DNA Binding Properties of Peroxisome Proliferator-activated Receptor Subtypes on Various Natural Peroxisome Proliferator Response Elements

IMPORTANCE OF THE 5'-FLANKING REGION*

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The three subtypes of the peroxisome proliferator-activated receptors (PPARα, β/δ, and γ) form heterodimers with the 9-cis-retinoic acid receptor (RXR) and bind to a common consensus response element, which consists of a direct repeat of two hexanucleotides spaced by one nucleotide (DR1). As a first step toward understanding the molecular mechanisms determining PPAR subtype specificity, we evaluated by electrophoretic mobility shift assays the binding properties of the three PPAR subtypes, in association with either RXRα or RXRγ, on 16 natural PPAR response elements (PPREs). The main results are as follows. (i) PPARγ in combination with either RXRα or RXRγ binds more strongly than PPARα or PPARβ to all natural PPREs tested. (ii) The binding of PPAR to strong elements is reinforced if the heterodimerization partner is RXRγ. In contrast, weak elements favor RXRα as heterodimerization partner. (iii) The ordering of the 16 natural PPREs from strong to weak elements does not depend on the core DR1 sequence, which has a relatively uniform degree of conservation, but correlates with the number of identities of the 5'-flanking nucleotides with respect to a consensus element. This 5'-flanking sequence is essential for PPARα binding and thus contributes to subtype specificity. As a demonstration of this, the PPARγ-specific element ARE6 PPRE is able to bind PPARα only if its 5'-flanking region is exchanged with that of the more promiscuous HMG PPRE.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamilly. Three PPAR subtypes (α, β/δ, and γ) with a high degree of sequence conservation of each subtype across various species have been characterized. Within a given species, the DNA binding domains of the three PPARs are 80% identical, while their ligand-binding domains exhibit a lower degree of identity, around 65% (reviewed in Desvergne and Wahli (1)). Consistent with this relatively high divergence between the subtype-specific ligand binding domains, differential activation by endogenous and exogenous compounds has been recently described and may account for the specific biological activity of the various PPAR subtypes. Fatty acids, such as arachidonic acid (and its analog 5,8,11,14-eicosatetraynoic acid or ETYA) and hypolipidemic drugs (e.g. fibrates) preferentially activate PPARs. We also recently demonstrated that leukotriene B4 and the hypolipidemic agent Wy144643 are bona fide ligands for PPARα (2), while the PPARγ subtype binds and is activated by the prostaglandin derivative 15-deoxy-D12,14-PGJ2 as well as by insulin-sensitizing thiazolidinediones, such as pioglitazone, troglitazone, and BRL49653 (reviewed in Lemberger et al. (3)). Recent studies have shown that various fatty acids, eicosanoids, and hypolipidemic compounds directly bind to PPARβ, γ (4–7).

Within the superfamilly of the nuclear receptors, PPARs belong to the type II subgroup that includes the vitamin D receptor, the thyroid hormone receptor, and the retinoic acid receptor (RAR). Members of this subgroup form heterodimers with the 9-cis-retinoic acid receptor (RXR) and typically bind to DNA elements containing two copies of an idealized consensus binding site –AGGTCA– arranged in a direct repeat array spaced by n nucleotides (DRn). The specificity with respect to the nuclear receptors is dictated by the number of nucleotides between the two hexanucleotides or two half-sites. For PPAR response elements (PPRE), the two copies are spaced by 1 base pair (DR1). However, a perfect DR1 element is quite promiscuous, since it allows the binding of RXR homodimers, PPAR/RXR, and RAR/RXR heterodimers as well as of orphan members of the nuclear receptor superfamilly (8). This illustrates that the spacing is not sufficient for conferring full specificity to a direct repeat element. Palmer et al. (9), using mutation analyses of the CYP4A6Z PPRE, recently demonstrated that the sequences upstream of the DR1, with the proposed consensus C(A/G)(A/G)(A/T)/CT, contribute to selective binding of PPARα/RXRα over other binding complexes such as RXR/RXR or the apolipoprotein regulatory protein-1. However, no PPAR subtype specific requirements have yet been studied.

The expression pattern of the PPAR subtypes can give some indications regarding their respective specific biological activi-
ity. In the adult rat, PPARs are mostly detected in liver, kidney, heart, brown adipose tissue, and the intestine, and its role in regulating hepatic lipid metabolism has been confirmed by the targeted disruption of the PPARγ gene in mice (10). PPARγ is predominantly expressed in adipose tissue, the small intestine, and lymphatic tissue and, in coordination with C/EBP α and β, is a key factor in inducing adipose differentiation (11). In contrast, the PPARβ/δ subtype, hereafter called PPARβ, is ubiquitously expressed, and its function is not yet known. Thus, many tissues co-express more than one PPAR subtype. Together with the fact that the three PPARs bind to a similar consensus sequence, this raises the question of the molecular mechanism of target gene specificity. One hypothesis is suggested by the example of adipose differentiation from fibroblasts which can be obtained through the forced expression of PPARγ whereas PPARα is much less adipogenic (12). One late target gene of PPAR in this process is the aP2 gene which contains a fat-specific enhancer bearing two response elements, ARE6 and ARE7. Interestingly, PPARα has a weak binding affinity to these elements compared with that of PPARγ, thereby providing an hypothesis for its poor ability to induce adipose differentiation. Moreover, it suggests that PPARs and γ have different sequence requirements that have yet to be characterized. Finally the role of the RXR subtype, as a heterodimeric partner of the various PPARs, in dictating target gene binding has hitherto not been examined.

In the present report, we examined 16 natural PPREs identified in 12 target gene promoters. These genes are involved in many aspects of fatty acid transport and metabolism (see Table I), and the regulation of their expression by PPARs has been previously characterized by in vitro binding assays as well as by functional assays (see references in Table I). Most of these genes are expressed in tissues where the different PPAR subtypes are present (adipose tissue, liver, and kidney), and hence illustrate the problem of subtype specificity. Thus, the relative DNA-binding capabilities of the three PPAR subtypes α, β, and γ were assessed on these 16 elements. Each natural element encompasses the DR1 core sequence and the seven nucleotides flanking its 5′ end, allowing the examination of the importance of this PPRE 5′-flanking sequence with respect to the PPAR subtype-binding specificity. In addition, we tested the possibility that the partner of heterodimerization, RXRα, or RXRγ, might also contribute to the DNA-binding specificity of the PPAR subtypes.

MATERIALS AND METHODS

Plasmids—The pSG5-xPPARs, xPPARβ, and xPPARγ expression plasmids have been previously described (13). pSG5-mRXRγ was generously provided by P. Chambon. For transient transfection assays, the reporter constructs pBL-1xPPRE-CAT8+ were obtained by cloning a single copy of double-stranded oligonucleotides corresponding to the tested PPREs (see Fig. 5) into the BamHI site of pBL-CAT8+. These reporter constructs were verified by DNA sequencing.

In Vitro Transcription/Translation of Receptors—Receptors were synthesized using the rabbit reticulocyte lysate transcription/translation kit TntT7 (Promega, Madison, WI) according to the manufacturer's instructions. Parallel reactions were performed using [35S]methionine. The labeled receptors were subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel. The gels were dried, and labeled receptors were quantitated by a PhosphorImager (Molecular Dynamics) for the purpose of standardization.

Electrophoretic Gel Mobility Shift Assay (EMSA)—Double-stranded oligonucleotides (sequences in Fig. 2) with 4-base pair 5′-overhangs corresponding to the overhang of the BamHI site were labeled by Klenow fragment filling-in in the presence of [α-32P]dATP. All probes were labeled at the same time in parallel, and their respective specific activities differed from each other by less than 10%. After correction for the number of methionines, standardized amounts of in vitro translated PPAR subtype and RXR with a molar ratio PPAR/RXR 3:1 were incubated with 20,000 cpm of labeled double-stranded oligonucleotides and 2 μg of poly[d(I-C)] in EMSA binding buffer (50 mM KCl, 20 mM Hepes, 20% glycerol, 0.05% Nonidet P-40, 10 mM β-mercaptoethanol, pH 7.5) in a final volume of 25 μL. For competition experiments, increasing concentrations of double-stranded oligonucleotides were added to the binding reaction. Incubation was performed at room temperature for 20 min, and the samples were electrophoresed on a 5% polyacrylamide gel for 80 min at 300 V. Gels were dried and exposed to a PhosphorImager screen (Molecular Dynamics) for quantification before autoradiography. Alternatively, the autoradiographies were quantified by densitometry. All quantifications were done after the same time of exposure.

Cell Culture and Transfection Studies—NH3T3 and HepG2 cells were maintained and transfected by electroporation and calcium-phosphate method, respectively (14, 15). After transfection, the medium was supplemented with either 1 μM ETYA, 100 μM bezafibrate (Sigma), 10−4 M BRL49653 (kind gift of S. Kliewer, Glaxo Wellcome), or vehicle as indicated. The chloramphenicol acetyltransferase (CAT) activity was determined in the lysate extracts as described previously (14, 15).

RESULTS

Comparison of PPARα, β, and γ Binding on Various Natural PPREs, Using RXRα as Heterodimeric Partner—Based on our own previous work and the literature, we selected 12 target genes whose direct regulation by PPAR has been characterized (see Table I). For the ACC, PEPC, and aP2 genes, two functional response elements have been identified within the promoter sequences. In the case of the ME gene, only one of the two DR1-like sequences, the proximal element hereafter called MEp, is functional, while the distal element or MEd is not functional on its own (16). The latter has been used in the present study as a negative control. Binding of the three PPAR
FIG. 1. A, EMSA of PPARα, PPARβ, and PPARγ as heterodimers with RXRα, on 16 natural PPREs. A standardized amount of in vitro translated PPAR was incubated with RXRα at a molar ratio of 3:1, together with 20,000 cpm of labeled double-stranded oligonucleotides corresponding to one of the PPREs described in Table I and Fig. 2. The arrowhead indicates the band corresponding to the bound PPAR/RXR complexes; PPRE, free probes, and *, nonspecific complexes. The first lane for each probe (−) corresponds to a binding reaction with unprogrammed lysate only. B, quantification of the bound complexes, PPARα/RXRα, PPARβ/RXRα, or PPARγ/RXRα, expressed in arbitrary units; *, no detectable binding. For both panels A and B, the PPREs are ordered as a function of PPARα binding. The overall mean of the binding activity, represented by a dotted line across the columns has been used to delimit three classes of elements of high, intermediate, and low PPAR binding activity. Independent EMSAs reproduce the same classification with permutation occurring only within a class. C, competition experiments. A perfect consensus DR1 (sequence given at the top of Fig. 2) was radiolabeled and used as a probe in EMSAs with PPARα, PPARβ, and PPARγ heterodimerized with RXRα. The numbers indicate the molar excess of cold oligonucleotide encompassing either the HMG or the ARE6 PPRE sequence that was required to obtain a 50% inhibition of PPAR binding. The results of two independent experiments are shown. D, transfection analyses. NIH3T3 cells were transfected by electroporation with 5 µg of the various PPRE-containing reporter genes, 3.2 µg of pSG5-xPPARα (left panel) pSG5-xPPARβ (middle panel), pSG5-xPPARγ (right panel), or 3.2 µg of the empty vector pSG5 and 5 µg of PUC19 as carrier DNA. 0.25 µg of pCMVβ expression vector were added as an internal control for transfection efficiency. Cells were cultured for 48 h in the absence (dark bars) or presence (light bars) of the PPAR ligands, as indicated, and CAT activity was measured in the cellular extracts and normalized to the β-galactosidase activity. For each reporter gene, the CAT activity of value 1 corresponds to the basal activity of the reporter gene in the absence of PPAR and ligands (transfection with the empty vector pSG5). Values are the mean ± S.E. of three independent experiments, each performed in duplicate. The PPREs used correspond to the high (HMG and Cyp4A1), intermediate (MEP and ACOA), and weak (ARE6) class of PPAR binding sites.
Receptor Specificity of Natural PPREs

subtypes with RXRa as the heterodimeric partner was thus tested by EMSA on a total of 16 natural DNA elements. The amount and specific activity of the radiolabeled oligonucleotide probes as well as the amount of in vitro translated proteins were standardized in all reactions as described under “Materials and Methods.” For the sake of simplicity, we use hereafter the name of the target gene for designating the corresponding PPRE.

 Autoradiograms of the EMSA are shown in Fig. 1A, and the rank order of binding obtained from this experiment is summarized in Fig. 1B. As expected from previous results, PPAR alone was unable to bind to any of the elements (data not shown), confirming that heterodimerization with RXR is a requirement for DNA binding. Interestingly, the order of DNA shown), confirming that heterodimerization with RXR is a re-

|        | HMG    | ARE6   |
|--------|--------|--------|
| PPARα  | 22     | 88     |
| PPARβ  | 46     | 92     |
| PPARγ  | 26     | 44     |

|        | Exp 1  | Exp 2  |
|--------|--------|--------|
| PPARα  | 22     | 22     |
| PPARβ  | 46     | 41     |
| PPARγ  | 26     | 23     |

In summary, both PPARα- and PPARβ-mediated transcriptional activities reflect their binding affinities to the target sequence, whereas PPARγ-mediated transactivation is less sensitive to subtle differences in binding properties.

In Fig. 2, we aligned the sequences of the 16 elements as a function of PPAR binding, with the top element as the strongest, and scored the number of identities between each element and the consensus sequence given at the top of the figure. This

terated with the ARE6 element from the aP2 gene where there is virtually no binding of PPARα and β while PPARγ still binds significantly.

We then assessed the functional relevance of this classification by testing in transfection two high class elements (HMG and CYP4A1), two intermediate class elements (MEp and ACOA) and one weak class element (ARE6) for their different abilities to mediate PPARα, β, and γ transactivation. As described previously for HeLa and CV1 cells, transfection of a vector expressing Xenopus PPARα in NIH3T3 results in an apparent ligand-independent stimulation of PPRE-containing reporter gene expression (17), likely due to the presence of an endogenous ligand. This increase in basal activity in NIH3T3 reaches a 15-fold stimulation for the HMG and Cyp4A1 reporter genes, while expression of MEp and ACOA reporter genes was increased by 8- and 12-fold, respectively, and that of ARE6 by 2-fold, thus reflecting the classification obtained from the binding studies (Fig. 1D, left panel). Addition of the specific ligand WY14,643 (2) does not further increase the activity of the various reporter genes. In contrast, transactivation mediated by PPARβ depends on the addition of bezafibrate, recently demonstrated as a bona fide ligand of PPARβ and PPARα (5). As seen in Fig. 1D, middle panel, the fold stimulation of the activity of the various reporter genes perfectly correlates with the classification of the corresponding PPRE. PPARγ in absence of added ligand increases the basal activity of the reporter genes by 2–5-fold. Addition of the PPARγ-specific ligand BRL49653 further increases this stimulation. However, Cyp4A1-, HMG-, and ACOA-containing reporter genes were equally activated (between 12- and 14-fold), while that containing MEp was increased by 22-fold, and ACOA-dependent transactivation is 4-fold. In summary, both PPARα- and PPARβ-mediated transcriptional activities reflect their binding affinities to the target sequence, whereas PPARγ-mediated transactivation is less sensitive to subtle differences in binding properties.
The consensus sequence is formed of two main parts, 13 nucleotides composing a perfect DR1 element and 7 nucleotides corresponding to the 5′-flank consensus as proposed by Palmer et al. (9). The best elements (HMG, FABP, CYP4A1, and BIF) in terms of binding for PPAR<sub>a</sub> have sequences with a high overall identity with the 5′ and the core consensus sequence (16 out of 20). Binding efficiency lowers with the increase of divergence between natural PPREs and the consensus element, dropping to no detectable binding with ARE6 that has a score of 11 out of 20. Very strikingly, the identity score of the 13-nucleotide long DR1 core sequence of the first ranking and of the last ranking PPRE, HMG, and MED, is 10 out of 13 nucleotides. Indeed, as seen in Fig. 2, the level of identity in the core element does not directly determine PPAR binding properties. In contrast, a clear increase of divergence is observed in the 5′-flanking sequence between the best elements (top) with a conservation of 6 out of 7 positions and the worst elements (bottom) with 1 or 2 positions out of 7. The same correlation between the score of the 5′-flanking sequence and binding activity is observed for the two other subtypes PPAR<sub>g</sub> and PPAR<sub>b</sub> (Fig. 2 and data not shown, respectively). However, in contrast to PPAR<sub>a</sub>, the absolute level of PPAR<sub>g</sub> binding to the weak elements remains relatively high, at around 20% of its highest binding activity. This suggests that the role of the 5′-flanking region is crucial for PPAR<sub>a</sub> binding while it is less important for PPAR<sub>g</sub>.

**Comparative Analysis of PPAR/RXR<sub>a</sub> and PPAR/RXR<sub>γ</sub> Heterodimer Binding Properties on Natural PPREs—**Three RXR subtypes, α, β, and γ, have been described. While they all can form heterodimer with PPARs, it is not known if different RXR partners affect the DNA binding properties of the PPAR/RXR heterodimer. We thus repeated the binding reactions of PPAR<sub>a</sub>, PPAR<sub>b</sub>, and PPAR<sub>g</sub> with RXR<sub>γ</sub> as the heterodimerization partner, using the 16 natural response elements of Fig. 2. As shown in Fig. 3, the general pattern resembles that of Fig. 1, as PPAR<sub>γ</sub> binds more efficiently than PPAR<sub>a</sub> and PPAR<sub>b</sub> to all PPREs and as the ranking order of the elements is similar. Interestingly, the differences in binding strength between PPAR<sub>γ</sub> on one hand and PPAR<sub>a</sub> and PPAR<sub>b</sub> on the other hand is increased by the presence of RXR<sub>γ</sub>. This is true for the strong PPREs such as HMG and FABP and is even more pronounced on the ACOA element, where the relative binding activity of PPAR<sub>γ</sub> is 3
times and 7.5 times that of PPARα when using RXRα and RXRγ as heterodimeric partners, respectively.

The binding strength of PPAR/RXRα as compared with PPAR/RXRγ is represented in Fig. 4A for HMG, ACOA, and Ti-LPT as examples. The results shown in Fig. 4B represent the ratio of binding strength of PPAR/RXRα over PPAR/RXRγ to each of the 16 elements. The line drawn across the columns corresponds to the value of one. Values smaller than one indicate that binding of the heterodimers PPAR/RXRγ is more efficient than that of PPAR/RXRα with respect to DNA binding affinity. Conversely, values above one indicate that PPAR/RXRα binds more efficiently than PPAR/RXRγ. This line divides the graph into two parts. On the left, corresponding to the high and intermediate binding sites, the ratio is below 1 and corresponds to a PPAR preference for RXRγ over RXRα. Conversely, values above one indicate that PPAR/RXRα binds more efficiently than PPAR/RXRγ. This line divides the graph into two parts. On the left, corresponding to the high and intermediate binding sites, the ratio is below 1 and corresponds to a PPAR preference for RXRγ over RXRα. Conversely, values above one indicate that PPAR/RXRα binds more efficiently than PPAR/RXRγ. This line divides the graph into two parts. On the left, corresponding to the high and intermediate binding sites, the ratio is below 1 and corresponds to a PPAR preference for RXRγ over RXRα. Conversely, values above one indicate that PPAR/RXRα binds more efficiently than PPAR/RXRγ.

Importance of the 5′-Flanking Sequences of the DR1 in PPAR Subtype Binding Specificity—As mentioned before, the HMG element displays equal binding of PPARα and PPARγ, and its 5′-flanking sequence is well conserved with respect to the consensus. In contrast, the ARE6 element has a very poor 5′-flank and is only able to significantly bind PPARγ but not PPARα. To evaluate the importance of the 5′-upstream nucleotides in the differential binding of the PPAR subtypes, we created a hybrid element containing the DR1 core sequence of ARE6 and the 5′-flanking region of HMG, which is called HMG/ARE6 (Fig. 5A). EMSAs were performed as before and demonstrate that the replacement of the 5′-flanking sequences in the ARE6 element by that of the HMG element dramatically increases the binding of PPARα and PPARβ (Fig. 5B and C). This supports the hypothesis that PPARα and β have sequence requirements different from PPARγ, and confirms the importance of the 5′-flanking region for PPARα and PPARβ binding.

We then performed transient transfection assays in HepG2 cells in which PPARα has less constitutive activity and can be further activated upon exogenous ligand addition. Expression vectors for PPARα or PPARγ and RXRα were transfected together with a reporter plasmid containing either the HMG, ARE6, or HMG/ARE6 elements (Fig. 6). The HMG-containing reporter genes is activated by PPARα and by PPARγ in presence of their respective ligands. As we previously observed in NIH3T3 (Fig. 1D), the ARE6-reporter gene is not activated by PPARα and is only weakly activated by PPARγ. Interestingly, replacement of the ARE6 5′-sequence by that of HMG, in the hybrid element HMG/ARE6, confers the ability to this sequence to mediate PPARα-dependent transcriptional activation, while also improving that mediated by PPARγ (Fig. 6).
were treated with ETYA (1 μM) or with BRL 49653 (1 μM) or with pSG5-PPARα, pSG5-PPARγ, and pSG5-RXRα together with an appropriate pBL-1xPPRE-CAT8 reporter gene, as indicated. Cells were transfected with pSG5-PPARα, pSG5-PPARγ, and pSG5-RXRα together with an appropriate pBL-1xPPRE-CAT8 reporter gene, as indicated. Cells were treated with ETYA (1 μM) or with BRL49653 (1 μM), as indicated, 24 h prior to harvesting and lysis. Transcriptional activity is expressed as relative CAT activity, normalized to protein content. Values are the mean ± S.E. of three independent experiments, each performed in triplicate.

Thus, these results further confirm a correlation between binding and transactivation properties.

**DISCUSSION**

Based on the analysis of 16 natural elements, we show that the PPRE can be defined as a bipartite element. One part is formed of the core DR1 sequence, the other part consists of the nucleotides which flank the DR1 sequence in 5′. This flanking sequence is crucial for PPARα binding and transactivation, and therefore contributes to PPAR subtype specificity.

At least four means can be used by a nuclear transcription factor to achieve gene or tissue specific activity: (i) restriction of its expression to a given tissue and/or at a given time, (ii) binding to specific DNA sequences and thus specific genes, (iii) activation by tissue specific ligands or activators, and (iv) modulation of the transactivation properties by physical or functional interactions with tissue specific co-factors. With respect to the role of the different PPARs as transcription factors, the predominant expression of PPARγ in the adipose tissue and the high expression of PPARα in the liver correlate with their specific role in adipose differentiation and fatty acid catabolism, respectively. Moreover, specific ligands for each of the PPARs are now emerging (2, 4–7, 18). However, the above arguments in favor of PPAR subtype specificities have to accommodate two observations. First, all tissues, including white adipose tissue and liver, also express PPARβ while in addition the brown adipose tissue, intestine, spleen, and retina also co-express PPARα and PPARγ (19). Second, the ligand specificity is relative, since natural molecules such as certain fatty acids can activate all three PPARs (20). Thus two main alternative pathways of specificity remain to be explored, specific interaction with target sequences and specific interaction with co-factor(s).

In the present work we compared the binding of the three PPAR subtypes to 16 previously identified DNA target sequences. When ordering the 16 elements according to their binding strength for each of the three PPAR subtypes, the three ranking orders obtained were quite similar. However, some significant specificities emerged, such as ARE6 binding exclusively PPARγ. Furthermore, the rank order of binding revealed that the overall efficiency of any given PPRE to bind PPAR is related to the 7-nucleotide sequence forming the 5′-flanking region of the core DR1. One notable exception is PEPCK1 which belongs to the intermediate group, according to its mean binding activity, although it has a very weak 5′-flanking sequence. One possible explanation is that some of the divergent bases, observed either within the DR1 or within the flanking sequence of PEPCK1, are up-mutation with respect to the consensus sequence that has been drawn from the compilation of native elements. A thorough comparative analysis of each of the divergent bases, alone or in various combinations, would be required to solve this question and could give further insight into PPAR binding requirements.

The importance of the sequence flanking the core DR1 element in 5′ was initially described by Palmer et al. (9) for the binding of PPARα/RXRα to the CYP4A6 element. These authors have shown that the presence of a particular flanking sequence together with a rather weak DR1 core element could allow binding discrimination between PPARα/RXRα and other DR1-binding proteins such as apolipoprotein regulatory protein-1 or RXR homodimers. In the present work, we demonstrate that PPARγ binding is less dependent on the 5′-flanking sequences than PPARα binding. This differential affinity is best exemplified by the weak PPREs, which bind only PPARγ, as is the case for ARE6. Exchanging the 5′-flanking sequence of ARE6 with that of HMG confers PPARα binding and transactivation. As mentioned previously, PPARγ is strongly adipogenic, which is compatible with its predominant binding to the ARE6 element of the apo2 gene (12). In contrast, the HMG, BIF, and FABP elements, which are predominantly regulated by PPARα in the liver, possess a conserved 5′-flanking sequence. Thus, the presence of a facilitating 5′-region corresponds to the first mechanism described that allows the differential use of a PPRE by two PPAR subtypes.
Interaction of the different PPARs with specific co-factors could be another mechanism for achieving specificity. An obligatory partner with which PPAR must interact to bind to DNA is RXR, for which three subtypes have been described, RXRa, which is mostly expressed in liver, kidney, lung, muscle, and spleen; RXRβ, which is ubiquitously expressed; and RXRγ, which is highly expressed in heart, muscle, and liver (21). Not only does the co-expression of at least two RXR subtypes in any tissue seem to be the rule, but all three RXR subtypes are able to form heterodimers with PPARs. So far, no analyses have been performed for determining specific sequence preferences of PPAR as a function of the RXR subtype. Our findings provide evidence that RXRγ augments PPAR binding compared with RXRa. However, formation of PPAR/RXR complexes on weak binding sites is better in the presence of RXRa compared with RXRγ, and this is particularly remarkable when the PPARa subtype is the partner. This suggests that heterodimeric complexes with RXRa have a less stringent sequence requirement and could thus compensate in part for the weak affinity of the PPARa partner on poor binding sites. Finally, it was generally observed on most elements that the difference in binding strength between PPARa and PPARγ is markedly increased in favor of PPARγ in the presence of RXRγ. This indicates that the differential activation from a given response element not only depends on the DNA sequence and PPAR subtype, but also on the RXR subtype present in a given target tissue. In that context, analyses of the RXRβ property as partner of PPAR will be of interest. What also remains to be explored are additional levels of combinatorial processes involving at least two other types of protein–protein interaction, interactions with non-DNA binding co-factors that can act as co-repressors or co-activators (reviewed in Horwitz et al. (22)), and interactions with other DNA-bound transcription factors such as SP1 (23).

Finally, we recently demonstrated that PPARa can undergo phosphorylation upon insulin treatment (24). The subtype specificity of this process as well as its role in PPAR activation may also be a route for specificity.

In conclusion, these studies on natural PPREs emphasize the crucial role of the 5′-nucleotides flanking the DR1 core sequence for efficient PPARa and PPARγ binding. Importantly, they also demonstrate that subtypes of both PPAR and RXR participate in the modulation of binding affinity, and that the resulting combinatorial binding property largely depends on the nature of the 5′-flanking sequence.

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