

The analysis of modified peroxisome proliferator responsive elements of the peroxisomal bifunctional enzyme in transfected HepG2 cells reveals two regulatory motifs

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Abstract Peroxisome proliferators (PPs) are non-genotoxic carcinogens in rodents. They can induce the expression of numerous genes via the heterodimerization of two members of the steroid hormone receptor superfamily, called the peroxisome proliferator-activated receptor (PPAR) and the 9-*cis* retinoic acid receptor (RXR). Many of the PP responsive genes possess a peroxisome proliferator response element (PPRE) formed by two TGACCT-related motifs. The bifunctional enzyme (HD) PPRE contains 3 such motifs, creating DR1 and DR2 sequences. PPAR and RXR regulate transcription via the DR1 element while DR2 modulates the expression of the gene via auxiliary factors in HepG2 cells.

Key words: Peroxisome proliferator-activated receptor; Peroxisome proliferator response element; 9-*cis* Retinoic acid receptor alpha; Rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; Transcriptional activation

1. Introduction

Peroxisomes are organelles involved in numerous important functions, including fatty acid β -oxidation. These reactions are essential for lipid homeostasis [1]. A group of compounds with diverse structures are able to provoke pleiotropic effects such as peroxisome proliferation and hepatocarcinogenesis in rodents: these compounds are termed peroxisome proliferators (PP). In rodents, PPs are able to regulate the transcription of several genes important for lipid metabolism, such as rat peroxisomal acyl-CoA oxidase (ACO) [2], rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) [3,4] and rabbit microsomal cytochrome *P*-450 4A6 (CYP4A6) [5]. They do this via a nuclear receptor termed the peroxisome proliferator activated receptor (PPAR), a member of the steroid hormone receptor superfamily [6]. These genes all contain a peroxisome proliferator response element (PPRE) which con-

sists of direct repeats of the sequence TGACCT, the consensus binding sequence for several members of the nuclear steroid hormone receptor superfamily [7]. PPAR binds to these PPREs through heterodimerization with the 9-*cis* retinoic acid receptor RXR α [4,8]. Transcriptional activation is regulated by diverse auxiliary transcription factors that can act as activators or repressors. For instance, the chicken ovalbumin upstream promoter-transcription factor (COUP-TF) can bind to the HD-PPRE and antagonizes peroxisome proliferators activation [9]. In fact, different studies on hormone response elements have shown that many known and unknown factors are able to bind promiscuous sequences [10–12]. Here we show that the PPAR-RXR heterodimer activates transcription via the DR1 part of the HD-PPRE and that other transcription factors modulate the level of induction via the most 5'-receptor motif in DR2.

2. Materials and methods

2.1. Plasmid construction

pG.Luc is a luciferase expression vector containing the rabbit β -globin promoter [4]. Mutations of the HD-PPRE were derived from the synthetic oligonucleotides used to create the HD-PPRE.G.Luc [4]. The TGACCT-like motifs were replaced by the unique sequence ATCTGG giving three different oligonucleotides called AB, AC, BC in reference to the three TGACCT-like motifs A,B,C, of the HD-PPRE (Fig. 1): AB top strand, 5'-AGCTTTCCTTTGACCTATTGAACAAATCTGGACATTGAG-3'; AC top strand, 5'-AGCTTTCCTTTGACCTATTCTGGATTACCTACATTGAG-3'; BC top strand, 5'-AGCTTTCCTTATCTGGATTGAACATTACCTACATTGAG-3'. Each double-stranded oligonucleotide was inserted between the *Hind*III and *Bam*HI sites of pG.Luc.

2.2. DNA-binding assays

The different oligonucleotides (AB, AC, BC or HD-PPRE) were annealed and labelled with 32 P using Klenow DNA polymerase and [32 P]dCTP. Receptor proteins were synthesised from pSG5 expression vectors using the TNT rabbit reticulocytes in vitro transcription system (Promega). A typical DNA-binding assay [4] contained approximately 75 fmol of mPPAR α and/or mRXR α in 10 mM HEPES pH 7.9, 50 mM KCl, 1 mM DTT, 2.5 mM MgCl₂, 2 μ g poly(dI·dC/dI·dC) (Pharmacia) and 10% glycerol in 10 μ l final volume. After 15 min on ice, radiolabelled oligonucleotides (30000 cpm, 25 fmol) and, when appropriate, unlabelled competitor oligonucleotides were added and incubation continued at room temperature for 10 min. Reactions were loaded onto a prerun (30 min) 5% polyacrylamide slab gel equilibrated in 0.5 \times TBE and electrophoresed at 10 mA at 4°C for 1 h without buffer recirculation. Finally, gels were soaked for 15 min in 5% (v/v) glycerol, dried and autoradiographed. The sequence of the unrelated competitor oligonucleotide is 5'-GATCTCCCAGGCTCTTCTACGGAAGTCCGGG-3'.

2.3. Methylation interference assays

A partially methylated probe was prepared by treating 0.5 pmol (2×10^6 cpm) [γ - 32 P]ATP end-labelled double stranded oligonucleotide with 2% (v/v) dimethylsulphate for 5 min at 25°C in 50 mM Na Ca-

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Abbreviations: ACO, rat peroxisomal acyl-CoA oxidase; ARP-1, ApoA1 regulatory protein; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; CRBP II, cellular retinol binding protein II; DR1, direct repeats elements with 1-base spacing; DR2, direct repeats elements with 2-base spacing; HD, rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; HNF4, hepatocyte nuclear factor 4; Luc, luciferase; PP, peroxisome proliferator; PPAR α , peroxisome proliferator-activated receptor alpha; PPRE, peroxisome proliferator response element; RXR α , 9-*cis* retinoic acid receptor alpha.

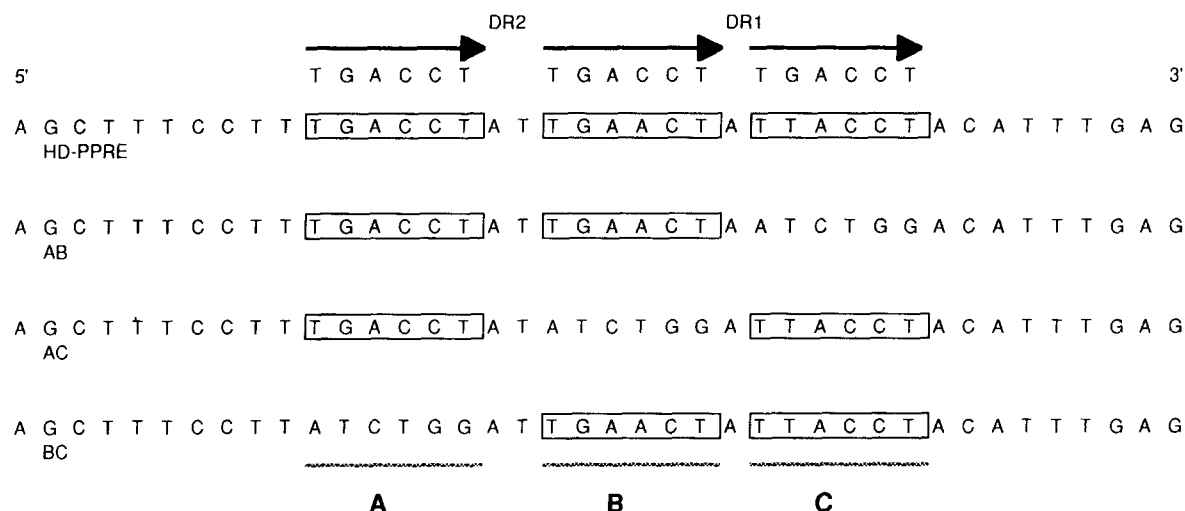
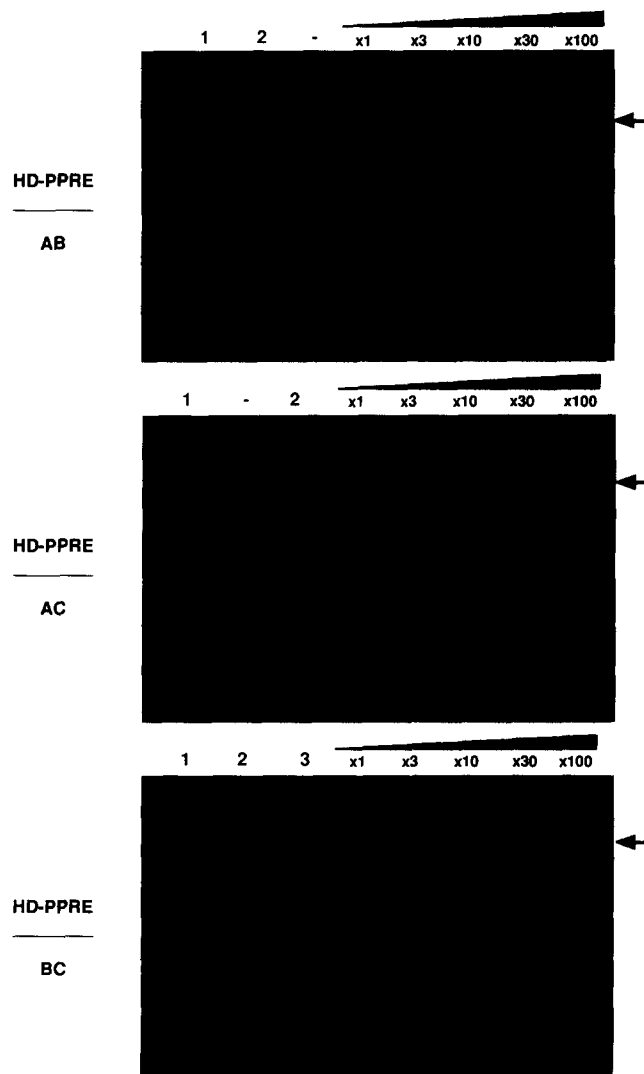


Fig. 1. Sequences of the modified oligonucleotides used in this study. AB, AC and BC are the names of the modified oligonucleotides. DR, direct repeat.



codylate pH 8.0, 1 mM EDTA. This probe was used in a preparative DNA-binding assay (scaled up 5×), with in vitro translated proteins (mPPAR α and mRXR α), both 'protein-bound' and 'free' oligonucleotides were isolated by polyacrylamide gel electrophoresis and electroelution. Oligonucleotides were then cleaved by 1 M piperidine (90°C, 30 min) and the resulting fragments separated on a 15% denaturing polyacrylamide gel (2000 Cerenkov cpm per lane). The gel was autoradiographed without drying. A Maxam and Gilbert G + A chemical sequencing reaction was performed in parallel [13].

2.4. Transfection assays

HepG2 cells (a human hepatoblastoma cell line) were seeded in 6-well dishes at 3×10^5 cells per well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 0.1 mg/ml gentamycin. At 80% confluence, the cells were washed in serum free medium (DMEM without Phenol red) and transfected by mixing 5 μ g of plasmid DNA (Qiagen column purified) with 10 μ g of Lipofectamine (Gibco-BRL) in 1 ml of serum-free and Phenol red-free medium which was applied to the cells [14]. Each well was transfected with 1 μ g of luciferase reporter plasmid, 1 μ g of expression vector (pSG5 or pSG5-mPPAR α) and 3 μ g of the β -galactosidase internal control plasmid pCH110 (Pharmacia). After 5 h, 1 ml of Phenol red-free medium containing 20% dextran coated charcoal treated FCS was added together with either vehicle alone (0.1% DMSO) or the PP Wy-14,643 (Wyeth-Ayerst, Princeton, New Jersey, USA) prepared as 1000× stocks in DMSO and used at 10^{-5} M. After 24 hours, the cells were harvested in 300 μ l of Reporter lysis buffer (Promega) and centrifuged after one freeze-thaw cycle. The cleared cytosol extract (10 μ l) was added to 50 μ l of Luciferase assay reagent (Promega) and the light emission measured for 10 seconds in a TLX1 luminometer (Dynatech). β -Galactosidase assays were performed using the Galacto-Light kit (Tropix, Bedford, MA, USA) as described by the supplier. The luciferase values were normalized using the β -galactosidase values.

3. Results

Each of the modified oligonucleotides (AB, AC, BC) were tested in DNA-binding competition assays against the original

Fig. 2. Gel-shift competition between labelled PPRE and unlabelled oligonucleotides AB, AC and BC. (1) Unprogrammed reticulocyte lysate; (2) in vitro translated PPAR and RXR; (3) competition with 200-fold excess of unrelated oligonucleotide; $\times 1$, $\times 3$, $\times 10$, $\times 30$ and $\times 100$, amount of unlabelled oligonucleotide added in the DNA-binding mix. The arrow indicates the position of the PPAR/RXR heterodimer.

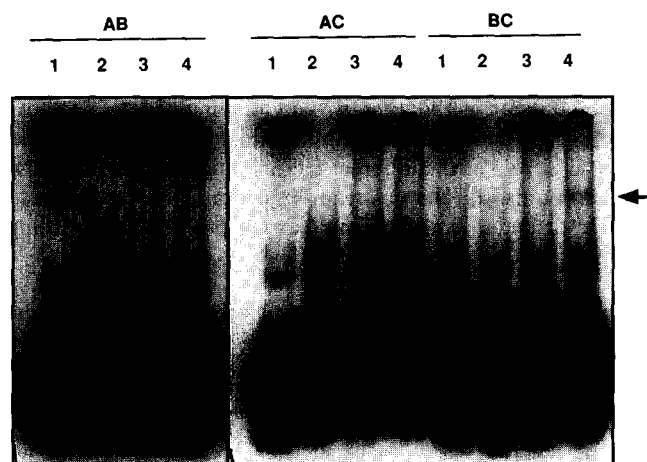


Fig. 3. Gel-shift with labelled oligonucleotides AB, AC and BC. (1) Unprogrammed reticulocyte lysate; (2) in vitro translated PPAR; (3) in vitro translated RXR; (4) in vitro translated PPAR and RXR. The arrow indicates the position of the PPAR/RXR heterodimer.

HD-PPRE sequence (Fig. 2). mPPAR α and mRXR α were synthesized in vitro and incubated with a mixture of radiolabelled HD-PPRE and an increasing amount of each mutated oligonucleotide. The PPAR/RXR heterodimer is visible in lane 2 of Fig. 2. The AB oligonucleotide was unable to decrease the

binding at 100-fold molar excess, whereas the AC oligonucleotide competed at a 30-fold molar excess, and the BC oligonucleotide competed for binding at 3-fold molar excess. The mutated oligonucleotides were then labelled and tested for DNA-binding activity with PPAR, RXR or both (Fig. 3). As expected, AB was unable to support binding, AC demonstrated weak binding, and BC bound to the PPAR-RXR heterodimer with high affinity. These results suggest that the presence of C is necessary for binding activity.

Nucleotides important for binding were identified by methylation interference. Using the AC oligonucleotide, based on 2 different experiments, guanosine residues at -2946 and -2934 were found to interact with the PPAR-RXR complex. Using the BC oligonucleotide, methylated guanosines at positions -2925, -2928, -2929 and -2935 prevented PPAR and RXR from binding, and methylated adenosine at position -2937 (Fig. 4).

Transfections activity of each sequence were carried out with the expression vector carrying mPPAR α , and the PP Wy-14,643 (Fig. 5). AB, having no DNA-binding ability, was unable to affect luciferase activity, as was AC. A striking effect was observed for BC, with a 70-fold induction over HD-PPRE.G.Luc or ACO(-570/-471).G.Luc activities, due to both a large increase in luciferase activity and a decrease of the basal expression of the reporter plasmid (excluding mPPAR α and Wy-14,643).

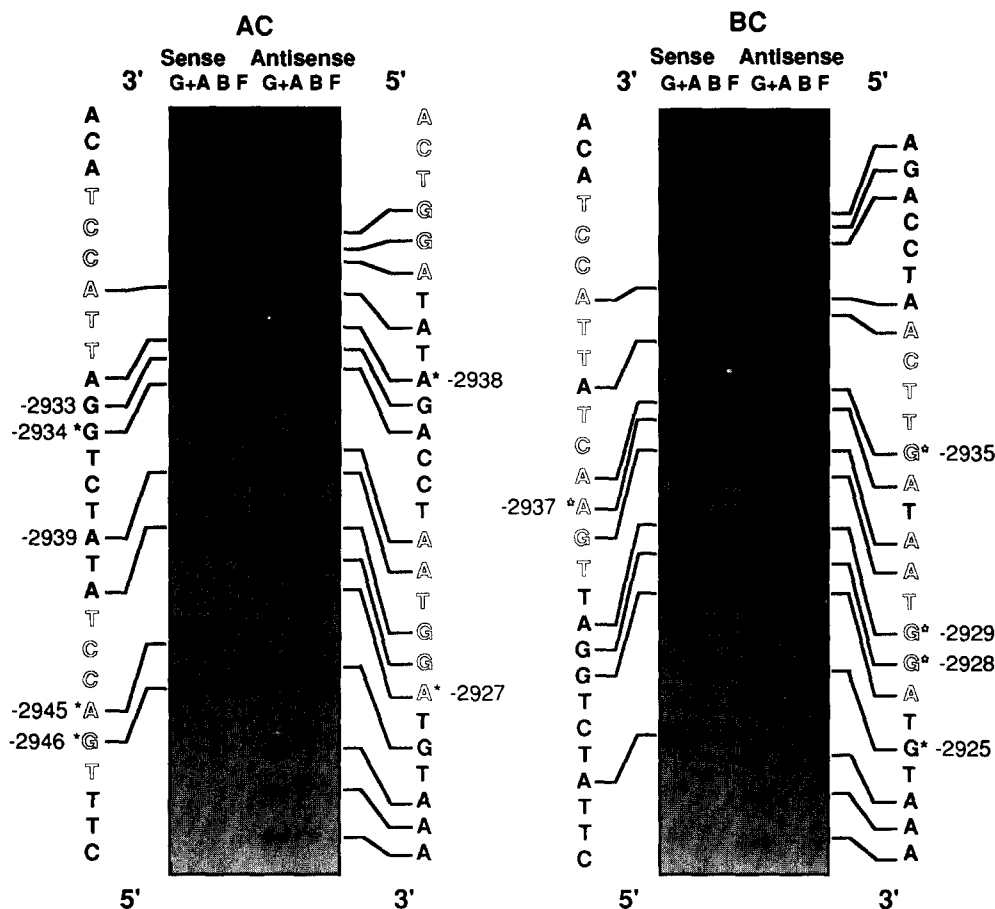


Fig. 4. Methylation interference of modified HD-PPRE: AC and BC. Sense and antisense sequences are indicated on each side. TGACCT-motifs are outlined. G + A, Maxam G + A sequencing; F, free oligonucleotides; B, bound oligonucleotides.

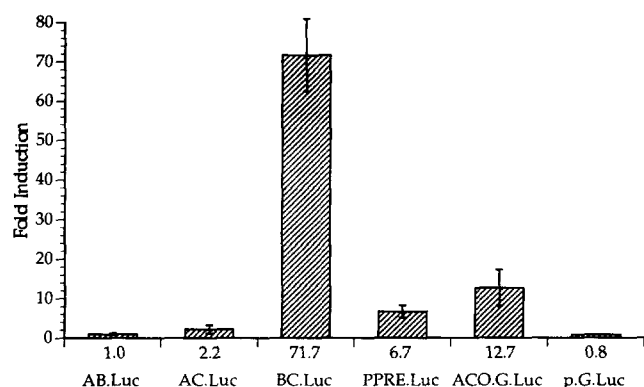


Fig. 5. Induction obtained with the different modified oligonucleotides in transfected HepG2 cells. The values indicated on x-axis are the fold induction of peroxisome proliferator (Wy-14,643) treated plates compared to the control plates (mean of three independent transfections; errors bars: standard deviation).

4. Discussion

These results support and extend recent studies by ourselves [4] and others [9,15], showing the importance of the TGACCT related motifs. Moreover, we show the presence of both an activating (DR1) and modulating (DR2) sequence in the same response element as an interesting feature of the transcriptional regulation of the HD gene. This phenomena is also observed in the regulation of the hApoA1 gene [16] and the mCRBP11 gene [12]. Recent findings show that COUP-TF is able to bind to the 2 first motifs of this response element [9]. Our present work now demonstrated that both TGACCT-like motifs in DR1 are critical and that no activation is possible if either is replaced. It is interesting that mPPAR α and mRXR α can bind on the AC oligonucleotide with only 2 TGACCT motifs spaced by 9 bp. However, the nucleotide methylation of this sequence showed that the receptors bind by different nucleotides. The striking induction observed with the BC oligonucleotide is especially interesting. This result contrasts with other results obtained with an oligonucleotide lacking the TGACCT-like motif A [9]. In the experiment by Miyata et al. (1993) [9], this oligonucleotide was shown to bind DNA but was unable to induce transcription of luciferase in transfections of rat hepatoma H4IIEC3 cells. Miyata's group has shown that COUP-TF binds as a homodimer to the HD-PPRE and does not form heterodimers with rPPAR α , RXR α or HNF4. But in HepG2 cells, it has been shown that HNF4 is present at higher levels than COUP-TF1 (Ear3) and COUP-TF2 (ARP-1) [17]. HNF4 is constitutively expressed and activates several promoters, such as mCRBP11 [12] or hApoA1, [18] when COUP-TF1 or COUP-TF2 act as negative modulator of transcription. So, we assume that in HepG2 cells the increase of induction for the BC oligonucleotide may be due to the change of the recognition site in favor of an activatory effect (probably by HNF4). These

results on HD-PPRE are consistent with a complex HRE formed of multiple recognition sites which interact with different transcription factors. The DR1 is a PPRE and DR2, acts as a modulator of PPRE activity by binding of others factors (repressor or activator), that can lead to a differential response to PP. The role of the different factors interacting on the HD-PPRE but also on the upstream sequence of this gene has to be investigated to explain the peroxisome proliferator signalling pathway.

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