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Transactivation by PPAR/RXR Heterodimers in Yeast Is Potentiated by Exogenous Fatty Acid Via a Pathway Requiring Intact Peroxisomes

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Peroxisome proliferator-activated receptors (PPARs) are orphan members of the nuclear hormone receptor superfamily. PPARs bind to cognate response elements through heterodimerization with retinoid X receptors (RXRs). Together PPAR/RXR regulate the transcription of genes for which products are involved in lipid homeostasis, cell growth, and differentiation. PPARs are activated by fatty acids and by nongenotoxic rodent hepatocarcinogens called peroxisome proliferators through as of yet undefined signal transduction pathways. In an effort to elucidate the requirements for PPAR function and the pathways of its activation, we expressed mouse PPARα and human RXRα in the yeast *Saccharomyces cerevisiae*. Mouse PPARα and human RXRα had little activity individually in yeast; however, when cosynthesized, they were able to synergistically activate transcription via cognate response elements. Transactivation was independent of exogenously added activators of either receptor but was potentiated by the addition of petroselinic acid, a fatty acid shown to activate PPARs in mammalian cells. Similar experiments were carried out in a mutant yeast strain lacking peroxisomes entirely or in a mutant strain deficient for 3-ketoacyl-CoA thiolase, the final enzyme of the peroxisomal β-oxidation cascade. The findings showed that constitutive transactivation by PPAR/RXR did not require the complete β-oxidation pathway or intact peroxisomes but required intact peroxisomes for potentiation by exogenously added petroselinic acid. This study demonstrates that at least part of the mammalian peroxisome proliferator-signaling pathway can be faithfully reconstituted in yeast and that activation of PPAR by at least one particular fatty acid requires the integrity of peroxisomes.

Peroxisome proliferator Nuclear receptor Yeast

PEROXISOMES are essential for the normal β-oxidation of fatty acids and thus play a key role in regulating lipid homeostasis in mammals (Vamecq and Draye, 1989; van den Bosch et al., 1992). Peroxisome proliferators, which include the fibrate family of hypolipidemic drugs, herbicides, and phthalate ester plasticizers, form a large group of xenobiotic compounds that increase both the number and metabolic capacity of hepatic peroxisomes (Reddy et al., 1980; Styles et al., 1988). Many peroxisome proliferators are nongenotoxic carcinogens that induce hepatocarcinogenesis in rodents (Reddy and Lalwani, 1983; Rao and Reddy, 1991; Lock et al., 1989; Bentley et al., 1993). Because of their ubiquity and potential for carcinogenesis, there is strong interest in understanding the mechanism of action of peroxisome proliferators and in assessing the possible health

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risks to humans due to exposure to these compounds.

The pleiotropic cellular effects of peroxisome proliferators are manifested in part by the transcriptional induction of a number of genes encoding peroxisomal and microsomal enzymes involved in lipid metabolism (Reddy et al., 1986; Sharma et al., 1988). These genes include those coding for fatty acyl-CoA oxidase (AOx) and hydratase-dehydrogenase (HD), the first two enzymes of the peroxisomal β-oxidation pathway, and the CYP4A6 gene coding for a member of the cytochrome P450 fatty acid ω-hydroxylase family. Transactivation of peroxisome proliferator-responsive genes is mediated by members of the steroid/thyroid hormone receptor superfamily called peroxisome proliferator-activated receptors (PPARs) that bind to specific peroxisome proliferator-responsive elements (PPREs) through heterodimerization with retinoid X receptors (RXRs) (Issemann and Green, 1990; Kliewer et al., 1992; Gearing et al., 1993; Keller et al., 1993a, 1993b; Marcus et al., 1993). PPREs have been identified in the 5′ flanking regions of the rat AOx (Osumi et al., 1991; Tugwood et al., 1992), rat HD (Zhang et al., 1992, 1993; Bardot et al., 1993), and rabbit CYP4A6 (Muerhoff et al., 1992) genes.

PPARs constitute a growing family of ligand-activated transcription factors, and multiple PPAR cDNAs have been cloned from several different species, including human (Schmidt et al., 1992; Sher et al., 1993), mouse (Issemann and Green, 1990; Zhu et al., 1993; Chen et al., 1993; Tontonoz et al., 1994), rat (Göttlicher et al., 1992), and *Xenopus* (Dreyer et al., 1992). It is becoming increasingly apparent that PPARs not only mediate the cellular response to hypolipidemic drugs and nongenotoxic carcinogens but also play fundamental roles in regulating the expression of a wide spectrum of genes involved in lipid homeostasis, differentiation, cell growth, and oncogenesis (Ockner et al., 1993; Auwerx, 1992; Chawla and Lazar, 1994; Tontonoz et al., 1994; Ledwith et al., 1993). PPARs can be activated by a variety of structurally diverse peroxisome proliferators as well as by several natural and synthetic fatty acids, demonstrating that regulation of gene expression by fatty acids and peroxisome proliferators can be linked and integrated through common, or convergent, regulatory circuits (Auwerx, 1992; Issemann et al., 1993; Keller et al., 1993a; Dreyer et al., 1993; Göttlicher et al., 1992). There is therefore considerable interest in elucidating the physiological roles of PPARs and their pathways of activation.

Much of our knowledge of PPAR function has come from transient transfection assays in mammalian cell cultures. However, the presence of endogenous nuclear hormone receptors and of putative activators of the peroxisome proliferator-response pathway precludes a direct investigation of the mechanisms of PPAR action in mammalian cells. The potential of various mammalian cellular proteins for heterodimerization with RXR, and possibly with PPAR, and the finding that other orphan receptors such as COUP-TF (Miyata et al., 1993) and HNF-4 (Winrow et al., 1994) can also bind to PPREs and modulate PPAR function have made it difficult to directly investigate the autonomous or cooperative functioning of individual PPARs and RXRs in the transcriptional activation of specific target genes. Indeed, although PPAR and RXR bind synergistically to PPREs in vitro, cosynthesis of receptors in mammalian cells results only in additive transcriptional effects, even in the presence of the RXRα-activating ligand 9-*cis*-retinoic acid and peroxisome proliferators (Keller et al., 1993a; Gearing et al., 1993). Moreover, ectopic synthesis of either receptor alone can stimulate PPRE-linked reporter genes (Keller et al., 1993a; Marcus et al., 1993), possibly due to cooperativity with endogenous cellular factors. Therefore, it has not yet been established whether PPAR functions exclusively, or necessarily, through cooperativity with RXR in vivo. Indeed, it has recently been demonstrated that mouse PPARα can also heterodimerize with the thyroid hormone receptor and differentially regulate specific thyroid hormone response genes (Bogazzi et al., 1994).

Despite the fact that a large number of compounds have been shown to be capable of activating PPARs in mammalian cells, none of these agents has been shown to specifically bind these receptors. Accordingly, the mechanisms of PPAR activation remain largely unknown. Studies carried out with metabolic inhibitors and non-β-oxidizable substrates have suggested that proximate PPAR activators are generated from peroxisome proliferators and fatty acids through their metabolic conversion to a common intermediate via the peroxisomal β-oxidation pathway or some enzymatic step prior to β-oxidation (Göttlicher et al., 1993; Bentley et al., 1993; Tomaszewski and Melnick, 1994). However, the role of the peroxisome in general, and the peroxisomal β-oxidation pathway in particular, in PPAR function and activation has not been addressed directly.

The yeast *Saccharomyces cerevisiae* is devoid of

endogenous nuclear receptors and retinoids. Various ectopically expressed mammalian hormone receptors have been shown to function in *S. cerevisiae* and to activate expression via cognate response elements (Metzger et al., 1988). Furthermore, the metabolic processes of yeast peroxisomes, such as β-oxidation, are mechanistically similar to their mammalian counterparts. Indeed, fatty acid β-oxidation in yeast is carried out exclusively in peroxisomes, whereas mitochondria lack this metabolic capacity (Lock et al., 1989; Mannaerts and DeBeer, 1982). We therefore asked whether PPAR could function in vivo in yeast. Our findings demonstrate that mouse (m) PPARα and human (h) RXRα cooperate in yeast to synergistically activate transcription via cognate PPREs in the absence of exogenously added ligands for either receptor and that transactivation is potentiated by at least one exogenously added fatty acid known to activate PPARs in mammalian cells. Moreover, we provide direct evidence that the integrity of peroxisomes is essential for stimulation of PPAR by fatty acid.

MATERIALS AND METHODS

Receptor Expression in S. cerevisiae

Yeast plasmids expressing nuclear hormone receptors were constructed as follows. The cDNA encoding mPPARα was excised from pPPAR/SG5 (Issemann and Green, 1990) as a 1.8-kb pair *Bam*HI fragment. This fragment was cloned into the *Bgl*II site of the phosphoglycerate kinase (PGK) promoter/terminator, which had been inserted into the *Hin*dIII site of the yeast shuttle vector pRS426 (Christianson et al., 1992). The entire mPPARα/PGK cassette was released as a *Bam*HI/*Xho*I fragment and cloned into the vector pRS423 to generate ymPPAR and into the *CEN*-vector pRS313 to generate cmPPAR. Vector yhRXRα expresses hRXRα under control of the PGK promoter. The PGK promoter was first cloned into the shuttle vector pRS425. The hRXRα cDNA was excised from pSRXR3-1 as a 1.8-kb pair *Eco*RI fragment, made blunt with the Klenow fragment of DNA polymerase I, and inserted into the blunted *Bgl*II site of pR425/PGK. mPPARα/PGK was released from pRS426 as a *Xho*I/*Bam*HI fragment and made blunt with Klenow fragment. *Xho*I linkers (5′-CCTCGAGG, New England Biolabs) were then ligated onto this blunt fragment, and the fragment was cut with *Xho*I. The resulting fragment was inserted into yhRXRα digested with *Xho*I to generate a plasmid, PPRXR425, expressing both receptors.

To construct the parental *lacZ* reporter plasmid ΔL1(ura+) from pLR1Δ20 (West et al., 1984), the *Xma*I-*Xho*I fragment upstream of the *GAL1* TATA box, which contains the four UASg elements, was removed. In its place were inserted synthetic *Xma*I-*Sal*I fragments from recombinant pSP73 plasmids containing one copy (1HDΔL1) or two copies (2HDΔL1) of the HD-PPRE oligonucleotide (5′-CCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA), one copy (1HDM3ΔL1) of the HD-PPRE in which the second direct repeat is mutated (5′-CCTCTCCTTTGACCTATTGAAgTATTACCTACATTTGA; Miyata et al., 1993), one copy (1HDM5ΔL1) of the HD-PPRE in which the most 5′ direct repeat is scrambled (5′-CCTCTCCTTTGACCTATTGAACTActattcACATTTGA; Miyata et al., 1993), one copy (1AOxΔL1) of the AOx-PPRE oligonucleotide (5′-CCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT), one copy (1AOxM1ΔL1) of the AOx-PPRE in which the 5′ direct repeat is scrambled (5′-CCTTTCCCGAACGctgcatTTGTCCTGGTCCCCTTTTGCT), and one copy (1AOxM2ΔL1) of the AOx-PPRE in which the 3′ direct repeat is scrambled (5′-CCTTTCCCGAACGTGACCTTgcttctGGTCCCCTTTTGCT). The underlined nucleotides indicate the directly repeated TGACCT motifs. Mutations in nucleotides of direct repeats are designated in lower case. To construct the his+, cen+ plasmid 2HD313, 2HDΔL1 was first digested with *Tth*111I and made blunt with Klenow fragment. *Xho*I linkers were ligated to the blunt ends and were then digested with *Xho*I. The plasmid was recircularized upon itself by ligation. The resulting vector was cut with *Xma*I/*Xho*I, and the insert was ligated into the corresponding restriction sites of pRS313 to generate 2HD313. *S. cerevisiae* strains DL-1 (*MATα*, *leu2,ura3,his3*; van Loon et al., 1983), YPH102 (*Matα,leu2,ura3,his3,lys2,ade2*; Sikorski and Hieter, 1989; van der Leij et al., 1992), and STUD (*Matα,leu2,ura3,his3,THI::URA3*; Glover et al., 1994) were transformed with the various plasmids and grown in 0.67% yeast nitrogen base/2% glucose supplemented with uracil, adenine, and lysine each at 20 $\mu g \cdot ml^{-1}$, as required. Yeast lysates were prepared and β-galactosidase activity was assayed (Ausubel et al., 1989; Himmelfarb et al., 1990).

Electrophoretic Mobility Shift Analysis

Electrophoretic mobility shift analysis using in vitro-translated mPPAR and hRXRα and radiola-

beled HD-PPRE and AOx-PPRE probes were performed as described by Marcus et al. (1993). Standard reactions contained 1 ng of labeled DNA probe, 8 μg of nonspecific competitor DNA [a 1 : 1 mixture of poly(dI-dC) · poly(dI-dC) and sonicated salmon sperm DNA], 60 μg of bovine serum albumin and 4 μl of breakage buffer [400 mM KCl/20 mM Tris-HCl (pH 7.5)/0.1 mM EDTA/20% glycerol/2 mM dithiothreitol/pepstatin (1 μg · ml^{-1})/chymostatin (0.1 μg · ml^{-1})/antipain (2.5 μg · ml^{-1})/leupeptin (0.5 μg · ml^{-1})/aprotinin (5 μg · ml^{-1})/0.2 mM phenylmethylsulfonyl fluoride] in a final volume of 15 μl. Where indicated in Fig. 2, reactions contained 2 μl of in vitro-translated mPPAR or hRXRα (or unprogrammed reticulocyte lysate) and 10 μg of yeast extract in breakage buffer expressing mPPAR and/or hRXRα. For supershift analysis, 1 μl of anti-mPPAR or anti-hRXRα antiserum or 1 μl of the corresponding preimmune serum was added. Reactions were incubated at 25°C for 20 min. Electrophoresis was performed at 4°C on prerun 3.5% polyacrylamide (30 : 1 acrylamide : *N*′,*N*′-methylenebisacrylamide weight ratio) gels with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer. Antisera to full-length mPPAR and hRXRα were raised in rabbits by injection of affinity-purified maltose binding protein fusions expressed in *Escherichia coli*.

RESULTS

mPPAR/hRXRα Synergistically Activate Transcription in Yeast via PPREs

cDNAs encoding mPPARα (hereafter called mPPAR) and hRXRα were linked to the constitutive phosphoglycerate kinase (PGK) promoter in high-copy yeast expression vectors containing different selectable auxotrophic markers. *LacZ* reporter gene constructs substituting one copy or two copies of the minimal PPRE of the rat HD gene (Zhang et al., 1992, 1993) or of the rat AOx gene (Tugwood et al., 1992) for the UASg located upstream of the yeast minimal *GAL1* promoter were also constructed. Cotransformation of yeast with the HD-PPRE-*lacZ* reporter gene construct 1HDΔL1 and with vectors expressing either hRXRα (yhRXRα) or mPPAR (ymPPAR) alone had little effect on the basal activity of the reporter gene construct (Table 1). However, there was a slight and reproducible mPPAR-dependent stimulation over control values when the 2HDΔL1 reporter construct was used (compare 2HDΔL1/ymPPAR to 2HDΔL1), suggesting that mPPAR may have some activity on its own on specific PPREs. Significantly, cotransformation with vectors expressing both mPPAR and hRXRα resulted in a greater than 100-fold and 800-fold stimulation of transcription of the reporter gene construct with one copy of the HD-PPRE and two copies of the HD-PPRE, respectively. Cosynthesis of both receptors had no effect on transcription of a reporter construct not containing a PPRE (ΔL1). Results similar to those obtained with the HD-PPRE were obtained with a reporter construct containing a single copy of the AOx-PPRE (1AOxΔL1). Expression of mPPAR and hRXRα individually had no effect on transcription from 1AOxΔL1, whereas coexpression of mPPAR and hRXRα resulted in a greater than fivefold stimulation of transcription over basal levels. A reporter construct containing two copies of the AOx-PPRE was induced approximately 15-fold by mPPAR/hRXRα expression (not shown). 1AOxΔL1 had a sixfold higher basal level of activity compared to 1HDΔL1 (Table 1). The absolute magnitude of induction by mPPARα/hRXRα of a reporter gene containing a single copy of the HD-PPRE was approximately threefold higher than with a reporter gene containing a single copy of the AOx-PPRE, indicating that the HD-PPRE is more efficiently activated by mPPAR/hRXRα. This result is in agreement with cell-based transfection assays showing that the HD-PPRE is also a more efficient response element than the AOx-PPRE in mammalian cells (Zhang et al., 1993; Bardot et al., 1993). Therefore, although mPPAR and hRXRα have little or no activity individually in yeast, they function synergistically to activate transcription of cognate PPRE-linked reporter genes, as in mammalian cells. Moreover, synergistic transcriptional activation was independent of exogenously added ligands. Ligand-independent transcriptional activation was not the result of expression of receptors from high-copy vectors, because significant transactivation was observed in yeast harboring a *CEN*-based plasmid expressing mPPAR in place of the high-copy expression plasmid (Table 2).

Studies carried out in mammalian cells using mutant PPREs have shown that the integrity of the DR1 repeats of both the AOx- and HD-PPREs are essential for activation by PPARs. To determine whether this target specificity is also required for activation in yeast, we made reporter gene constructs containing a single-copy PPRE in which the DR1 repeats were individually mutated. Dis-

TABLE 1
ACTIVATION OF TRANSCRIPTION BY mPPAR AND hRXRα IN *Saccharomyces Cerevisiae*

Reporter Construct	yhRXRα	ymPPAR	β-Galactosidase Activity (U/mg Protein)*
ΔL1	+	+	12
1HDΔL1	−	−	13
	+	−	13
	−	+	19
	+	+	1510
2HDΔL1	−	−	3.3
	+	−	5.0
	−	+	52
	+	+	2708
1AOxΔL1	−	−	78
	+	−	71
	−	+	69
	+	+	439
2AOxΔL1	−	−	87
	+	−	72
	−	+	80
	+	+	1238

**S. cerevisiae* harboring the plasmids indicated (see Materials and Methods) were grown, harvested, lysed, and assayed for β-galactosidase activity (Ausubel et al., 1989; Himmelfarb et al., 1990). Units are given as the $\Delta A_{420} \times 10^3$/min. The values reported are the averages of at least two independent assays done in duplicate. Values did not vary by more than 10%.

ruption of either the first or second TGACCT repeat in the AOx-PPRE (1AOxM1ΔL1 and 1AOxM2ΔL1, respectively) abolished transactivation by mPPAR/hRXRα (Fig. 1A). Similarly, transactivation of HD-PPRE-linked reporter genes was dramatically reduced by mutating either of the DR1 repeat motifs of the HD-PPRE (1HDM3ΔL1 and 1HDM5ΔL1, respectively) (Fig. 1B). Mutation of the most 3′ TGACCT repeat (1HDM5ΔL1) in the HD-PPRE also eliminated the modest mPPAR-dependent, hRXRα-independent induction observed with this PPRE. These data show that the activation of HD-PPRE-linked reporter genes observed with mPPAR on its own depends on the integrity of the PPRE and is not the result of some nonspecific effect. Moreover, because PPRE mutations similar to those described above have been shown to disrupt transactivation by PPAR in mammalian cells (Zhang et al., 1993), PPAR/RXR transactivation displays similar target site requirements in both yeast and mammalian cells.

TABLE 2
TRANSACTIVATION BY mPPAR EXPRESSED FROM A LOW-COPY VECTOR

yhRXRα	ymPPAR	cmPPAR	β-Galactosidase Activity (U/mg Protein)*
−	−		2.5
+	−		5.3
−	+		33
+	+		2260
−		−	6.6
+		−	6.7
−		+	5.2
+		+	460

**β-Galactosidase activity was measured as described in Table 1. The reporter plasmid was 2HDΔL1. Plasmid constructs are defined in Materials and Methods.*

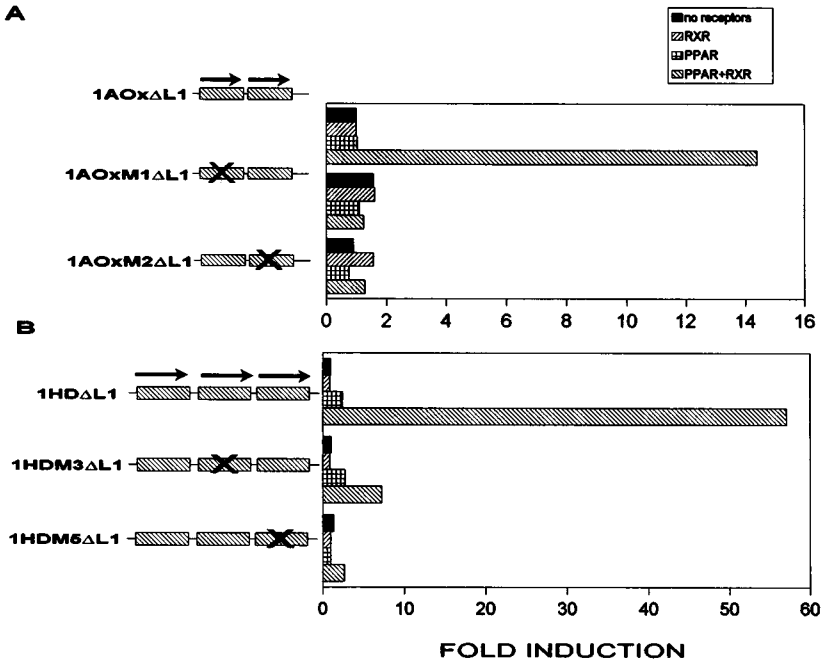

FIG. 1. The integrity of repeats in the AOx-PPRE (A) and HD-PPRE (B) is essential for activation by mPPAR/hRXRα in yeast. Reporter gene constructs containing one copy of either the wild-type AOx- or HD-PPRE or mutant PPREs in which individual TGACCT repeats were mutated, as indicated, were introduced into *S. cerevisiae* strain DL-1 in the absence or presence of plasmids expressing hRXRα and/or mPPAR. Cells were grown as described in Materials and Methods, and β-galactosidase activity was measured (Ausubel et al., 1989; Himmelfarb et al., 1990). The values reported are the averages of at least two independent transformants assayed in duplicate normalized to the activity obtained with 1AOxΔL1 (A) and 1HDΔL1 (B), which was taken as 1 in each case. The sequences of the wild-type and mutant PPREs in the various plasmid constructs are given in Materials and Methods.

mPPAR and hRXRα Expressed in Yeast Bind Cooperatively to PPREs In Vitro

In vitro-synthesized PPARs and RXRs bind cooperatively to the AOx- and HD-PPREs in vitro (Kliewer et al., 1992; Marcus et al., 1993) (Fig. 2A,B, lanes c). To determine whether mPPAR and hRXRα synthesized in yeast also cooperate in DNA binding, gel retardation assays were performed using yeast extracts and labeled AOx-PPRE and HD-PPRE probes. Only extracts from yeast synthesizing both receptors generated a specific protein/DNA complex on the HD-PPRE (compare Fig. 2B, lanes d and e, with Fig. 2C, lane c) and on the AOx-PPRE (Fig. 2A, lanes d and e, and data not shown). The presence of both receptors in the complex formed with the HD-PPRE was verified by supershift analysis with specific anti-mPPAR and anti-hRXRα antisera (Fig. 2C, lanes f and h, respectively). mPPAR synthesized in yeast cooperated with in vitro-translated hRXRα to bind DNA (Fig. 2A,B, lanes f) and vice versa (lanes g). The small amount of complex seen with in vitro-translated mPPAR alone (Fig. 2A,B, lanes b) or when mixed with untransformed yeast extract (Fig. 2A,B, lanes i) is due to the interaction of mPPAR with endogenous RXR present in rabbit reticulocyte extract (Marcus et al., 1993). Extracts from untransformed yeast contain an endogenous factor that binds to the AOx-PPRE but not the HD-PPRE (Fig. 2A, arrow). The nature of this factor is unknown; however, if it is a transcription factor, its presence and ability to bind to the AOx-PPRE might explain the higher basal β-galactosidase activity observed with the AOx-PPRE reporter construct vis-à-vis the HD-PPRE reporter construct (see Table 1). The above results show that mPPAR and hRXRα synthesized in yeast bind cooperatively to PPREs, as has been observed with these receptors synthesized in mammalian cells or in vitro (Marcus et al., 1993). We have recently demonstrated that mPPARα and hRXRα physically interact in vivo in yeast in the absence of a cognate target site using the two-

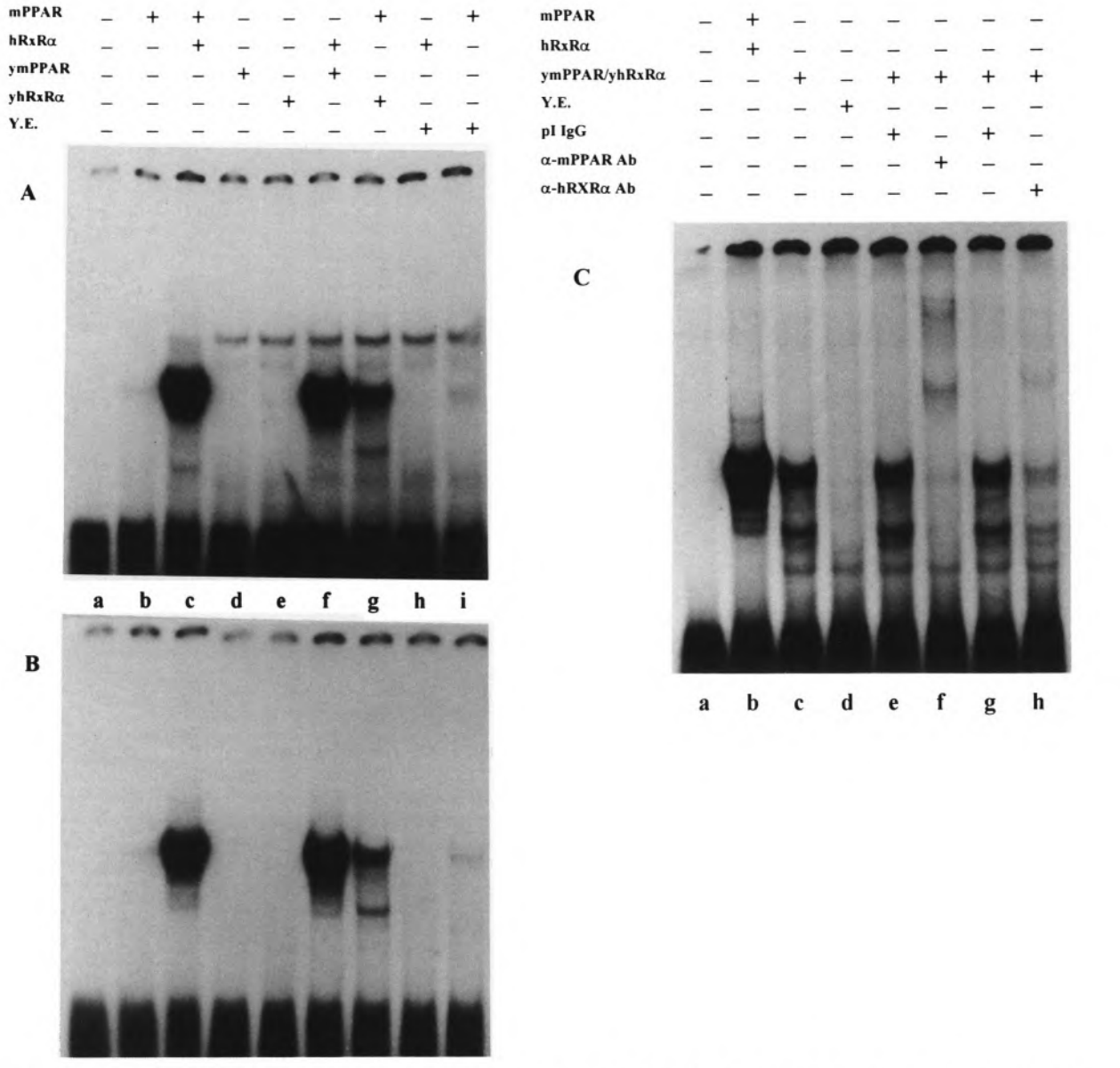

FIG. 2. mPPAR and hRXRα expressed in *S. cerevisiae* bind cooperatively to the AOx- and HD-PPREs. Extracts prepared from *S. cerevisiae* expressing mPPAR or hRXRα were used in mobility shift assays with labeled double-stranded oligonucleotide probes corresponding to the rat AOx-PPRE (A) or HD-PPRE (B). Additions are indicated at the top of each lane. Lanes a contain only labeled probe. mPPAR and hRXRα are receptors synthesized in vitro in rabbit reticulocyte lysate. ymPPAR and yhRXRα are receptors synthesized from corresponding cDNAs expressed in yeast. The arrow in (A) indicates a nonspecific complex that is generated with the AOx-PPRE. The small amount of protein/DNA complex observed with mPPAR in lanes b of (A) and (B) is due to the presence of endogenous RXR in reticulocyte lysate (Marcus et al., 1993). (C) Mobility shift assays were carried out with the HD-PPRE probe as in (B) using receptors translated in vitro (mPPAR, hRXRα) or yeast extracts coexpressing mPPAR and hRXRα (ymPPAR/yhRXRα) as indicated at the top of each lane. Lane a contains only labeled HD-PPRE probe. Where indicated, reactions were supplemented with 1 μl of anti-mPPAR (lane f) or anti-hRXRα (lane h) serum. Control lanes e and g contained 1 μl of preimmune serum from the rabbit giving the corresponding immune serum. The results show that both mPPAR and hRXRα coexpressed in yeast are present in the protein/DNA complex formed on the HD-PPRE. Similar results were obtained with the AOx-PPRE (data not presented).

hybrid protein interaction system (Miyata et al., 1994). Thus, synergistic transcriptional activation by mPPAR and RXR in yeast results from cooperative protein–protein and protein–DNA interaction.

Exogenously Added Fatty Acid Potentiates PPAR Transactivation in Yeast

Because a large number of hypolipidemic agents as well as polyunsaturated and monounsa-

turated fatty acids have been shown to activate PPARs in mammalian cells (Dreyer et al., 1993; Keller et al., 1993b; Issemann et al., 1993), we investigated whether some of these agents could also potentiate mPPAR/hRXRα-dependent transactivation in yeast. The potent peroxisome proliferators Wy-14,643 and nafenopin (Fig. 3A) or the fibrate drugs clofibrate and ciprofibrate (not presented) had no effect on transcription of the reporter gene construct containing a single copy of the AOx-PPRE. The inclusion of 9-*cis*-retinoic acid (0.1 μM) along with these peroxisome proliferators had no effect (data not presented). Similarly, the polyunsaturated fatty acids docosahexaenoic acid (C22 : 6ω3) and linoleic acid (C18 : 2ω6), or the monounsaturated fatty acids oleic acid (C18 : 1ω6) and elaidic acid (C18 : 1ω6 *trans*), did not further stimulate mPPAR/RXR activity. Among the fatty acids tested, only petroselinic acid, C18 : 1ω12, showed an effect on mPPAR/hRXRα-dependent transcription in yeast (Fig. 3A). Growth of yeast expressing mPPAR and hRXRα in medium containing 0.01% (w/v) petroselinic acid resulted in an approximately threefold induction of the AOx-PPRE reporter gene construct. Similar results were obtained using the HD-PPRE reporter construct, although in this case induction by petroselinic acid was more modest (1.5- to 2-fold). Potentiation by petroselinic acid required the presence of both receptors (Fig. 3B) and was dose dependent (Fig. 3C). The addition of 9-*cis*-retinoic acid (0.1 μM) did not increase the petroselinic acid response (data not shown). Petroselinic acid caused only a very slight but variable (10–20%) stimulation of transcription of the reporter genes in the absence of either receptor.

Peroxisomes Are Not Required for Constitutive PPAR/RXR Transactivation But Are Necessary for Stimulation by Exogenously Added Fatty Acid

The relaxed structural specificity of PPAR activators is paradoxical with the concept of high-specificity ligand interactions for nuclear receptors. It has been suggested that the true proximate PPAR ligand(s) may be a common metabolic derivative of peroxisome proliferators and naturally occurring fatty acids, perhaps generated via peroxisomal β-oxidation or some step prior to β-oxidation such as thioesterification (Göttlicher et al., 1993, Bentley et al., 1993). The results given in Tables 1 and 2 demonstrate that mPPAR/hRXRα potently and synergistically stimulates expression of PPRE-linked reporter genes in the absence of exogenously added activators or ligands for either receptor. These results suggest that PPAR is a constitutive transcriptional activator, or alternatively, that yeast contain endogenous activators of this receptor.

The availability of yeast strains that lack peroxisomes entirely or that are defective in various peroxisome-associated enzymatic activities provides an opportunity to explore the role of this organelle in PPAR activation. Therefore, we expressed mPPAR and hRXRα in YPH102, a peroxisome assembly mutant of *S. cerevisiae* that lacks peroxisomes (van der Leij et al., 1992). In this set of experiments, mPPARα and hRXRα were carried on a single plasmid (pRS425, 2 μm) and 2HDΔL1 was expressed from a *CEN* plasmid (pRS313) because of the lack of appropriate auxotrophic markers in the mutant strains. As shown in Table 3, expression of 2HDΔL1 was strongly activated by mPPAR/hRXRα in YPH102. Thus, constitutive transcriptional activation by mPPAR/RXRα does not require intact peroxisomes or an intact peroxisomal β-oxidation pathway. Similar results were obtained using the AOx-PPRE reporter gene (not shown).

In contrast, intact peroxisomes appear to be necessary for stimulation of mPPAR/RXR activity by petroselinic acid. Thus, as shown in Table 3, addition of petroselinic acid to DL-1 cells expressing mPPAR and hRXRα resulted in a twofold induction of the HD-PPRE reporter, as was previously shown in Fig. 3. In contrast, petroseli-

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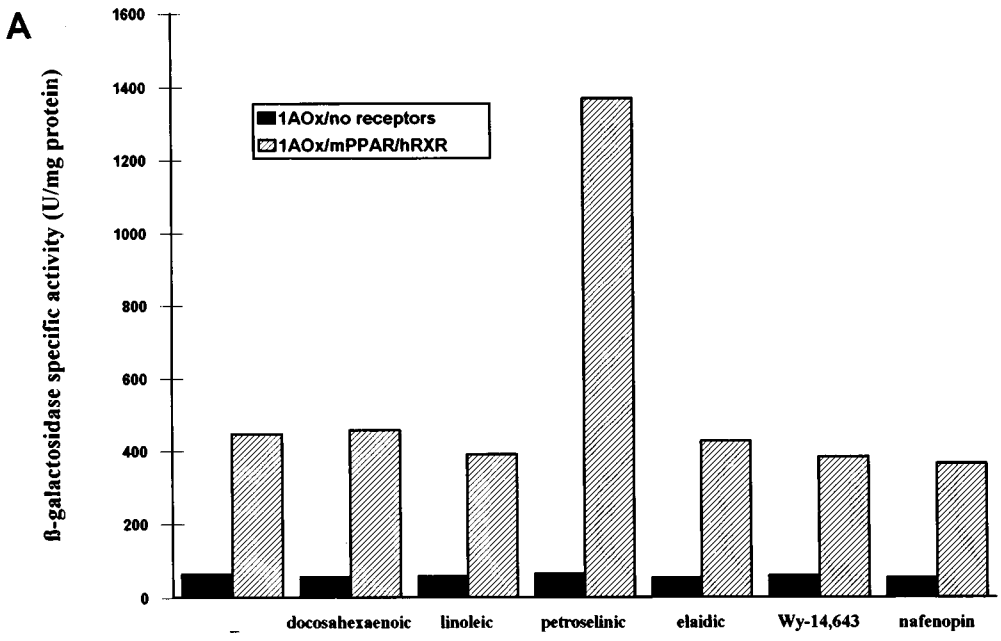
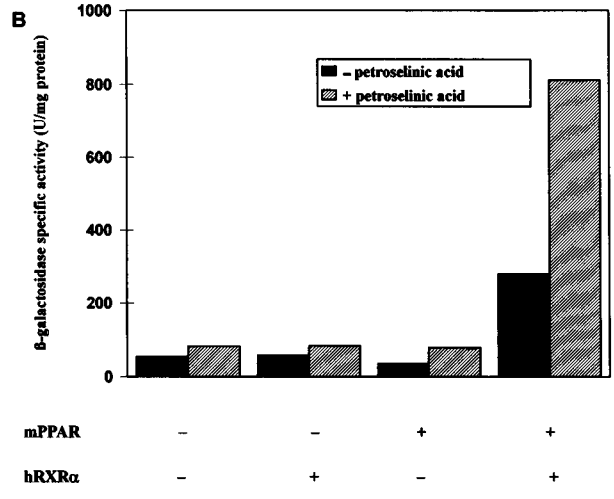
FIG. 3. mPPAR/hRXRα-dependent transactivation in yeast is potentiated by petroselinic acid. (A) Effects of various fatty acids and peroxisome proliferators on transcription of the AOx-PPRE in *S. cerevisiae*. Yeast transformed with mPPAR and hRXRα expression plasmids (or the corresponding empty vectors) were grown to an A600 of 0.5 in 0.67% yeast nitrogen base/2% glucose, pelleted, washed in water, and resuspended in 0.67% yeast nitrogen base/1% glucose/0.02% Tween 40. Fatty acids (docosahexaenoic, linoleic, petroselinic, elaidic) and peroxisome proliferators (Wy-14,643 and nafenopin) (all stock solutions 100 mg · ml^{-1} in ethanol) were added to a final concentration of 0.01%. Cells were grown for a further 6 h, harvested, lysed, and assayed for β-galactosidase (Ausubel et al., 1989; Himmelfarb et al., 1990). (B) Effects of petroselinic acid addition on transcription of the AOx PPRE by mPPAR or hRXRα. Cells were grown in petroselinic acid and assayed for β-galactosidase activity as in (A). (C) Dose-response curve for petroselinic acid addition. Cells transformed with mPPAR and hRXRα expression plasmids and reporter genes containing either the AOx PPRE or HD-PPRE, as indicated, were grown in increasing concentrations of petroselinic acid and assayed for β-galactosidase activity as above.


A
ß-galactosidase specific activity (U/mg protein)
1600
1400
1200
1000
800
600
400
200
0
1AOx/no receptors
1AOx/mPPAR/hRXR
–
docosahexaenoic
linoleic
petroselinic
elaidic
Wy-14,643
nafenopin


B
ß-galactosidase specific activity (U/mg protein)
1000
800
600
400
200
0
– petroselinic acid
+ petroselinic acid
mPPAR – – + +
hRXRα – + – +

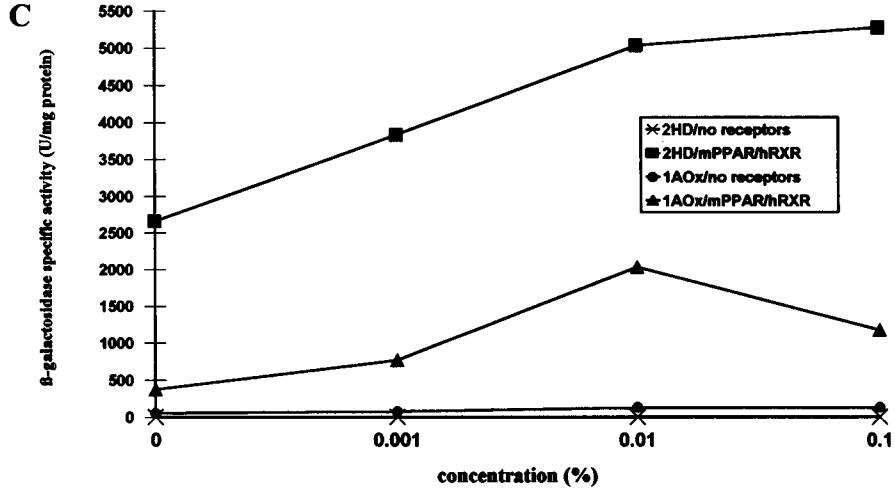

C
ß-galactosidase specific activity (U/mg protein)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
2HD/no receptors
2HD/mPPAR/hRXR
1AOx/no receptors
1AOx/mPPAR/hRXR
0
0.001
0.01
0.1
concentration (%)

TABLE 3
ACTIVATION OF mPPAR BY PETROSELINIC ACID REQUIRES INTACT PEROXISOMES BUT NOT AN INTACT β-OXIDATION PATHWAY

Yeast Strain	mPPAR/hRXRα	β-Galactosidase Activity (U/mg Protein)	
		− Petro	+ Petro
DL1	−	1.9	2.3
	+	149	331
YPH102	−	1.1	1.2
	+	190	186
STUD	−	1.7	1.6
	+	134	266

Strains DL-1, YPH102, and STUD were transformed with the reporter gene plasmid 2HD313 and low-copy plasmid PP.RXR.425, expressing both mPPAR and hRXRα (see Materials and Methods for details). The strains were grown in the absence or presence of 0.01% (w/v) petroselinic acid as described in the legend to Fig. 3. Transformants were assayed for β-galactosidase activity as in Table 1. Control transformants contained the corresponding empty vectors.

nic acid had no additional stimulatory effects on transactivation by mPPAR/RXRα in YPH102. It is not clear what aspect of peroxisomal function is required for this effect because peroxisomal assembly mutants fall into at least nine complementation groups. To explore the requirement for β-oxidation, we used the yeast strain STUD, a DL-1 derivative that carries a disruption in the 3-ketoacyl-CoA thiolase gene (Glover et al., 1994). Thiolase is the third enzyme of the β-oxidation pathway and catalyzes the cleavage of 3-ketoacyl-CoA into acetyl-CoA and an acyl-CoA that is two carbons shorter and that is refed back into the pathway. As demonstrated in Table 3, petroselinic acid was able to stimulate mPPAR/RXRα function in STUD as effectively as in DL-1. Therefore, the requirement of intact peroxisomes for the petroselinic acid response does not appear to reflect a need for the integrity of the peroxisomal β-oxidation pathway.

DISCUSSION

We have shown that mPPAR potently and synergistically activates transcription in yeast through cooperative interaction with hRXRα. Moreover, this activity can be stimulated by at least one natural fatty acid known to activate mPPAR in mammalian cells. These findings demonstrate that at least part of the mammalian peroxisome proliferator signaling pathway can be faithfully reconstituted in yeast, thereby providing a powerful experimental model system with which to systematically investigate the properties of PPARs and their mechanisms of activation.

Transcriptional activation by PPAR/RXR in yeast was dependent upon the integrity of the cognate PPREs. Interestingly, the HD-PPRE was more efficiently activated than the AOx-PPRE, as has also been observed in mammalian cells (Marcus et al., 1993; Zhang et al., 1993; Bardot et al., 1993). This finding supports the contention that the nature of the PPRE plays a significant role in the induction response (Miyata et al., 1993). Activation by PPAR/RXR did not require the addition of exogenous ligands or activators of the receptors. This is not entirely surprising because several other nuclear hormone receptors, including RAR/RXRα, have been shown to function in yeast in the absence of exogenously added cognate ligands (Heery et al., 1993; Hall et al., 1993). Our findings are consistent with the possibility that mPPARα is an intrinsic constitutive transcriptional activator for which activation function and target site binding in vivo do not require ligand engagement. Alternatively, overexpression of receptors may abrogate the requirement of ligand for efficient activation. PPARs also display significant ligand-independent activity in mammalian cells. This is usually attributed to the presence of endogenous activators present in these cells (Dreyer et al., 1992; Marcus et al., 1993). Thus, it remains possible that yeast fortuitously contain endogenous PPAR activators. The issue of whether PPARs require specific high-affinity ligands for activity can only be clarified once the proximate activators of PPARs are identified.

9-*cis*-Retinoic acid, which is capable of stimulating transactivation by RAR/RXR heterodimers and RXR homodimers in yeast (Allegretto et al., 1993), had no effect on PPAR/RXR function in yeast. It is possible that PPAR/RXR heterodimers respond differently to 9-*cis*-retinoic acid compared with RXR homodimers in yeast. Alternatively, the extent of transactivation in yeast resulting from overexpression of mPPAR and hRXRα may be beyond the threshold level at which 9-*cis*-retinoic acid and/or peroxisome proliferators may be expected to have some effect. Our finding that at least one fatty acid can significantly potentiate mPPAR activity in yeast argues against this possibility (see below). In mammalian cells, the stimulatory effect of 9-*cis*-retinoic acid on PPAR/RXR activation is modest and depends on the particular PPRE tested (Kliewer et al., 1992). Accordingly, although maximal PPAR/RXR-dependent activation of AOx-PPRE-linked reporter genes in mam-

malian cells is observed in the presence of both peroxisome proliferators and 9-*cis*-retinoic acid (Gearing et al., 1993; Kliewer et al., 1992), 9-*cis*-retinoic acid has no additional stimulatory effects on activation of HD-PPRE-linked reporter genes by PPAR/RXR (Bardot et al., 1993). Furthermore, even with the AOx-PPRE, where a stimulatory response is observed in the presence of 9-*cis*-retinoic acid, it is not clear if the ligand plays a direct or indirect role in transactivation. As we demonstrate here, RXRα is required for transactivation by PPAR in yeast, but ligand activation of RXRα is apparently not necessary.

With the exception of petroselinic acid (see below), potent peroxisome proliferators and fatty acids previously shown to activate mPPAR in mammalian cells were unable to do so in yeast. The reason for this finding is not yet clear but could be due to poor uptake of these compounds into yeast or their rapid clearance and/or metabolism in yeast. The failure of the peroxisome proliferators and most of the fatty acids tested to activate mPPAR in yeast may reflect the lack of capability of converting these compounds to proximate PPAR activators in this organism. For some of the fibrate hypolipidemic drugs, the ultimate PPAR-activating molecule appears to be an acyl-CoA ester derivative or other derivative generated prior to β-oxidation rather than the free peroxisome proliferator itself (Göttlicher et al., 1993). Similarly, there is evidence that the metabolism of free fatty acids to thioester derivatives prior to β-oxidation or to dicarboxylic acids via cytochrome P450 ω-hydroxylases may be important for PPAR activation (Auwerx, 1992; Gibson, 1993).

The observation that petroselinic acid stimulates PPAR function in yeast is an important step toward deciphering the pathways of PPAR activation and in understanding the role of the peroxisome in this process. Stimulation of mPPAR/RXRα function by petroselinic acid in yeast was modest (two- to threefold) but is comparable to the extent of PPAR activation by this fatty acid observed in mammalian cells. It is possible that petroselinic acid is a true proximate ligand for PPAR or that yeast is capable of converting this particular fatty acid into an activating derivative.

Elucidating the role of the peroxisome in PPAR activation is central to our understanding of the role of PPARs in modulating the pleiotropic cellular responses to peroxisome proliferators and to perturbation in lipid homeostasis. Until now, such questions have been addressed principally through the use of inhibitors of peroxisomal β-oxidation and of other lipid metabolic pathways or with nonoxidizable substrates and suicide inactivators. We have examined directly for the first time the requirement of intact peroxisomes and a functional β-oxidation pathway in modulating PPAR function. Our findings show that neither intact peroxisomes nor β-oxidation is necessary for constitutive activity of PPAR/RXR heterodimers. However, intact peroxisomes, but not necessarily the integrity of the peroxisomal β-oxidation pathway, is necessary for specific activation of PPAR by petroselinic acid. Petroselinic acid stimulated induction by mPPAR/RXRα in STUD, a yeast strain deleted for 3-ketoacyl-CoA thiolase, but not in YPH102, a strain devoid of intact peroxisomes. This result indicates that stimulation of PPAR function by petroselinic acid may be dependent upon the direct or indirect formation of intermediates generated by peroxisomes, perhaps prior to β-oxidation. It is surprising that oleic acid (C18 : 1ω9), which differs from petroselinic acid only with respect to the position of the double bond, had no effect on PPAR function. This may indicate that under our experimental conditions, petroselinic acid, but not oleic acid, is poorly metabolized in yeast, therefore resulting in the accumulation of intermediates that are proximate PPAR activators. Indeed, yeast are able to grow on oleic acid but not petroselinic acid when these compounds are used as the sole carbon source (S. L. Marcus, R. A, Rachubinski, and J. P. Capone, unpublished observations). Our findings are consistent with the observation that poorly metabolized fatty acids or derivatized fatty acids that cannot undergo β-oxidation are more potent substrate inducers of PPARs in mammalian cells. Indeed, Göttlicher et al. (1993) have shown that blocking β-oxidation stimulated activation of rat PPAR by fatty acid. These authors suggested that the proximate PPAR activator is either the CoA ester or some other derivative thereof of the fatty acid prior to β-oxidation. We are currently testing a broader spectrum of known PPAR activators and using fox mutants of *S. cerevisiae* (Erdmann et al., 1989) that are defective in specific steps of fatty acid activation and peroxisomal β-oxidation to gain further insights into the structural and metabolic requirements for PPAR activation.

The central role of PPARs in regulating lipid homeostasis in vertebrates and in mediating the pleiotropic physiological responses to a wide spectrum of xenobiotic compounds and nongenotoxic carcinogens is becoming increasingly recognized. The ability to functionally reconstitute PPAR ac-

tivity in yeast affords a unique biochemical and genetic approach to investigate the mechanisms of action and pathways of signal transduction of this growing family of important transcription factors.

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