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DNA Binding Preferences of PPARα/RXRα Heterodimers

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The regulatory elements mediating the transcriptional effects of the Peroxisome Proliferator Activated Receptor (PPAR)/Retinoid X Receptor heterodimers consist of a direct repeat of a variant of the consensus hexamer AGGTCA with an interspacing of 1 basepair (DR1). A binding site selection was performed to investigate whether any further constraints for PPAR/RXR binding to DR1 elements exist and/or whether other high affinity binding sites for these heterodimers can be identified. One half of the recovered sequences contained two hexamers related to the consensus halfsite organised as DR1, DR2, PAL0 or as DR3, in diminishing order of frequency. The other binding sites consisted of three hexamer repeats with the number of interspacing bases varying between 0 and 7. An element with three consecutive hexamer sequences each spaced by 1 basepair was most efficient in mediating the effects of peroxisome proliferators. The results indicate that the upstream flanking sequence of a DR1 differentially influences the binding of PPARα/RXRα heterodimers and of RXRα homodimers.

Peroxisome proliferators are a diverse class of xenobiotics that induce a vast increase in the number of peroxisomes in rodent liver. This organel proliferation is accompanied by an induction of multiple peroxisomal, mitochondrial, microsomal and cytosolic enzymes (1). The enzyme induction was shown to be a transcriptional process mediated by heterodimers of the Peroxisome Proliferator Activated Receptor α (PPARα) and RXR, both members of the thyroid/retinoid subfamily of ligand dependent transcription factors (2). A dozen of peroxisome proliferator responsive genes have been characterized to date. In their promoters, repeats of AGGTCA, the common recognition site for all members of this receptor subfamily, were identified. In all these regulatory elements the hexamers were arranged as direct repeats with an interspacing of 1 basepair (DR1) (2). Exceptionally, a complex element present in the Medium Chain Acyl CoA Dehydrogenase promoter that consists of 4 hexamers not encompassing a DR1 was shown to confer responsiveness to peroxisome proliferators (3).

The strict recognition of DR1 elements by PPARα/RXRα heterodimers contrasts with the promiscuous binding of RAR/RXR heterodimers to DR1, DR2, DR5 and ER8 (everted repeat with 8 interspacing basepairs). In order to address the question whether other hexamer configurations can constitute recognition sites for PPARα/RXRα heterodimers, binding experiments have been conducted of PPARα/RXRα heterodimers with synthetic oligonucleotides containing direct repeats of the consensus hexamer AGGTCA with interspacings varying between 0 and 6. According to Green (4) and ourselves (unpublished observations) PPARα/RXRα bound with highest affinity to DR1 elements and to a lesser extent to DR0 and DR2 elements. However, other hexamer configurations such as inverted and everted repeats have not been considered for binding PPARα/RXRα heterodimers.

On the other hand, DR1 elements constitute binding sites not only for PPARα/RXRα but also for homodimers of RXR (5), HNF-4 (6) and COUP-TF (7) and for the heterodimers COUP-TF/RXR and RAR/RXR (5). The question arises whether any discrimination exists between these receptor pairs for recognition of the DR1 elements with regard to optimal hexamer sequences and/or their flanking bases. In previous work we determined the optimal binding sites for RXRα homodimers and found that RXRα preferentially binds DR1 elements in which the upstream hexamer is preceded by A or G, and in which the interspacing base is A, G or T but not C (8). We have now extended this study and examined whether similar or other requirements apply for binding of PPARα/RXRα to DR1 sequences.

MATERIALS AND METHODS

Oligonucleotides. All oligonucleotides have been used in previous studies (8). The randomized oligonucleotide contained the following

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Abbreviations: COUP-TF, chicken ovalbumin upstream promoter-transcription factor; DR, direct repeat; EMSA, electrophoretic mobility shift assay; ER, everted repeat; HNF, hepatocyte nuclear factor; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator responsive element; RXR, retinoic X receptor.

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sequence: CACGTTACTTCGGCGGATCCTGTCG**(A/C/G/T)**$_{25}$GAGGCTCTAGAAGTGCAAGTGCAGC

Production of recombinant proteins. All recombinant proteins used were of bacterial origin. The bacterial expression vectors encoding the mouse PPARα and human RXRα, were generously provided by D.D. Moore, Department of Molecular Biology, Boston, USA. The PPARα protein was N-terminally tagged with the 9E10 c-*myc* epitope and the RXRα was preceded by 6 histidines and the *Haemophilus influenza flu* epitope as described (9,10). The fusion proteins were generated in BL21(DE3) cells after induction with isopropylthiogalacto-pyranoside and released by sonication (9).

Binding reactions. The random oligonucleotide was rendered double-stranded by annealing with 300 ng of the reverse primer and filling-in with Klenow DNA polymerase in a 20 μl reaction containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 200 μM each of dATP, dCTP, dGTP and dTTP and 1 U of Klenow fragment for 3 h at room temperature. Binding reactions included 25 ng double-stranded DNA in EMSA buffer and *myc*PPARα (100 ng) and His*flu*RXRα (10 ng) respectively (receptor concentrations as estimated by silver staining and Western blot analysis in comparison with proteins of known concentrations) in a total volume of 20 μl for 15 min at room temperature. The *myc*PPARα and His*flu*RXRα proteins were preincubated for 15 min at room temperature.

Isolation of receptor-bound oligonucleotides. Anti-*myc* antibody-coated magnetic beads (20 μl, 10 mg of beads per ml) were added to the binding reaction and the mixture was gently agitated for 1 h at room temperature. Then 500 μl of PBS containing 0.1% BSA was added, and the bead/receptor/DNA complexes were recovered by holding a magnet against the side of the microfuge tube for 1 min before withdrawing the supernatant. Following two washes with the same solution, the beads were resuspended in 40 μl 1 $\times$ PCR buffer, boiled for 5 min in order to disrupt protein-DNA interactions, and briefly centrifuged. Anti-*myc* antibody-coated magnetic beads were prepared by incubating 1 mg of sheep anti-mouse-coated beads (Dynabeads M-280 Sheep anti-Mouse IgG, Dynal Inc., Skøyen, Norway) overnight at 4 °C with 10 μg monoclonal mouse anti-*myc* antibodies followed by four washes for 30 min at 4 °C with PBS containing 0.1% BSA.

Amplification of selected sites. A 10 μl portion of the bead supernatant was subjected to 15 PCR cycles in a reaction containing 1 $\times$ PCR buffer, 100 ng forward primer (primer 13), 100 ng reverse primer (primer 14), 250 μM each of dATP, dCTP, dGTP and dTTP, and 2.5 U AmpliTaq DNA polymerase. One fourth of the PCR was used in the next round of selection and amplification.

Analysis of selected sites. After six rounds the selected oligonucleotides were digested with *Bam*HI and *Xba*I, purified on a 2% agarose gel and ligated to an equally treated and dephosphorylated pUTKAT vector. Two hundred indiviual colonies were obtained and the nucleotide sequence of 31 inserts was determined using the dideoxy chain termination technique.

To monitor the enrichment of high affinity binding sites, the material obtained from each cycle was radioactively labeled by PCR using 10 μCi [α-^{32}P]dCTP (3000Ci/mmol), 50 μM each of dATP, dGTP and dTTP, 20 μM dCTP and used as probes. In other gel-shift experiments synthetic oligonucleotides were used as a probe after end labelling with [γ^{32}P]-ATP. The binding reactions and gelshift analysis were performed as previously described (11) using bacterially expressed, partially purified RXRα and *myc*PPARα.

Cotransfection experiments. Cotransfection experiments were performed in COS cells as described (8). Cells were treated with ciprofibrate (100 μM) or with 9-cis RA (1 μM).

RESULTS AND DISCUSSION

Isolation of PPARα/RXRα Binding Oligonucleotides

In order to select the oligonucleotides that bind PPARα/RXRα and not those that bind RXRα homodimers, an immunoaffinity method was used to isolate the bound oligonucleotides. Antibodies directed to the N terminal *myc* epitope tag of the PPARα were fixed on magnetic beads and used to recover the heterodimeric complexes formed. The enrichment of binding sites after each round of selection and amplification was tested in an electrophoretic mobility shift assay (EMSA). As shown in Fig 1, binding activity to PPARα/RXRα could be detected after 3 rounds of selection and amplification. Three additional rounds were performed to select for sites that bind with highest affinity. The recovered oligonucleotides were cloned in the pUTKAT reporter and individual sequences were determined (Table 1). The oligonucleotides selected with PPARα/RXRα heterodimers all contained two or three copies of the halfsite sequence AGGTCA. These were most often organised as DR1 elements, the established configuration of regulatory elements in peroxisome proliferator target genes. However, also DR2 sites, palindromic elements without interspacing (PAL0) and one DR3 configuration were recovered. In the elements with triple hexamer repeats, the half site sequences appeared to deviate more from the consensus AGGTCA. This distribution of hexamer configurations (DR1, DR2, PAL0) is very similar to the one we ob-

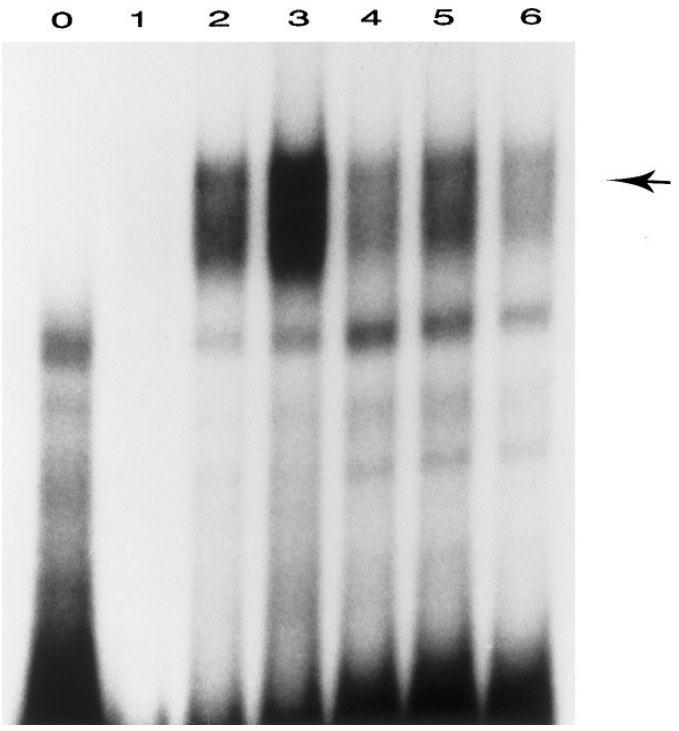

FIG. 1. Enrichment of binding sites for PPARα/RXRα heterodimers. The isolated oligonucleotides obtained after each selection round were radiolabeled in a 1 cycle PCR, incubated with bacterially expressed *myc*PPARα and RXRα and separated on a 5% non-denaturing polyacrylamide gel. The cycle numbers are indicated, 0 stands for the original randomer. The arrow indicates the retarded band.

TABLE 1

Sequences of Recovered PPREs

DR1	P11		CT	GGGTCA	T	AGGTGA	GG	
	P17		GT	CGGTCA	A	GGGTGA	GG	
	P20		CA	AGGTCA	G	GGGTCG	GT	
	P41		TG	GGGTCA	G	GGGTCA	TG	
	P5		AT	AGGTTA	G	AGGTCA	G	GGGTGA
	P32		CG	GGGTCG	G	GGGTTA	CATA	TGGTCG
	P18		TA	GGGTCA	G	GGGTGT	ATGTC	GGGTGA
	P24		TA	CGGCCA	C	AGGTTA	CTG	GGGTCA
	P16	AGGTCA	TCCTG	TGGTGA	T	CGGGCA	CG	
				NGGTCA	**N**	**GGGTNA**		
DR2	P8		GT	AGGTCG	GG	GGGTCG	AG	
	P14		AG	GGGTCA	TG	GGGTCG	AG	
	P25		AC	AGGTCA	TG	GGGTGA	GG	
	P44		TC	GGGTCA	TA	GGGTGT		AGGTTG
	P15		TC	GGGTAA	AG	GGGTCA	GT	AGGTCG
	P26		CA	AGGTGC	TG	GGGTGG		TGACCT
	P3		CA	GGGGCA	GC	AGGGCT	ACA	TGACCC
	P2	TGGTCA	CTGTC	GGGTTA	AG	GGGTGA	GG	
	P38	GGGTCA	CTCC	TGGTCA	AT	GGGTCA	TG	
				GGGTCA	**NN**	**GGGTCN**		
PAL0	P34		AG	GGGTTA		TAACCC	TG	
	P35		G	GGGTCA		TGACCT	G	
	P12		CA	GGGTCG		TGGCCT	G	GGGTCG
	P47		CG	AGGTCA		TGACCG	T	GGGTAT
	P22	GGGCAA	CATGA	AGGTCA		TGACCC	TA	
				GGGTCA		**TGACCN**		
DR3	P21		TC	AGGTTA	TTG	GGGTCA		
	P37		TC	GGGTGT	AGG	GGGTCA	N7	GGGTGA
DR0	P19	GGGTTA	N7	GGGTGG		AGGTCA		
	P27			GGGTCT		CGGTAA	N6	GGGTCG
Other	P33		G	GGGTCA	N13	GGGTGA		
	P23		C	GGGTGT	N12	GGGTCA		
	P31		GGG	TCACCT	N6	AGGTCA		
	P28	TGCCCA	G	AGGTCA	N4	GGGTCG		

After six cycles of binding, selection and amplification, the oligonucleotides were cloned in the pUTKAT vector and sequenced using the dideoxy chain termination technique. To facilitate the location of the hexamer sequences these were printed on grey background. Consensus sequences are indicated in bold.

tained after a RXRα homodimer binding site selection (8). No obvious sequence conservation was found upstream of the first hexamer or in between the two hexamers. To functionally test the recovered binding sites, EMSA and transactivation experiments were performed.

Functional Tests of Elements with Two Hexamer Repeats

In previous experiments, we and others showed that a DR1 functions optimally as an RXRE when the upstream hexamer is preceded by a G or A and starts with G or A (8,12). In order to test whether the same requirement apply for PPREs, we compared the activities of the recovered response elements P41 (**G** GGGTCA G GGGTCA) and P11 (**T** GGGTCA T AGGTGA) and of a wild type PPRE present in the malic enzyme promoter (**T** GGGTCA A AGTTGA)(ME-PPRE). The activation of these elements by PPARα/RXRα in the presence of ciprofibrate and by RXRα in the presence of 9-cis RA was evaluated (Fig 2). As expected, the P41 element representing an ideal RXRE, was activated to a greater extent by 9 cis RA than the elements with T preceding the upstream hexamer. Interestingly, an opposite rank order of activity of these elements was observed after cotransfection with PPARα/RXRα. The element with 'G' preceding the upstream hexamer conferred indeed lower responses to ciprofibrate than those preceded by 'T'. These experiments indicate that RXRα homodimers and PPARα/RXRα heterodimers have divergent preferences for flanking bases when binding to DR1 elements. However, no definite conclusions on the optimal flanking bases for PPARα/RXRα binding can be drawn because of the limited number of selected DR1 elements and because the exact halfsite sequences

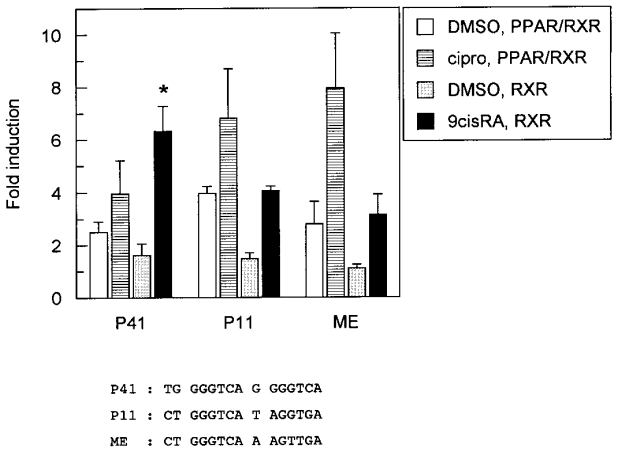

FIG. 2. Transactivation of selected DR1 with PPARα/RXRα and with RXRα. Cotransfection experiments of a RXRα expressing plasmid; a combination of PPARα and RXRα expressing plasmids or the parental expression vector with reporters containing the selected DR1 elements P11 and P41 or the wild type malic enzyme DR1 PPRE in COS cells. The CAT activity after cotransfection with the parental expression vector was considered to be the basal level of CAT activity for each element and was set at one. The induction of CAT activity by RXRα and by PPARα/RXRα in the absence and presence of the respective ligands vs. the activity in the presence of the parental expression vector is represented. Cells were treated with either vehicle (DMSO), 1 μM 9-cis RA (cotransfections with RXRα) or 100 μM ciprofibrate (cotransfections with PPARα/RXRα). The mean $\pm$ SEM of three independent experiments is shown. * significantly different from the activation of P11 and ME-PPRE by RXRα in the presence of 9-cis RA, $P < 0.05$ according to one way analysis of variance.

of the downstream hexamers in these response elements slightly differ (GGGTCA in P41, AGGTGA in P11 and AGTTGA in ME-PPRE).

We have previously shown that a 'C' as intervening base in a DR1 RXRE strongly reduces the binding affinity for RXRα homodimers. Because almost no elements were recovered with a 'C' as intervening base in

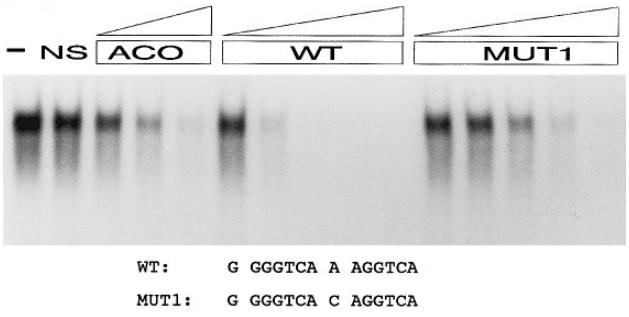

FIG. 3. 'C' as intervening base reduces the binding affinity of a DR1 PPRE. The malic enzyme DR1 was used as a probe in an EMSA with bacterially expressed *myc*PPARα and RXRα. Binding competition was performed by coincubation with either an excess of nonspecific oligonucleotide (NS, 1000 fold excess), the ACO element at 10-, 30-, and 100-fold excess, a perfect DR1 or a mutant DR1 with C as intervening base (MUT 1), both at 10-,30-, 100-, 300-, and 1000-fold excess.

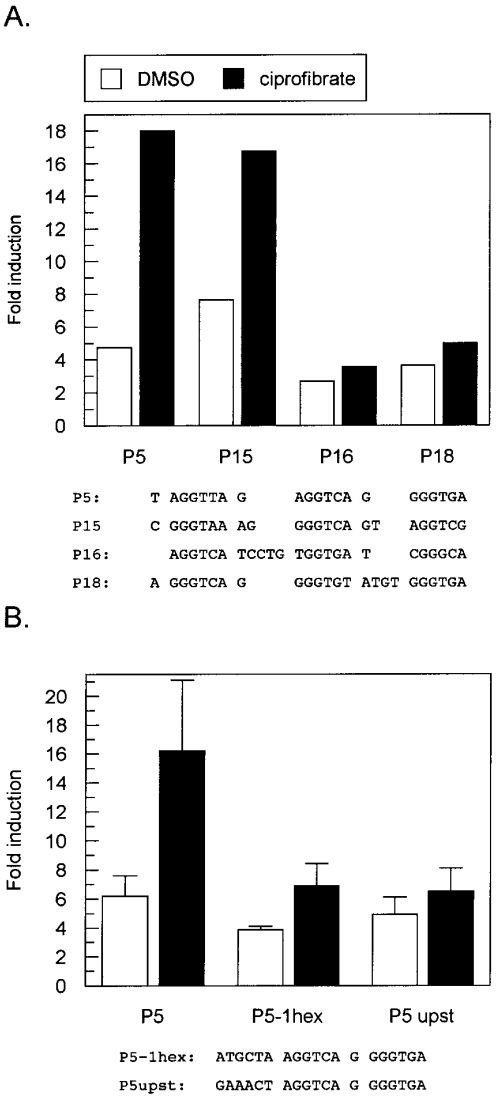

FIG. 4. Efficacy of PPREs with triple hexamer repeats. Cotransfection experiments were conducted in COS cells with reporters containing the indicated elements and either the parental expression vector or a combination of PPARα and RXRα expression plasmids. Cells were treated with DMSO or with 100 μM ciprofibrate as indicated. The level of CAT activity in the presence of the parental expression vector was set at one for each element tested. (A) Comparison of the efficacy of four selected PPREs with triple hexamer repeats. One representative experiment out of two is represented. (B) Comparison of the efficacy of a selected double DR1 (P5) with two mutant forms in which the upstream hexamer was either deleted (P5 - 1 hex) or replaced by the consensus upstream sequence (P5 upstream) defined by Palmer (13). The mean $\pm$ SEM of three independent experiments is shown. * significantly different from activation of the mutated P5 elements by PPARα/RXRα in the presence of ciprofibrate; $P < 0.05$ according to one way analysis of variance.

the PPARα/RXRα selection, we tested the possibility that 'C' in this position is also disadvantageous for PPARα/RXRα binding. A competition EMSA was performed with the radiolabeled malic enzyme DR1 PPRE as a probe and either a perfect (GGGTCA A AGGTCA)

or a mutant (GGGTCA **C** AGGTCA) DR1 as competitors. As shown in Fig 3, the amount of oligonucleotide required for inhibiting complex formation with the radiolabeled probe was ten times higher for the mutant than for the wild type DR1. These results demonstrate that 'C' as intervening base in a DR1 is indeed unfavorable for binding PPARα/RXRα heterodimers.

Functional Tests of Elements with Three Hexamer Repeats

Because elements consisting of three hexamer repeats were frequently recovered, we investigated the importance of this finding. As shown in fig 4A, the peroxisome proliferator responsiveness of these elements widely varied depending on the configuration of the three hexamers. Because a double DR1 or DR2 induced a markedly greater transactivation than the single DR1 elements (see fig 2), we hypothesized that the presence of a hird hexamer can improve activation of a DR1 by PPARα/RXRα. It is noteworthy that in several wild type DR1 elements an additional hexamer can be recognized downstream, upstream or partly overlapping with the DR1 (2). On the other hand, Palmer et al. previously showed (13) that a conserved presequence C(A/G)(A/G)A(A/T)CT can enhance binding of PPARα/RXRα to wild type DR1 elements. However, this only applies if these PPREs are composed of hexamers diverging from the consensus. In our selected elements -whether these consist of perfect or imperfect hexamers- the 5′ extended region was not found. In order to evaluate the importance of the sequence preceding a DR1, the activity of the double DR1 (P5) was compared with that of a mutant in which the upstream hexamer was deleted (P5 -1hex) and with another mutant in which the upstream hexamer was replaced with the consensus upstream sequence defined by Palmer (P5 upst). As shown in fig 4B, the selected double DR1 was 2 to 3 fold more active than each of the mutants. These experiments indicate that the presence of an additional hexamer improves the efficacy of a DR1. Remarkably, opposite results were obtained by other investigators with the element in the bifunctional enzyme gene promoter consisting of a DR1 followed by a DR2 (14). Deletion of the hexamer that was only part of the DR2 resulted in a 10 fold increase in PPRE activity. The latter hexamer was considered to have a modulator function of PPRE activity, binding either a repressor or an activator. The occupation of the 3 hexamers in complex PPREs by PPARα, RXRα and possibly other nuclear factors needs to be elucidated in order to clarify these contradictory results.

In conclusion, the upstream base of a DR1 element might modulate the binding specificities of RXRα homodimers and PPARα/RXRα heterodimers. Furthermore, additional half site sequences might increase the potency of a DR1 element for activation by PPARα/RXRα. These notions will need to be corroborated by the identification of new wild type RXREs and PPREs.

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