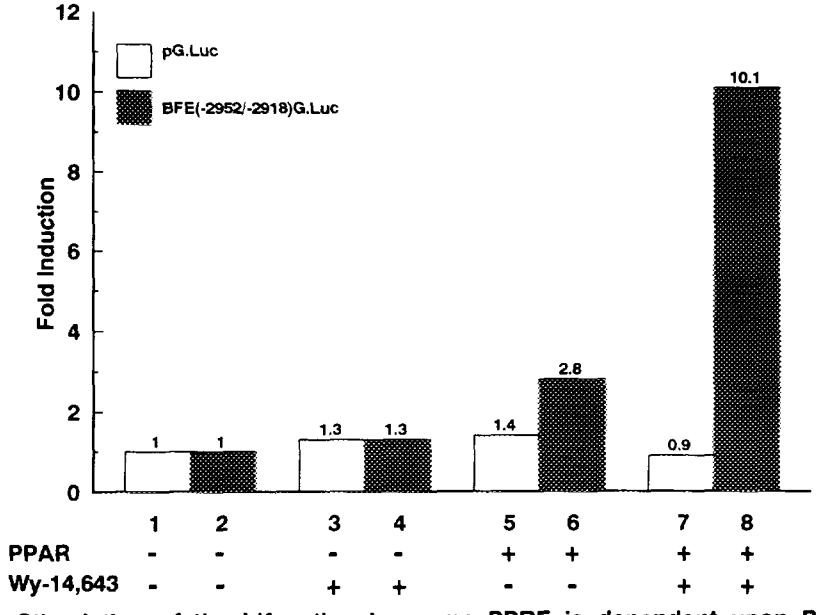

Fig.2. Stimulation of the bifunctional enzyme PPRE is dependent upon PPAR. Hepa1 cells were co-transfected with either the BFE(-2952/-2918)G.Luc reporter plasmid (1μg) or the parent vector pG.Luc (1μg) with either pSG5 (1μg, lanes 1-4) or pSG5-mPPARα (1μg, lanes 5-8) in the absence (lanes 1,2,5,6) or presence (lanes 3,4,7,8) of Wy-14,643 (10^{-5}M). The fold stimulation of luciferase activity, comparing transfections in the absence and presence of PPARα and Wy-14,643, is shown. Each experiment was performed in triplicate.

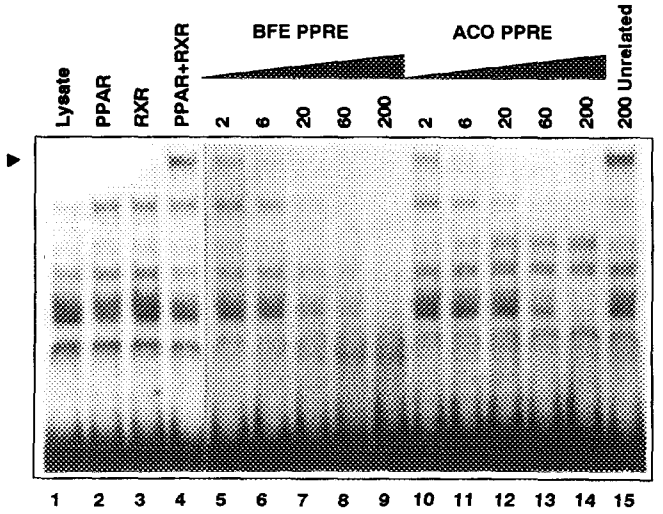

Fig.3. PPARα and RXRα bind co-operatively to the bifunctional enzyme PPRE. *In vitro* translated PPARα and RXRα (approximately 75 fmoles) were incubated with the labelled oligonucleotide (45,000 cpm, 25 fmoles) representing the -2952/-2918 region of the bifunctional enzyme upstream sequence and the protein-DNA complexes resolved by polyacrylamide gel electrophoresis. The arrowhead indicates the specific PPAR-RXR-PPRE ternary complex. Competitions were performed using an increasing ratio of unlabelled double-stranded oligonucleotide representing either the bifunctional enzyme PPRE (-2952/-2918) in lanes 5-9, the acyl CoA oxidase PPRE (-580/-543) in lanes 10-14, or an unrelated sequence that does not contain TGACCT motifs in lane 15.

lanes 2 and 3), however combining both PPARα and RXRα enhanced DNA binding dramatically (lane 4) indicating that RXRα is essential for PPARα to bind to this response element and suggesting that RXRα and PPARα bind as a heterodimer. Interestingly, the addition of the RXR ligand, 9-*cis* retinoic acid, or the putative PPARα ligand, Wy-14,643, had no effect upon the PPAR-RXR heterodimer binding to DNA indicating that ligands are not required for DNA binding (data not shown). To demonstrate that the DNA binding was specific, competition experiments were performed using oligonucleotides containing either the bifunctional enzyme or acyl CoA oxidase PPREs or an unrelated DNA sequence. Competition for PPAR-RXR binding to the bifunctional enzyme PPRE was only observed when using the bifunctional enzyme PPRE (lanes 5-9) or acyl CoA oxidase PPRE (lanes 10-14) and not with the unrelated sequence (lane 15). Furthermore, the bifunctional enzyme PPRE appeared to be a more efficient competitor than the acyl CoA oxidase PPRE suggesting that the affinity of the PPAR-RXR heterodimer was approximately three times greater for the bifunctional enzyme PPRE (compare lanes 7 and 13).

Since RXRα was required for PPARα to bind to DNA it was of interest to determine whether 9-*cis* retinoic acid binding to RXRα contributes to stimulate the BFE(-2952/-2918)G.Luc reporter plasmid. Co-transfection of the reporter with PPARα alone produced a 2.6 fold increase in luciferase activity (Fig.4, lane 5 and see also Fig.2, lane 6) that is most probably due to the presence of endogenous PPAR ligands. The addition of Wy-14,643 produced a 7.8 fold increase (lane 6) that was unaffected by the further addition of 9-*cis* retinoic acid (lane 8). Interestingly, a 4.5 fold stimulation was observed in the presence of 9-*cis* retinoic acid (lane 7) that is probably due to endogenous RXR interacting with the transfected PPARα (see lane 3). As anticipated, Wy-14,643 had no effect when the reporter was co-transfected with RXRα (lane 10) whereas 9-*cis* retinoic acid produced a 4.4 fold stimulation (lane 11) that was unaffected by the addition of Wy-14,643 (lane 12). When the reporter was co-transfected with both PPARα and RXRα, the response to Wy-14,643 was slightly reduced to 6.2 fold (lane 14) and that to 9-*cis* retinoic acid increased to 6.8 fold (lane 15). Importantly, however, the

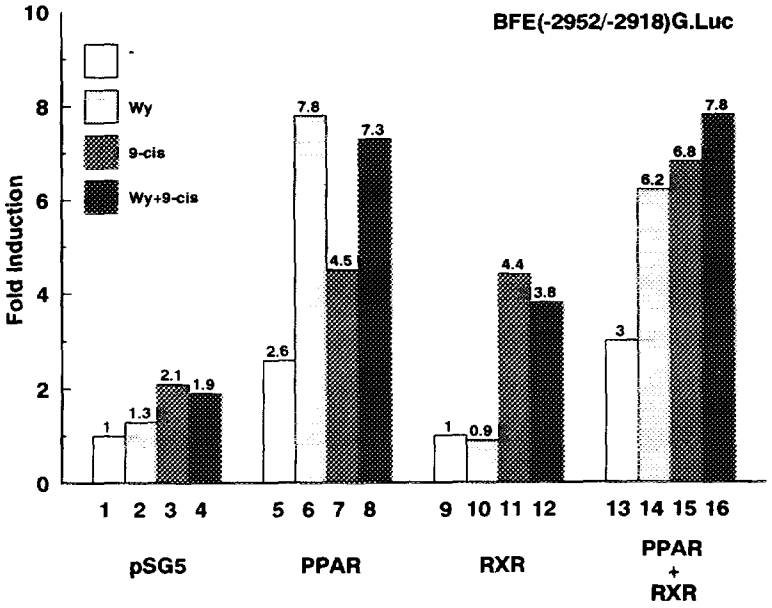

Fig.4. 9-*cis* retinoic acid does not enhance Wy-14,643 stimulation of the bifunctional enzyme PPRE. The BFE(-2952/-2918)G.Luc reporter plasmid (1μg) was transfected into Hepa1 cells with pSG5 (2μg, lanes 1-4), pSG5 (1μg) and pSG5-mPPARα (1μg, lanes 5-8), pSG5 (1μg) and pSG5-mRXRα (1μg, lanes 9-12) or both receptor expression vectors (1μg each, lanes 13-16) in either the absence or presence of Wy-14,643 (10^{-5}M) and/or 9-*cis* retinoic acid (10^{-6}M). The average fold induction of triplicate results is shown.

response to both activators was the same as observed with PPAR and Wy-14,643 alone (compare lanes 6 and 16).

Discussion

Our results confirm and extend recent studies (17) demonstrating that the upstream region of the rat peroxisomal bifunctional enzyme contains a sequence (PPRE) that confers responsiveness to peroxisome proliferators. We show here that this sequence contains three TGACCT related motifs that are very similar to the PPRE identified in the upstream region of the rat peroxisomal acyl CoA oxidase gene (14). Importantly we show that the response to the peroxisome proliferator Wy-14,643 is dependent upon the presence of PPARα and that PPARα binds specifically to the bifunctional enzyme PPRE. Recent findings indicate that PPARα binds to the acyl CoA oxidase PPRE as a heterodimer with RXRα (12, 18) and we show here that this is also true for the bifunctional enzyme PPRE. However, unlike the results obtained with the acyl CoA oxidase upstream regulatory sequences, 9-*cis* retinoic acid had no additional transcriptional effect compared with Wy-14,643 alone (Fig.4) suggesting that RXRα is required for DNA binding but may not contribute to activate transcription. Note that similar results were obtained when using a BFE(-3400/+20)CAT reporter plasmid (data not shown) suggesting that the absence of an additive or synergistic effect of 9-*cis* retinoic acid and Wy-14,643 is not due to the use of the β-globin promoter or the absence of other upstream sequences. It is intriguing that the bifunctional enzyme PPRE contains three TGACCT related repeats; a direct repeat spaced by 2 bp and a third direct repeat spaced by 1bp. Since the acyl CoA oxidase PPRE with its 1 base pair spacing between the direct repeat is optimal for PPAR-RXR to bind and activate gene transcription (12) it will be interesting to determine whether all three repeats in the bifunctional enzyme PPRE contribute to regulate gene transcription.

We have suggested that PPARα could mediate the pleiotypic response to peroxisome proliferators since PPARα is activated by a wide range of peroxisome proliferators and there is a good correlation between the ability of a chemical to activate PPARα and produce both peroxisome proliferation and liver cancer in rodents (1). The finding that PPARα binds to specific sequences upstream of several peroxisome proliferator responsive genes (14, 19), including the bifunctional enzyme gene, provides

strong support for this suggestion. Therefore, understanding more about the role and function of PPAR could help determine the carcinogenic and hypolipidemic mechanism of action of peroxisome proliferators.

Acknowledgments

We are especially grateful to P. Chambon for providing the mouse RXRα expression plasmid, A. Levin and J. Grippo (Hoffmann-La Roche) for the 9-*cis* retinoic acid, and J. Whitlock for the Hepa1 cells. We also thank the Biographics unit for help in preparing the figures, R. Moore for synthesising the DOTMA reagent and D. Holland for synthesising the oligonucleotides. OB was a recipient of a LAVOISIER fellowship from the French ministry of foreign affairs in 1992.

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