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Inhibition of Peroxisome Proliferator Signaling Pathways by Thyroid Hormone Receptor

COMPETITIVE BINDING TO THE RESPONSE ELEMENT*

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Takahide Miyamoto‡§, Atsuko Kaneko‡, Tomoko Kakizawa, Hiroki Yajima, Keiju Kamijo¶, Rieko Sekine, Kunihide Hiramatsu, Yutaka Nishii, Takashi Hashimoto¶, and Kiyoshi Hashizume

From the Department of Geriatrics, Endocrinology and Metabolism, ¶Department of Biochemistry, Shinshu University School of Medicine, Matsumoto 390, Japan

Peroxisome proliferators (*e.g.* clofibric acid) and thyroid hormone play an important role in the metabolism of lipids. These effectors display their action through their own nuclear receptors, peroxisome proliferator-activated receptor (PPAR) and thyroid hormone receptor (TR). PPAR and TR are ligand-dependent, DNA binding, trans-acting transcriptional factors belonging to the *erbA*-related nuclear receptor superfamily. The present study focused on the convergence of the effectors on the peroxisome proliferator response element (PPRE). Transcriptional activation induced by PPAR through a PPRE was significantly suppressed by cotransfection of TR in transient transfection assays. The inhibition, however, was not affected by adding 3,5,3′-triiodo-L-thyronine (T3). Furthermore, the inhibition was not observed in cells cotransfected with retinoic acid receptor or vitamin D3 receptor. The inhibitory action by TR was lost by introducing a mutation in the DNA binding domain of TR, indicating that competition for DNA binding is involved in the molecular basis of this functional interaction. Gel shift assays revealed that TRs, expressed in insect cells, specifically bound to the ^{32}P-labeled PPRE as heterodimers with the retinoid X receptor (RXR). Both PPAR and TR bind to PPRE, although only PPAR mediates transcriptional activation via PPRE. TR·RXR heterodimers are potential competitors with PPAR·RXR for binding to PPREs. It is concluded that PPAR-mediated gene expression is negatively controlled by TR at the level of PPAR binding to PPRE. We report here the novel action of thyroid hormone receptor in controlling gene expression through PPREs.

Peroxisomes are cytoplasmic organelles that are important in mammalian lipid homeostasis (1). The structurally diverse xenobiotic peroxisome proliferators (PPs),[1] such as clofibrate, nafenopin, and WY-14,643 stimulate the proliferation of peroxisomes (2–5) and cause tumorigenic transformation of hepatic cells in rodents (6, 7). Some of these compounds have been used in man as hypolipidemic agents. PPs have been shown to induce peroxisomal and microsomal enzymes involved in lipid metabolism through activation of the peroxisome proliferator-activated receptor (PPAR) (8, 9). The PPAR is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors and is structurally related to the subfamily of receptors that includes the thyroid hormone receptor (TR), retinoic acid receptor (RAR), and vitamin D3 receptor (VDR) (10). To date, three subtypes of PPARs have been identified in amphibians, rodents, and humans, PPARα, -β, and -γ (8, 9, 11–14). Further investigation revealed that natural fatty acids are also potent activators of PPARα (14, 15), although no direct interaction of PPARα with either PPs or fatty acids has been described so far. Recently, ligands for PPARγ have been identified that are potent inducers of adipogenesis *in vivo*. These include thiazolidine diones, a class of anti-diabetic drugs, and the arachidonic acid derivative 15-deoxy-D12,14-prostaglandin J2 (16–18).

PPARs regulate gene expression by binding to DNA sequence elements termed PPAR response elements (PPRE). PPREs have recently been identified in the 5′-flanking sequences of peroxisome proliferator-inducible genes such as the rat acyl-CoA oxidase (*aox*) gene (19, 20), and the gene for cytochrome P450 CYP4A6 (21). The product of the former is the key enzyme in peroxisomal β-oxidation and that of latter catalyzes ω and ω-1 hydroxylation of fatty acids. PPREs are composed of two direct AGG(A/T)CA repeats separated by a single nucleotide (DR1), which is similar to previously described retinoid X response elements (22). These direct repeat motifs are also found in a number of other nuclear receptor response elements, *e.g.* the TRE, RARE, VDRE (22). Each receptor can recognize the same half-site motif. However, they discriminate between target elements through the spacing between the half-site motifs.

It is well established that heterodimerization with retinoid X receptor (RXR) strongly enhances binding of the TR, RAR, and VDR to their cognate response elements (23–26). Like other members of this subfamily, it has been demonstrated that PPAR binds to the PPRE by forming a heterodimer with RXR (27, 28). Therefore, it is possible that PPAR may exhibit promiscuous cross-talk with other members of the nuclear receptor family (29). Thyroid hormone is another effector that influences lipid metabolism including fatty acid β-oxidation (30–33). TRs and PPAR appear to play an important role in lipid metabolism, and their signaling pathways might be coupled.

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‡ Contributed equally to this work and should be considered first authors.

§ To whom correspondence should be addressed: Dept. of Geriatrics, Endocrinology and Metabolism, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, 390 Japan. Tel.: 81-263-37-2686; Fax: 81-263-37-2710.

[1] The abbreviations used are: PPs, peroxisome proliferators; PPAR, peroxisome proliferator-activated receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D3 receptor; AOX, acyl-CoA oxidase; PPRE, peroxisome response element; TRE, thyroid hormone response element; TK promoter, herpes simplex thymidine kinase promoter; T3, 3,5,3′-triiodo-L-thyronine; DBD, DNA binding domain; CMV, cytomegalovirus.

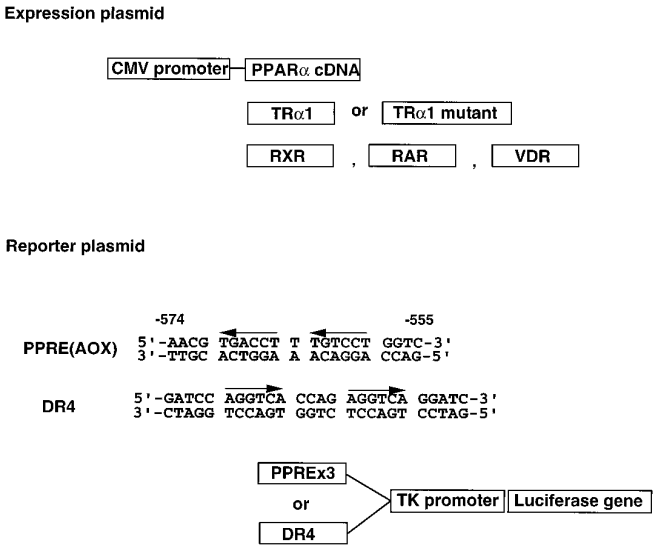

FIG. 1. **Construction of expression vector and luciferase reporter used in this study.** Full-length rPPARα was inserted into the *Bam*HI site of pCMV expression plasmid using *Bam*HI linker. TRα1 and mutant TRs were also expressed under the control of CMV promoter (pCDM) (26). PPRE-TK-luciferase reporter plasmid harbors three copies of PPRE from the *aox* promoter in front of the TK promoter (19). DR4-TK-luciferase reporter contains one copy of DR4-TRE.

Based on the considerations described above, we focused on the convergence of TRα1 and PPARα signaling pathways on PPREs. We have examined the suppressive effects of TRα1 expression on PPARα-mediated transcriptional activation of peroxisome proliferator response genes. We demonstrated that TRα1 negatively regulates PPARα action on PPREs through competition for DNA binding, and this negative regulation occurs in a ligand (T3) -independent manner.

In addition, we present evidence that PPRE (DR1) is a high affinity binding site for TRα1 but not a functional response element for TRα1. TRα1 cannot activate PPRE in the presence of T3, despite its specific and high affinity binding to the element as a heterodimer with RXR. These results imply that binding to DNA is necessary but not sufficient for T3-dependent transcriptional regulation by TR.

MATERIALS AND METHODS

cDNA Isolation and Plasmids Constructions—Total RNA was extracted from rat liver using a guanidinium thiocyanate method. A cDNA pool was made by reverse transcriptase and $(dT)_{17}$ primer. Reverse transcriptase-polymerase chain reaction technique was applied to amplify the rat PPARα cDNA using reported primers as follows: 5′-ATGGTGGACACAGAGAGCCCCATCTGTCCT-3′ as sense primer and 5′-TCAGTACATGTCTCTGTAGATCTCTTGCAA-3′ as antisense primer (14). The nucleotide sequence of the isolated rat PPARα cDNA was confirmed by sequencing (34). Fig. 1 illustrates the plasmid constructs used in this study. Full-length rat PPARα was inserted into the *Bam*HI site of pCMV expression plasmid using *Bam*HI linkers. Human TRα1 and mutant TR were also expressed under the control of the CMV promoter (pCDM) (35, 36). The TRα1 DNA binding domain mutant (DBD mutant) was made by introducing a cysteine to serine substitution at amino acid 73 using a site-directed mutagenesis system (pSELECT vector, Promega) as described previously (36). RARα and VDR cDNA are kind gifts from Dr. R. M. Evans (The Salk Institute, La Jolla, CA) and Dr. B. W. O'Malley (Baylor College, TX), respectively. The coding sequences of RARα or VDR was amplified by PCR and inserted into the *Bam*HI site of pCMV expression vector. PPRE-TK-luciferase reporter plasmid harbors three copies of PPRE from the *aox* promoter in front of the TK promoter (27). The reporter employing the native promoter for *aox* (27) is kindly provided by Dr. R. M. Evans. The DR4-TK-luciferase reporter plasmid contains one copy of the DR4-TRE sequence, 5′-GGATCCAGGTCACAGGAGGTCAGGATCC-3′.

Cell Culture and Transfection—COS1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected by the calcium phosphate precipitation technique as described previously (36). Transfections were performed in 24-well plates. In general, each dish received 250 ng of reporter construct, 0.8–50 ng of expression vector, 50 ng of β-galactosidase expression vector (pCH110, Pharmacia Biotech Inc.) to monitor the efficiency of transfection and, if necessary, carrier DNA (pBluescript, Stratagene) to reach a total of 450 ng of DNA. Twenty hours after transfection, the medium was replaced by that containing T3 (10^{-7} M) or clofibric acids (10^{-3} M), and an additional 24 h later, cells were harvested and assayed for β-galactosidase and luciferase activity (36, 37).

β-Galactosidase and Luciferase Assays—β-Galactosidase was measured by the method previously described (38). Luciferase assays were performed using the PicaGene Luciferase Assay System (Toyo Inki, Tokyo). Cells were harvested by adding 50 μl/well Cell Culture Lysis Reagent buffer. Samples were centrifuged (12,000 × *g*) at 4° C for 10 min, and the supernatant was retained for assay. Luciferase assays were performed by adding 30 μl of cell extract to 100 μl of Luciferase Assay Reagent. The reactions were performed at room temperature and assessed using Lumat LB9501 (Berthold Japan K.K., Tokyo, Japan) and expressed as relative light units. Luciferase activities were corrected for the β-galactosidase activity present. Assays were conducted in triplicate, and data represent the mean ± S.E. of more than three individual experiments.

Gel Mobility Shift DNA Binding Assay—Gel mobility shift assays were carried out as described in several reports (38). In standard conditions, synthetic oligonucleotides representing each strand of sequences were purified by polyacrylamide gel electrophoresis, eluted, and annealed. Double-stranded oligonucleotides were radiolabeled with [^{32}P]dCTP (>3300 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) by fill-in reactions using Klenow large fragment DNA polymerase. Labeled probes were separated from unincorporated nucleotides by centrifugation through a Sephadex G-25 column, which was equilibrated with 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 150 mM NaCl. Radiolabeled probes (10 fmol, 20,000–30,000 cpm) were then incubated with binding proteins in 30 μl of reaction mixture containing 10 mM KPO_4 (pH 8.0) buffer, 1 mM EDTA, 80 mM KCl, 1 μg of poly(dI-dC), 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 5 μg of bovine serum albumin, and 10% glycerol. These reactions were incubated for 30 min at room temperature and analyzed on a 5% nondenaturing polyacrylamide gel in TAE buffer. Electrophoresis was performed at a constant voltage of 200 V at 4 °C in the same buffer. Gels were dried under vacuum and autoradiographed for 6–12 h at room temperature. Complexes were quantified by densitometric scanning of autoradiographs and by liquid scintillation counting of excised gel slices. Both methods gave essentially identical results. The sequences of the probes used in this study are listed as follows: PPRE 5′-gatccTGACCTTTGTCCTg-3′ for sense strand; 5′-gatccAGGACAAAGGTCAg-3′ for antisense strand; DR4 5′-gatccAGGTCACAGGAGGTCAg-3′ for sense strand; 5′-gatccTGACCTCCTGTGACCTg-3′ for antisense strand. The source of PPRE and DR4 are from *aox* gene and rat malic enzyme gene, respectively.

RESULTS

TRα1 Inhibits the PPAR Action on PPRE but RAR or VDR Do Not—In a transient transfection system using COS1 cells, coexpression of TRα1 suppressed the PPRE-TK luciferase activity induced by PPARα in the presence of clofibric acid, whereas equivalent amounts of the empty expression vector did not inhibit PPARα-mediated transcription (Fig. 2). In the absence of PPRE, TK-luciferase reporter was not affected by cotransfection of PPARα or TRα1 in the presence of clofibric acid or T3, respectively (Fig. 2). Cotransfection of increasing amounts of TRα1 expression vector showed that the inhibitory effect of TRα1 on PPRE occurred at doses of TRα1 similar to those necessary for activation of a T3 response element (DR4-TRE) (Fig. 3), suggesting a physiological role of TRα1 in negative control of gene expression through PPRE. Then we tested the specificity of the inhibitory effect among other members of the nuclear receptor family. Expression plasmids for RARα or VDR were cotransfected with the expression vector for PPARα and PPRE-TK-luciferase reporter construct. As shown in Fig. 4, RARα or VDR did not inhibit the trans-activation of PPRE-TK-luciferase reporter by PPARα, whereas TRα1 effectively blocked the transcriptional activation of the reporter.

Negative Effect of TRα1 Was Not Reversed by Coexpression of

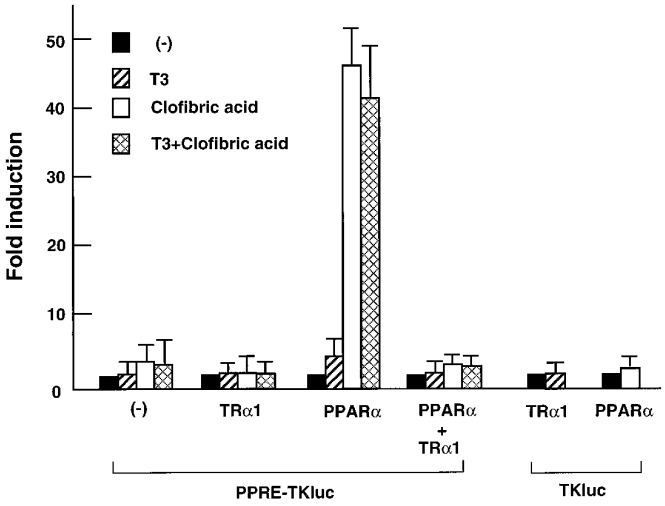

FIG. 2. **TRα1 inhibits PPAR action on PPRE.** COS1 cells were transfected with either TK-luciferase (*TKluc*) or PPRE-TK-luciferase reporter plasmid (250 ng). Twenty five ng of expression plasmid consisting of either the parental CDM expression vector or 12.5 ng of TRα1 expression vector and 12.5 ng of the parental CDM vector or a combination of PPAR and TRα1 expression vectors (12.5 ng each) was cotransfected. Cells were treated with either dimethyl sulfoxide as vehicle or 10^{-3} M clofibrate in the presence or absence of 10^{-7} M T3, as indicated. Cell extracts were assayed for luciferase activity. All luciferase activity was corrected for transfection efficiency by measuring β-galactosidase activity. Normalized luciferase activity was expressed as fold induction relative to untreated cells. Assays were conducted in triplicate, and data represent the mean ± S.E. of five individual transfection experiments. *Error bars* are indicated.

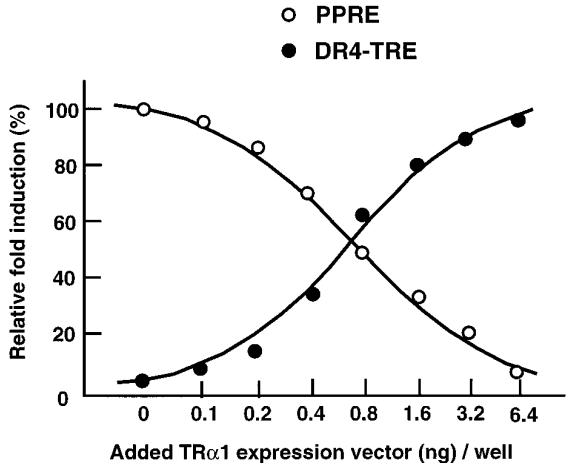

FIG. 3. **Dose dependence of TR expression plasmid for PPAR inhibition and TRE activation.** COS1 cells were transfected with PPRE-TK-luciferase reporter plasmid (250 ng) and 12.5 ng of pCMV-PPAR expression vector (*A*) or DR4-TK-luciferase reporter plasmid (250 ng) (*B*). Indicated amounts of pCDM-TRα1 expression plasmid were cotransfected. Cells were treated with vehicle or 10^{-3} M clofibric acid (*A*) or T3 (10^{-3} M) (*B*). Normalized luciferase activity was expressed as fold induction relative to untreated cells. Assays were conducted in triplicate, and data represent the mean of three individual experiments.

RXRα—Chu *et al.* (39) reported a similar inhibition of TR on the PPAR-regulated peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA hydrogenase gene. They showed that inhibition of this gene by TR was through titration of limiting amounts of RXR. To test this possibility, RXRa expression plasmid was cotransfected into COS1 cells in which inhibitory activity of TRα1 occurred. As shown in Fig. 4, the inhibitory effect of TRα1 is not obliterated by cotransfection of expression plasmid for RXRα, suggesting that inhibition is not due to squelching of the cofactor, RXR. This *in vivo* study indicates that sequestering of RXR is not responsible for the inhibitory effect of TRα1.

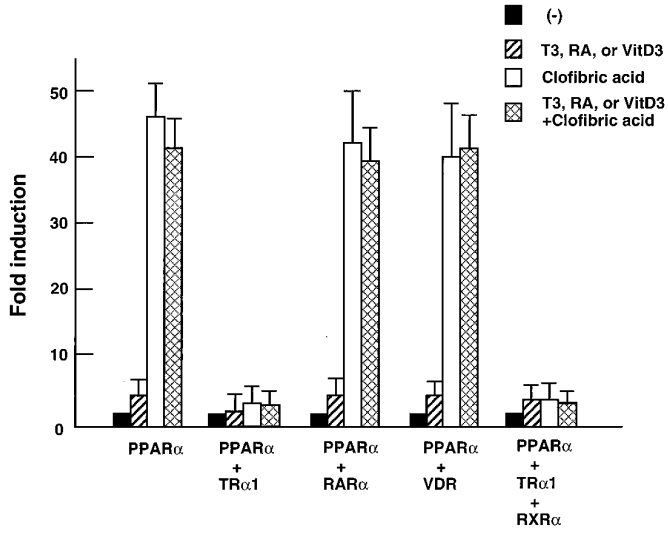

FIG. 4. **Effect of RAR and VDR on PPAR activity.** COS1 cells were transfected with PPRE-TK-luciferase reporter plasmid (250 ng) and 12.5 ng of pCMV-PPAR. Indicated parental expression vector or receptor expression vector (12.5 ng each) was cotransfected. Cells were treated with either dimethyl sulfoxide as vehicle or $10-3$ M clofibrate in the presence or absence of 10^{-3} M of T3 for TR, or 10^{-3} M of all *trans*-retinoic acid (*RA*) for RAR, or 10^{-3} M of 1–25-OH vitamin D3 (*VitD3*) for VDR. Cell extracts were assayed for luciferase activity. All luciferase activities were corrected for transfection efficiency by measuring β-galactosidase activities. Normalized luciferase activity was expressed as fold induction relative to untreated cells. Assays were conducted in triplicate, and data represent the mean ± S.E. of three individual experiments.

Introduction of a Mutation in the DNA Binding Domain of TRα1 Eliminates the Inhibitory Effect of TR on PPAR—To define the mechanism for inhibitory effect of TRα1 on PPARα action, we examined the contribution of the DNA binding domain of TRα1 to this inhibition. A mutation introduced into the P box in the DNA binding domain (DBD) of the TRα1 was designed to prevent its binding to DNA (TRα1 DBD mutant) (36). In transient cotransfection assay, as shown in Fig. 5, the DBD mutant did not show an inhibitory effect on PPARα, indicating that competition for DNA binding is involved. These results indicate that TRα1 regulates PPARα-mediated transcriptional activation of genes containing PPRE through competing binding to PPRE.

Expression of TRα1 DBD Mutant Protein—Expression of wild type and DBD mutant proteins, as determined by Western blot, has been previously reported (36). Expression of the wild type and mutant proteins was examined by transfection of expression plasmids into COS1 cells. T3 binding capacity of protein expressed by pCDα1 DBD mutant was, as expected, equivalent to wild type and was not influenced by cotransfection of PPAR expression vector (Table I). It is logical to consider that the lack of inhibition by the DBD mutant receptor is not due to the amounts of mutant protein present in COS1 cells in transient transfection assays.

TR Modulates PP-dependent Transcriptional Activation by PPAR of the aox Gene—We tested whether TRα1 can inhibit PPARα activity on a native promoter as well as an heterogeneous promoter (TK promoter). We confirmed transcriptional regulation by TR of the gene containing a PPRE, using a rat acyl-CoA oxidase (*aox*) gene promoter. *aox*-luciferase reporter plasmid was activated by PPARα in the presence of clofibric acid. This activation was completely suppressed by cotransfection of TRα1 expression plasmid (Fig. 6). These results are similar to those observed when PPRE-TK luciferase was used.

Binding of TRα1 to PPAR and TRE—To test the ability of TRα1 to bind to PPRE *in vitro*, gel mobility shift assay was

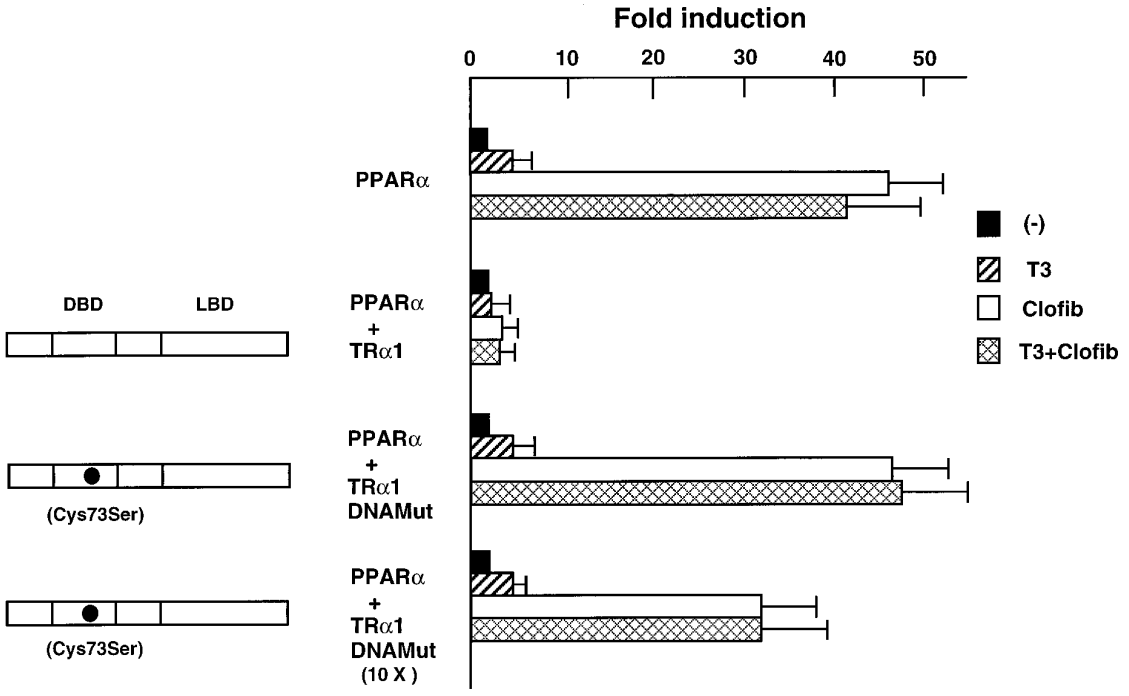

FIG. 5. **Mutation in DNA binding domain of TRα1 restored the inhibition of PPAR.** COS1 cells were transfected with PPRE-TK-luciferase reporter plasmid (250 ng) and 12.5 ng of pCMV-PPAR. Parental expression vector or receptor expression vector was cotransfected as indicated. Cells were treated with either dimethyl sulfoxide as vehicle or 10^{-3} M clofibrate in the presence or absence of 10^{-7} M T3. Cell extracts were assayed for luciferase activity. All luciferase activities were corrected for transfection efficiency by measuring β-galactosidase activities. Normalized luciferase activity was expressed as fold induction relative to untreated cells. Assays were conducted in triplicate, and data represent the mean ± S.E. of four individual experiments.

TABLE I

Protein expression of TRα1 and its DBD mutant in COS1 cells

TRα1 or TRα1 DBD mutant expression vector (2 μg) were transfected into COS1 cells in 10-cm plates with or without PPAR expression vector (5 μg) using the calcium phosphate precipitation method. Twelve hours after transfection, the medium was changed, and cells were incubated another 36 h. Cell extracts were prepared, and T3 binding assays were performed as described previously (61). T3 binding is shown as specific binding per total T3 added, corrected for β-galactosidase activity.

	Minus PPAR	PPAR	PPAR + clofibric acid (+)
TRα1	31.4 ± 4.1	30.0 ± 3.4	33.2 ± 4.8
TRα1(BDB)mut	32.5 ± 3.5	33.4 ± 4.6	34.2 ± 4.2

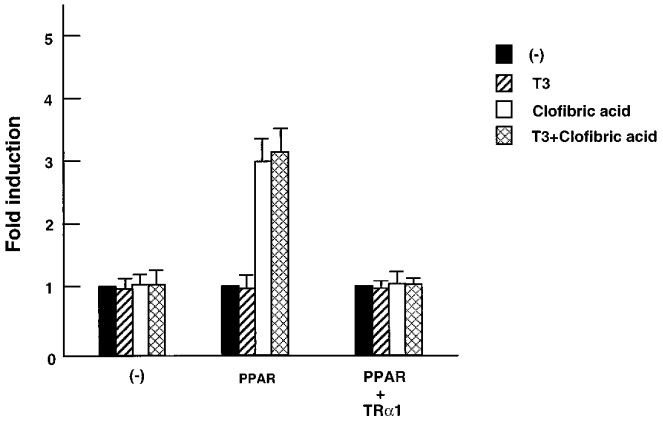

FIG. 6. **TR modulates PP-dependent transcriptional activation by PPAR in *aox* gene.** COS1 cells were transfected with the *aox* promoter in a luciferase reporter plasmid. Twenty five ng of expression plasmid consisting of either the parental CDM expression vector or 12.5 ng of receptor and 12.5 ng of the parental CDM vector or a combination of PPAR and TRα1 expression vector (12.5 ng each) was cotransfected. Cells were treated with either dimethyl sulfoxide as vehicle or 10^{-3} M clofibrate in the presence or absence of 10^{-7} M T3. Cell extracts were assayed for luciferase activity. All luciferase activities were corrected for transfection efficiency by measuring β-galactosidase activities. Normalized luciferase activity was expressed as fold induction relative to untreated cells. Assays were conducted in triplicate, and data represent the mean ± S.E. of three individual experiments.

performed using TRα1 and RXRα expressed in Sf9 insect cells. The results of these experiments employing TRα1, RXRa, and ^{32}P-labeled probes are shown in Fig. 7. TRα1 plus RXRa produced a retarded heterodimeric band with PPRE, and increasing amounts of unlabeled probe displaced the binding, as observed when a classical TRE (DR4) was utilized as a probe. To achieve high concentrations of DNA, we diluted a fixed amount of the particular radioactive probe with increasing amounts of unlabeled DNA and approximately corrected the specific activity in calculations. Bound and free complexes were quantified by a densitometric analysis. The TRα1·RXRα binding to DNA increased with increasing concentrations of DNA and approached saturation. Scatchard plots of data obtained using the PPRE and DR4 probes could be interpreted as a straight line and a single biomolecular reaction. The K_a was calculated from the slope of the Scatchard plots. The K_a values for binding of TRα1·RXRα heterodimers to PPRE and TRE (DR4) are (2.2×10^9 M^{-1}) and (2.5×10^9 M^{-1}), respectively.

DISCUSSION

It is well documented that hypolipidemic drugs, such as clofibrate, induce peroxisome proliferation in rodent liver and increase the activity of enzymes involved in peroxisomal β-oxidation of fatty acids (1–5). Regulation of the expression of genes involved in lipid metabolism by hypolipidemic drugs and hormones is of great physiological and clinical interest. In this paper we show that TRα1 negatively regulates PPRE containing genes by competing for DNA binding with PPARα. TRα1 inhibits the binding of PPARα to *aox*-PPRE, resulting in the suppression of peroxisome proliferator-dependent activation by PPARα. This is a novel mechanism of actions of TR, to regulate gene expression through the DR1 motif (PPRE).

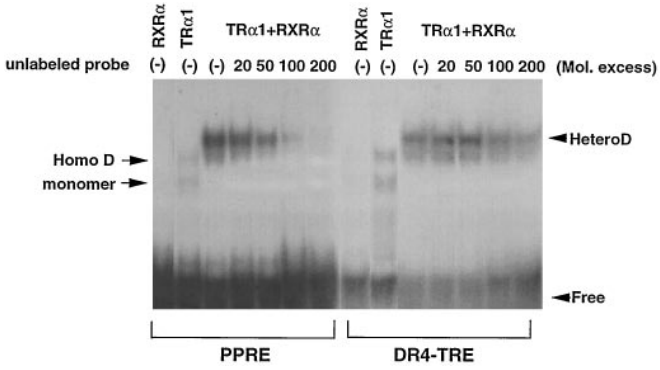

FIG. 7. **TR homodimers and heterodimers with RXR can bind to PPRE(DR1).** Ability of TR to bind DR4-TRE and DR1-PPRE from *aox* gene was tested. Sf9 cells extracts containing 50 fmol of TRα1 were incubated with 20 fmol of ^{32}P-labeled DR1-PPRE (*A*) or DR4-TRE (*B*). Increasing amounts (0, 200, 400, 1000–2000 fmol) of each unlabeled probe were coincubated. Reactions were incubated for 30 min at room temperature and analyzed on a 5% nondenaturing polyacrylamide gel in TAE buffer. Electrophoresis was performed at a constant voltage of 200 V at 4 °C in the same buffer. Gels were dried under vacuum and autoradiographed for 6–12 h at room temperature. The results shown are representative of three experiments.

To date, several enzymes, which are involved in peroxisomal β-oxidation, have been shown to be regulated by PPAR through a PPRE in the promoter region. These include the peroxisomal fatty acid acyl-CoA oxidase (9, 19, 20), peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme) (40, 41), the liver fatty acid binding protein (42), and the rabbit P450 4A6 fatty acid ω-hydroxylase (21). Our results strongly suggest that these enzymes might be modulated by TRs through convergence of PPAR signaling pathways.

The mechanism for negative regulation of PPARα-mediated transcription by TRα1 was clarified in this paper. In general, three different mechanisms are proposed for inhibition of transcription factors: 1) competition for binding to a response element, 2) formation of inactive heterodimers, and 3) squelching a cofactor. It has been reported that PPARα is able to modulate TRβ1 activity by forming TRβ1·PPARα heterodimers (29) or by competing for heterodimerization with RXR (43). In this study, the importance of DNA binding activity of TRα1 for negative regulation of PPARα was demonstrated using an artificial mutant receptor (TRα1 DBD mutant). A mutation at a base coding for a cysteine residue in the P box (44) of the first zinc finger of the DNA binding domain in TRα1 destroyed binding to DNA. This artificial DBD mutant TRα1 reveals no inhibitory effect on PPARα action in transient transfection assay, whereas wild type TRα1 acts as a strong suppressor of PPARα (Fig. 2). Previously, we confirmed by Western blotting that both wild type TRα1 and TRα1 DBD mutant are expressed at similar levels in COS1 cells when identical amounts of expression plasmids are transfected (36). It is important to know whether the TRα1 DBD mutant is appropriately expressed in COS1 cells, since differences in suppression of PPAR could be due to expression of different amounts of wild type TRα1 or TRα1 DBD mutant in COS1 cells in the transient transfection assay. In this paper, we reconfirmed the similar expression levels of wild type and DBD mutant TRα1 by T3 binding analysis (Table I). We have now shown that DNA binding is required for inhibition of PPARα activity by TRα1.

A second possible inhibitory mechanism is formation of inactive TRα1·PPARα heterodimers. Interestingly, weak suppression of PP-dependent transcription by PPARα was observed when an excess amount of TRα1 DBD mutant was cotransfected (Fig. 5). The suppression was weak but statistically significant. We speculate that this inhibition by the DBD mutant TR could be mediated by formation of inactive heterodimers. The DBD mutant receptor could form TRα1 DBD mutant-PPARα heterodimers and decreased the number of functional PPARαs. Indeed, Bogazzi *et al.* (29) reported that TRβ1 and PPARα form heterodimers in solution (29), resulting in the inhibition of transcriptional activation by TRβ1. TRα1·PPARα heterodimers may be inactive in PP-dependent trans-activation on PPRE (DR1). In fact, introduction of a second mutation into the TRα1 DBD mutant (TRα1DBD+9th heptad mutant), adds an artificial mutation in the 9th heptad region of TRα1, and this TR has no inhibitory effect on PPARα signaling even when present in excess amounts (data not shown). The 9th heptad region is thought to be a domain important for dimer formation with partner proteins. This formation of inactive heterodimers is possibly involved in inhibition of PPARα by TRα1. This could explain the inhibition seen with excess amounts of the TRα1 DBD mutant, which still retains the activity for dimerization, although the inhibition is weaker than by wild type TRα1 (Fig. 5).

It must be noted that a much higher concentration of TR is required for inhibition by TRα1 DBD mutant than by wild type TRα1. These results indicate that a supraphysiological concentration of TR is required for inhibition of PPAR activity by forming inactive TR·PPAR heterodimers. Inhibition through DNA binding competition occurs at a lower concentration of TRα1 than inhibition through formation of inactive TRα1·PPAR heterodimers. Therefore, the mechanism of competition for DNA binding appears to be most important in the physiological situation.

A third possible mechanism is a squelching effect. Recently Chu *et al.* (39) reported inhibition by TR of the PPAR-regulated peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene. Their findings indicated that inhibition of this gene by TR was ligand-dependent and through titration of limiting amounts of RXR. This finding is in agreement with reports by Juge-Aubry *et al.* (43) and Hunter *et al.* (45). Their observations appear to conflict with our data. However, at least on a PPRE from the acyl-CoA oxidase gene, coexpression of RXRα cannot reverse the inhibitory effect of TRα1 (Fig. 4), suggesting that inhibition by TRα1 was not mediated through the sequestration of limiting amounts of RXR by TRα1. Further evidence to support this notion is the absence of inhibitory effect by VDR and RAR. In cotransfection studies, neither VDR nor RAR influences PPAR activity, regardless of their capacity to heterodimerization with RXR. Differences between their data and ours may be due to use of PPREs from different genes. Our observations suggest that competition for DNA binding must be the main mechanism for inhibition of PPARα by TRα1 on the PPRE from acyl-CoA oxidase gene.

To confirm that inhibition occurred under physiological conditions, it was important to titrate the dose of TR expression plasmid necessary for inhibition of PPARα. We compared the titration curve of TRα1 expression plasmid which was necessary for inhibition of PPARα on PPRE with that necessary for T3-dependent trans-activation of TRE. Experiments with cotransfection of an increasing amount of TRα1 expression vector showed that the inhibitory effect by TRα1 on PPRE occurred with a dose of TRα1 similar to that necessary for activation of the T3 response element. This suggests a physiological role for TRα1 in controlling gene expression through the PPRE *in vivo*.

We confirmed the high affinity binding of TRα1 to a PPRE in gel shift assays. Umesono *et al.* (46) have shown that VDR, TR, and RAR specifically activate transcription of genes containing AGGTCA direct repeats with spacings of 3, 4, and 5 base pairs, respectively. Thus, the ability of a receptor to recognize, discriminate, and bind to variants of the AGGTCA core sequence

is critical to its function. Analysis of natural PPAR response elements has shown that PPARα can bind to core elements with a spacing 1 base pair (27). The results of DNA binding experiments clearly show that TRα1·RXRa heterodimers can bind to PPRE(DR1) as well as to a classical TRE(DR4). TRα1, however, cannot activate the PPRE in the presence of T3, even with high affinity binding as a heterodimer with RXRα.

The convergence of retinoid and PPAR signaling pathways has been analyzed by several groups (27, 28, 47, 48), and it has been shown that both PPARα and RXRα stimulate the acyl-CoA oxidase gene through PPARα·RXRα heterodimers that bind PPREs. Therefore, most probably PPARα·RXR heterodimers are competed on PPRE by TRα1·RXR heterodimers, resulting in the suppression of PP-dependent transcriptional activation.

Several lines of evidence suggest direct competition of nuclear receptors for target DNA sequence. The estrogen receptor and thyroid hormone receptor each bind to a palindromic estrogen response element, although only the estrogen receptor mediates transcriptional activation via this sequence (49). We show in this paper that both the PPARα and TRα1 molecule bind to PPRE (DR1), although only the PPARα mediates transcriptional activation through PPRE, and TRα1 inhibits the PPARα activity on PPRE. These results suggest that DNA binding, even it is specific and with high affinity *in vitro*, is not enough for trans-activation. The DNA sequence of the regulatory element itself contains information regulating trans-activation by TRs.

Kurokawa *et al.* (50) demonstrated that RXR·RAR heterodimers bind to DR1 motifs but do not activate transcription, whereas they bind in the opposite polarity on natural RAREs containing DR5 motifs, where they are functional. Thus, RAR·RXR heterodimers also seem to be potential competitors with PPAR·RXR for binding to PPREs which are also DR1 motifs. However, our results demonstrated that RARα does not influence the PPARα mediated trans-activation of PPRE (Fig. 4). Furthermore, the PPRE luciferase reporter was not activated by cotransfection of RXRα in the presence of 9-*cis*-retinoic acid, whereas RXRE, which also consists of a DR1 motif, was activated by RXRα in the presence of 9-*cis*-retinoic acid (data not shown). Differences of the flanking sequence or the sequence between the hexamers might discriminate between PPRE and RXRE. Thus, we can suppose that RAR·RXR or RXR·RXR dimers are not able to bind to PPRE so efficiently as PPAR·RXR or TR·RXR heterodimers.

Results in this study strongly suggest that alteration of TR expression level influences the transcriptional activity of genes that are regulated by PPAR via PPREs. Several conditions that alter the TR expression are reported. For example, fasting decreased the maximal T3 binding capacity (51–53) and increased the fatty acid turnover. Our results possibly connect the relationship between fasting and activated fatty acid metabolism. PPAR activity may be released from suppression by TR due to decreased number of TR during fasting, resulting in the increased transcriptional levels of enzymes regulating fatty acid β-oxidation such as *aox* gene. Furthermore, TR expression is regulated by hormones (54) and strictly controlled during ontogeny and development (55–58). TRs exert their effects on lipid metabolism through convergence of PPAR signaling pathways. Recently, PPAR has shown to be involved in the activation of the adipocyte-specific *AP2* gene through PPRE (59, 60), and PPARγ plays an important role in differentiation of adipocytes. It is possible that TR might regulate the differentiation of adipocytes through controlling PPAR-mediated transcription.

In conclusion, we demonstrate the remarkable potential of TRα1 to compete with PPARα signaling pathway regulating lipid metabolism, cell growth, and differentiation. Nuclear receptors appeared to have a great diversity of actions and promiscuous interaction. We presented further evidence for cross-talk among nuclear receptor signaling pathways.

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REFERENCES

1. Vamecq, J. & Draye, J. P. (1989) *Essays Biochem.* **24,** 115–225
2. Reddy, J. K. & Mannaerts, G. P. (1994) *Annu. Rev. Nutr.* **14,** 343–370
3. Nemali, M. R., Reddy, M. K., Usuda, N., Reddy, P. G., Comeau, L. D., Rao, M. S. & Reddy, J. K. (1989) *Toxicol. Appl. Pharmacol.* **97,** 72–87
4. Lock, E. A., Mitchell, A. M. & Elcombe, C. R. (1989) *Annu. Rev. Pharmacol. Toxicol.* **29,** 145–163
5. Berge, R. K., Hosoy, L. H., Aarsland, A., Bakke, O. M. & Farstad, M. (1984) *Toxicol. Appl. Pharmacol.* **73,** 35–41
6. Marsman, D. S., Cattley, R. C., Conway, J. G. & Popp, J. A. (1988) *Cancer Res.* **48,** 6739–6744
7. Cattley, R. C. & Popp, J. A. (1989) *Cancer Res.* **49,** 3246–3251
8. Issemann, I. & Green, S. (1990) *Nature* **347,** 645–650
9. Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. & Wahli, W. (1992) *Cell* **68,** 879–887
10. Laudet, V., Hanni, C., Coll, J., Catzeflis, F. & Stehelin, D. (1992) *EMBO J.* **11,** 1003–1013
11. Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K. & Evans, R. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7355–7359
12. Sher, T., Yi, H. F., McBride, O. W. & Gonzalez, F. J. (1993) *Biochemistry* **32,** 5598–5604
13. Schmidt, A., Endo, N., Rutledge, S. J., Vogel, R., Shinar, D. & Rodan, G. A. (1992) *Mol. Endocrinol.* **6,** 1634–1641
14. Gottlicher, M., Widmark, E., Li, Q. & Gustafsson, J. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4653–4657
15. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. & Wahli, W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2160–2164
16. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M. & Kliewer, S. A. (1995) *J. Biol. Chem.* **270,** 12953–12956
17. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M. & Evans, R. M. (1995) *Cell* **83,** 803–812
18. Kliewer, S. A., Lenhard, J. M., Wilson, T. M., Patel, I., Morris, D. C. & Lehmann, J. M. (1995) *Cell* **83,** 813–819
19. Osumi, T., Wen, J. K. & Hashimoto, T. (1991) *Biochem. Biophys. Res. Commun.* **175,** 866–871
20. Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPheat, W. L. & Green, S. (1992) *EMBO J.* **11,** 433–439
21. Muerhoff, A. S., Griffin, K. J. & Johnson, E. F. (1992) *J. Biol. Chem.* **267,** 19051–19053
22. Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S. & Evans, R. M. (1991) *Cell* **66,** 555–561
23. Yu, V. C., Delsert, C., Andersen, B., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K. & Rosenfeld, M. G. (1991) *Cell* **67,** 1251–1266
24. Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. & Evans, R. M. (1992) *Nature* **355,** 446–449
25. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Garnier, J. M., Mader, S. Chambon, P. (1992) *Cell* **68,** 377–395
26. Zhang, X. K., Hoffmann, B., Tran, P. B., Graupner, G. & Pfahl, M. (1992) *Nature* **355,** 441–446
27. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. & Evans, R. M. (1992) *Nature* **358,** 771–774
28. Gearing, K. L., Gottlicher, M., Teboul, M., Widmark, E. & Gustafsson, J. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1440–1444
29. Bogazzi, F., Hudson, L. D. & Nikodem, V. M. (1994) *J. Biol. Chem.* **269,** 11683–11686
30. Heimberg, M., Olubadewo, J. O. & Wilcox, H. G. (1985) *Endocr. Rev.* **6,** 590–607
31. Ichikawa, K., Hashizume, K., Yamada, T. & Hashimoto, T. (1987) *Endocrinol. Jpn.* **34,** 245–250
32. Takeda, T., Ichikawa, K., Miyamoto, T., Kobayashi, M., Nishii, Y., Suzuki, S., Sakurai, A. & Hashizume, K. (1992) *Biochem. Biophys. Res. Commun.* **185,** 211–216
33. Blennemann, B., Moon, Y. K. & Freake, H. C. (1992) *Endocrinology* **130,** 637–643
34. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
35. Miyamoto, T., Suzuki, S. & DeGroot, L. J. (1994) *Mol. Cell. Endocrinol.* **102,** 111–117
36. Liu, R.-T., Suzuki, S., Miyamoto, T. & DeGroot, L. J. (1995) *Mol. Endocrinol.* **9,** 86–95
37. Suzuki, S., Miyamoto, T., Opsahl, A., Sakurai, A. & DeGroot, L. J. (1994) *Mol. Endocrinol.* **8,** 305–314
38. Miyamoto, T., Suzuki, S. & DeGroot, L. J. (1993) *Mol. Endocrinol.* **7,** 224–231
39. Chu, R., Madison, L. D., Lin, Y., Kopp, P., Rao, M. S., Jameson, J. L. & Reddy,

J. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11593–11597
40. Zhang, B., Marcus, S. L., Miyata, K. S., Subramani, S., Capone, J. P. & Rachubinski, R. A. (1993) *J. Biol. Chem.* **268,** 12939–12945
41. Bardot, O., Aldridge, T. C., Latruffe, N. & Green, S. (1993) *Biochem. Biophys. Res. Commun.* **192,** 37–45
42. Issemann, I., Prince, R., Tugwood, J. & Green, S. (1992) *Biochem. Soc. Trans.* **20,** 824–827
43. Juge-Aubry, C. E., Gorla-Bajszczak, A., Pernin, A., Lemberger, T., Wahli, W., Burger, A. G. & Meier, C. A. (1995) *J. Biol. Chem.* **270,** 18117–18122
44. Umesono, K. & Evans, R. M. (1989) *Cell* **57,** 1139–1146
45. Hunter, J., Kassam, A., Winrow, C. J., Rachubinski, R. A. & Capone, J. P. (1996) *Mol. Cell. Endocrinol.* **116,** 213–221
46. Umesono, K., Murakami, K. K., Thompson, C. C. & Evans, R. M. (1991) *Cell* **65,** 1255–1266
47. Rodriguez, J. C., Gil-Gomez, G., Hegardt, F. G. & Haro, D. (1994) *J. Biol. Chem.* **269,** 18767–18772
48. Palmer, C. N. A., Hsu, M.-H., Muerhoff, A. S., Griffin, K. J. & Johnson, E. F. (1994) *J. Biol. Chem.* **269,** 18083–18089
49. Graupner, G., Zhang, X. K., Tzukerman, M., Wills, K., Hermann, T. & Pfahl, M. (1991) *Mol. Endocrinol.* **5,** 365–372
50. Kurakawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gloss, B., Rosenfeld, M. G., Heyman, R. A. & Glass, C. K. (1994) *Nature* **371,** 528–531
51. Rodriguez, M., Rodriguez, F. & Jolin, T. (1992) *Endocrinology* **131,** 1612–1618
52. Lane, J. T., Godbole, M., Strait, K. A., Schwartz, H. L. & Oppenheimer, J. H. (1991) *Endocrinology* **129,** 2881–2885
53. Thrall, C. L. (1983) *Brain Res.* **279,** 177–183
54. Hodin, R. A., Lazar, M. A. & Chin, W. W. (1990) *J. Clin. Invest.* **85,** 101–105
55. Falcone, M., Miyamoto, T., Fierro, R. F., Macchia, E. & DeGroot, L. J. (1994) *Eur. J. Endocrinol.* **130,** 97–106
56. Tuca, A., Giralt, M., Villarroya, F., Vinas, O., Mampel, T. & Iglesias, R. (1993) *Endocrinology* **132,** 1913–1920
57. Rodd, C., Schwartz, H. L., Strait, K. A. & Oppenheimer, J. H. (1992) *Endocrinology* **131,** 2559–2564
58. North, D., Lakshmanan, J., Reviczky, A., Kaser, M. & Fisher, D. A. (1992) *Pediatr. Res.* **31,** 330–334
59. Tontonoz, P., Graves, R. A., Budavari, A. I., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P. & Spiegelman, B. M. (1994) *Nucleic Acids Res.* **22,** 5628–5634
60. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I. & Spiegelman, B. M. (1994) *Genes Dev.* **8,** 1224–1234
61. Miyamoto, T., Sakurai, A. & DeGroot, L. J. (1991) *Endocrinology* **129,** 3027–3033