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TRANSCRIPTIONAL REGULATION OF RAT PEROXISOMAL ACYL-CoA OXIDASE AND ENOYL-CoA HYDRATASE/3-HYDROXYACYL-CoA DEHYDROGENASE BY PEROXISOME PROLIFERATORS

by

Sandra L. Marcus 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Cell Biology and Anatomy

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ABSTRACT

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated members of the nuclear hormone receptor superfamily that regulate the transcription of genes involved in lipid homeostasis. PPARs activate transcription by binding to peroxisome proliferator response elements (PPRE) located upstream of target genes.

In Chapter 2 of this thesis, PPARs were shown to activate the expression of PPRE-linked reporter genes *in vivo* in response to peroxisome proliferators. Binding of PPARs to PPREs requires the presence of auxiliary cellular cofactors, one of which is the 9-*cis* retinoic acid receptor (RXRα). Cooperative DNA binding and heterodimerization between RXRα and several PPAR subtypes were seen with PPREs from the genes encoding peroxisomal acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; however, PPAR/PPRE binding and cooperativity with RXRα are obligatory, but not necessarily sufficient, for transcriptional activation *in vivo*.

In Chapter 3, PPAR function is investigated further. Mouse PPARα and human RXRα were expressed in the yeast *Saccharomyces cerevisiae*. Cosynthesis of both receptors resulted in synergistic transcriptional activation via PPREs. Transactivation was potentiated by the addition of petroselinic acid, a fatty acid shown to activate PPARs in mammalian cells.

Other cellular factors, including additional nuclear hormone receptors, also interact with PPREs and modulate PPAR function. Chapter 4 describes the development of a selection strategy in yeast to identify mammalian cellular factors that functionally interact with PPREs. Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII),

an orphan member of the nuclear hormone receptor superfamily, was identified as a PPRE-binding factor. COUP-TFII was a potent activator of PPRE-linked reporter gene expression in yeast. Significantly, COUP-TFII did not activate transcription of PPRE-linked reporter genes in mammalian cells, but strongly inhibited induction mediated by PPAR/RXR.

We hypothesized that the differential activity of COUP-TFII in yeast versus mammalian cells was due to auxiliary cellular cofactors absent in yeast. Chapter 5 describes the identification of a cellular factor that bound to COUP-TFII *in vitro* and apparently allowed COUP-TFII to function as a transcriptional activator in mammalian cells. This factor is identical to a ligand of the tyrosine kinase signaling molecule $p56^{lck}$, suggesting that it mediates crosstalk between mitogenic and nuclear hormone receptor signal transduction pathways.

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AOx	acyl-CoA oxidase
Amp^r	ampicillin resistance gene
AP-1	activator protein-1
CYC1	gene encoding the CYC1 gene product
CARLA	coactivator-dependent receptor ligand assay
CBP	CREB-binding protein
CEN	centromeric sequence
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CPS	carbamoyl phosphate synthetase
CREB	cAMP response element-binding protein
CoA	coenzyme A
CMV	cytomegalovirus
CYP	cytochrome P450
DBD	DNA-binding domain
DMEM	Dulbecco's modified Eagle's medium
DRx	direct repeat of TGACCT-like motifs with x nucleotides in between
EDTA	ethylenediamine tetraacetic acid
ETYA	5,8,11,14-eicosatetraynoic acid
GAL1	gene encoding the GAL1 protein; an enzyme involved in galactose metabolism
GR	glucocorticoid receptor
GBD	Gal4 DNA-binding domain
GRIP-1	GR-interacting protein-1
HA	influenza hemmagglutinin antigen (epitope tag)
HD	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
hNUC1	human NUC1 (a PPAR isoform)
hRXRα	human RXRα
HETE	hydroxyeicosatetraenoic acid
HIS	gene encoding the HIS3 protein; an enzyme involved in histidine biosynthesis
HNF	hepatocyte nuclear factor
HRE	hormone response element
LBD	ligand-binding domain
LEU2	gene encoding the LEU2 protein, an enzyme involved in leucine biosynthesis
LTB_4	leukotriene B_4
luc	luciferase gene
MAP kinase	mitogen-activated protein kinase
MBP	maltose binding protein
mPPAR	mouse PPAR
NCoA	nuclear receptor coactivator
NCoR	nuclear receptor corepressor

Oct	octamer nucleotide binding protein
ORCA	orphan receptor coactivator
PGK	phosphoglycerate kinase
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-response element
rPPAR	rat PPAR
RAR	retinoic acid receptor
RXR	retinoid X receptor
RXRα	9-*cis* retinoic acid receptor
SDS	sodium dodecyl sulfate
SH2	*src* homology 2
SMRT	silencing mediator for RARs and TRs
Sp1	specificity factor-1
SRC-1	steroid receptor coactivator-1
SV40	*Simian* virus 40
TK	thymidine kinase
TR	thyroid hormone receptor
TRP1	gene encoding the TRP1 protein, an enzyme involved in tryptophan biosynthesis
UAS_g	upstream activating sequence of the GAL1 promoter
VDR	vitamin D_3 receptor
xPPAR	*Xenopus* PPAR

CHAPTER 1

INTRODUCTION

1.1 Overview

Regulating gene expression is a fundamental problem for all cells. A cell must be able to adapt to its changing environment and respond to changes in nutritional status to avoid sustaining futile opposing anabolic and catabolic pathways. A cell can control the extent to which a particular gene is expressed at three levels: the concentration of its transcribed mRNA in the cell, the efficiency with which that mRNA is translated, and the stability of the encoded protein in the cell. The study of gene expression has greatly advanced in the last two decades with the tools of molecular biology, particularly at the level of gene transcription.

Transcriptional regulation of the genes encoding peroxisomal proteins is an interesting and useful model for studying the mechanisms controlling gene expression. Chemically induced overexpression of peroxisomal genes can lead to profound changes in fatty acid metabolism, peroxisome proliferation, hepatomegaly, and carcinogenesis (Reddy and Lalwani, 1983). Peroxisome proliferators are a diverse group of xenobiotic chemicals that include the clinically important hypolipidemic drugs and industrial phthalate ester plasticizers. Studies of the promoter regions of the genes encoding the peroxisomal β-oxidation enzymes acyl-CoA oxidase (AOx) (Osumi *et al.*, 1991; Tugwood *et al.*, 1992); and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) (Zhang *et al.*, 1992; 1993) led to the identification of *cis*-acting peroxisome proliferator-response elements (PPRE). Transcriptional induction by peroxisome proliferators is mediated by members of the nuclear hormone receptor superfamily termed peroxisome proliferator-activated receptors (PPAR), which activate transcription by binding directly to PPREs. The finding that certain fatty acids, and metabolites of

fatty acids, are PPAR ligands that can induce the formation of peroxisomes suggests that peroxisome proliferation is ultimately a natural adaptation of the cell to changes in nutritional status. Furthermore, fatty acids can no longer be considered simply as biological substrates or inert structural entities, since they possess hormone-like properties (Krey *et al.*, 1997). In addition to peroxisomal genes, PPARs regulate the expression of genes involved in multiple metabolic pathways, as well as genes important for differentiation. PPREs are also subject to regulation by other members of the nuclear hormone receptor superfamily. Thus, the study of gene regulation by peroxisome proliferators has led to much insight into how different hormonal signaling pathways communicate with one another, as well as how mammals maintain energy balance.

This thesis focuses on the mechanisms of transcriptional activation by PPARs, the interplay of nuclear hormone receptors with PPREs, and their combined mechanisms of action. The following review includes a brief introduction to the biological effects of peroxisome proliferators, and outlines the factors that regulate gene transcription via PPREs and how these factors may communicate with the basal transcription machinery.

1.2 Functions of Peroxisomes

Peroxisomes are ubiquitous subcellular organelles that carry out a diverse set of metabolic functions, which vary depending on the organism, tissue, or cell in which they are found, and on growth conditions (Lazarow and Fujiki, 1985; Tolbert, 1981). Mammalian peroxisomes are most abundant in liver and kidney and are involved mainly with the respiration and metabolism of carbohydrates, amino acids, purines and lipids (Small *et al.*, 1990). Over half of the known peroxisomal enzymes are involved in lipid metabolism. Peroxisomes are involved in cholesterol biosynthesis (Appelkvist *et al.*,

1990), the formation of bile acids (Pedersen and Gustafsson, 1980), plasmalogen biosynthesis (Hajra *et al.*, 1979) and the β-oxidation of fatty acids (Lazarow and de Duve, 1976).

Prior to β-oxidation, fatty acids are first activated to their acyl-CoA derivatives by acyl-CoA synthetase, located in the peroxisomal membrane. After transport of acyl-CoAs across the peroxisomal membrane, the remaining steps take place in the peroxisomal matrix (reviewed in van den Bosch *et al.*, 1992). The first and rate-limiting step of β-oxidation is catalyzed by acyl-CoA oxidase (AOx), yielding H_2O_2 that is removed by catalase. The second and third reactions are catalyzed by bifunctional enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD). The final reaction is catalyzed by 3-ketoacyl-CoA thiolase. Peroxisomal β-oxidation is incomplete, because it cannot metabolize fatty acids shorter than 8 carbons. Thus, acyl-CoAs are converted by peroxisomal carnitine acyltransferase to carnitine esters and fed into the mitochondrial β-oxidation pathway.

The reactions of peroxisomal β-oxidation resemble those of mitochondrial β-oxidation, but the enzymes catalyzing them are different and are encoded by entirely different sets of genes. Therefore, their regulation and substrate specificities are also different (reviewed in Mannaerts and DeBeer, 1982; van den Bosch *et al.*, 1992). Peroxisomes preferentially oxidize medium-, long-, and very long-chain fatty acids (C_{10} to C_{30}). In contrast, mitochondria oxidize short-, medium-, and long-chain fatty acids (up to C_{18}). Peroxisomal β-oxidation can also use other substrates that are not efficiently oxidized by mitochondria, such as unsaturated fatty acids (Osmundsen, 1982; Osmundsen

and Hovik, 1988) and medium- and long-chain dicarboxylic acids (van Hoof *et al.*, 1988).

Mitochondrial β-oxidation is the major pathway for fatty acid oxidation under normal conditions (Mannaerts and DeBeer, 1982). However, under conditions in which energy balance is perturbed, such as starvation, high fat diet, diabetes, or administration of peroxisome proliferators, the peroxisomal β-oxidation pathway is notably enhanced (reviewed in Lock *et al.*, 1989). In light of the following discussion of the cellular effects of peroxisome proliferators, two properties of the peroxisomal β-oxidation pathway are worth noting (Ockner *et al.*, 1993). First, unlike mitochondrial β-oxidation, peroxisomal β-oxidation is not subject to metabolic constraints such as coupling to oxidative phosphorylation and metabolic regulation of carnitine palmitoyltransferase I activity. Therefore, under conditions in which fatty acid flux rates exceed the capacity of the normally dominant esterfication and mitochondrial β-oxidation pathways, the peroxisomal β-oxidation pathway becomes important. Second, peroxisome proliferators cause an imbalance in H_2O_2-generating β-oxidation versus H_2O_2-degrading detoxification enzymes. This has been proposed to result in oxidative stress that may damage DNA and/or alter gene expression, leading to tumour promotion (reviewed in Ockner *et al.*, 1993).

1.3 Peroxisome Proliferators and Their Biological Effects

Peroxisome proliferators cause a dramatic increase in the number and metabolic capacity of peroxisomes and alter the expression of many genes involved in lipid metabolism and cell proliferation. These compounds also induce a host of other

biochemical and morphological changes in several tissues, including hepatomegaly and tumorigenesis. The effects of peroxisome proliferators are most striking in the liver cells of highly sensitive species such as rats and mice. Much of our knowledge of the effects of peroxisome proliferators comes from research with these experimental animals; however, marked species differences do exist (Lock *et al.*, 1989; Bentley *et al.*, 1993).

1.3.1 Peroxisome Proliferators

The hypolipidemic drugs clofibrate and ciprofibrate are widely used clinically to prevent cardiovascular morbidity (Reddy *et al.*, 1980). They are extremely effective in lowering elevated plasma triglyceride and cholesterol levels. Other hypolipidemc agents, including Wy-14,643, nafenopin, fenofibrate, gemfibrozil and their analogues, as well as a wide variety of structurally unrelated xenobiotic chemicals, have been shown to act as peroxisome proliferators (Reddy and Lalwani, 1983; Lock *et al.*, 1989; Bentley *et al.*, 1993). Some examples are phthalate ester plasticizers (*e.g.* di(2-ethylhexyl)phthalate) chlorophenoxy acid herbicides, halogenated hydrocarbon solvents (*e.g.* trichloroethylene), and anti-inflammatory agents (*e.g.* aspirin). Dose-response studies have shown that the potencies of peroxisome proliferators vary over several orders of magnitude. Hypolipidemic drugs are among the strongest, whereas plasticizers and chlorinated hydrocarbons are relatively weak.

1.3.2 Peroxisome Proliferation

Administration of hypolipidemic drugs to rats causes an increase in the number of hepatic peroxisomes by 4- to 10-fold, and an even larger increase (10- to 30-fold) in peroxisomal β-oxidation activity (Lazarow and deDuve, 1976; Hess *et al.*, 1965;

Lazarow, 1977). The increase in β-oxidation activity is accompanied by a parallel increase in the levels of all three peroxisomal β-oxidation enzymes (Lazarow *et al.*, 1982), primarily due to the transcriptional induction of their corresponding nuclear genes (Furuta *et al.*, 1982; Reddy *et al.*, 1986; Osumi, 1993). Also induced are a number of other peroxisomal enzymes, including long-chain acyl-CoA synthetase (Suzuki *et al.*, 1990). Increases in hepatic mRNAs encoding peroxisomal β-oxidation enzymes are rapid and can be observed within a few hours of a single dose of fibrate administration. Liver cells are most affected; however, smaller increases (2- to 4-fold) are evident in kidney, heart, and small intestine (Nemali *et al.*, 1988). The precise relationship between peroxisome proliferation and the induction of the peroxisomal β-oxidation enzymes is unknown; however, in certain cases the two effects can be uncoupled. For example, 4-(2-[4-(chlorocinnamyl)piperazine-1yl]ethyl)benzoic acid and BM 15766 induce marked proliferation of peroxisomes without the simultaneous induction of β-oxidation (Baumgart *et al.*, 1990), suggesting that peroxisome proliferation and β-oxidation may be regulated separately. The rapid, coordinated, and cell type-restricted increase in transcription of the genes encoding β-oxidation enzymes suggests a common mechanism of induction. As discussed below, peroxisome proliferator-responsive genes are activated through receptor-based mechanisms of transcriptional activation by members of the nuclear hormone receptor superfamily.

1.3.3 Induction of Cytochrome P450IVA

Clofibrate has also been shown to produce an approximately 10-fold increase in the cytochrome P450IVA family of hepatic microsomal enzymes at the level of

transcription (Sharma *et al.*, 1988; Bars *et al.*, 1993). Among them, cytochrome P452 (also known as P450IVA1, CYP 4A1) and P450IVA6 (CYP 4A6) carry out the ω-hydroxylation of long-chain fatty acids for subsequent oxidation into the corresponding dicarboxylic fatty acids. The increase in rat liver P450IVA1 precedes the induction of peroxisomal β-oxidation in the livers of rats administered a high-fat diet or clofibrate (Small *et al.*, 1990; Kaikaus *et al.*, 1993). The induction kinetics of both the P450IVA1 enzyme and mRNA were shown to be biphasic, peaking 1 and 24 hours after administration. The second peak coincided with the induction of the peroxisomal β-oxidation enzymes (Small *et al.*, 1990). Furthermore, pretreatment of rats with the protein synthesis inhibitor, cycloheximide, blocked the clofibrate-induced increase in peroxisomal AOx mRNA but had only a small effect on the induction of P450IVA1 mRNA (Small *et al.*, 1990). Finally, clofibrate-induced peroxisome proliferation was blocked by an inhibitor of P450IVA enzymes, 1-aminobenzotriazole (Kaikaus *et al.*, 1993). Therefore, the induction of P450IVA enzymes may be an obligatory event for peroxisome proliferation (Gibson, 1992, 1993).

1.3.4 Effects on Non-Peroxisomal and Non-Microsomal Enzymes

The transcription of a number of genes encoding non-peroxisomal and non-microsomal enzymes is also induced by peroxisome proliferators. Many of these enzymes are involved in lipid metabolism and include liver fatty acid binding protein (Brandes *et al.*, 1990) and acyl-CoA binding protein (Vanden Heuvel *et al.*, 1993). Furthermore, apolipoprotein A-IV mRNA is down-regulated by fibrate drugs in a tissue-specific manner (Staels *et al.*, 1990). Peroxisome proliferators also down-regulate certain genes regulated by steroid hormones, such as the thyroid hormone binding protein

transthyretin, suggesting that these chemicals influence hormone signaling pathways (Motojima *et al.*, 1992). With respect to carcinogenesis, there is evidence that peroxisome proliferators induce the expression of several proto-oncogenes that are also induced during liver regeneration, including Jun-fos, H-ras, c-myc and c-raf (Hsieh *et al.*, 1991; Cherkaoui Malki *et al.*, 1990). Together, these observations suggest that peroxisome proliferators not only evoke profound changes in lipid metabolism, but also influence a wide spectrum of cellular functions including cell proliferation and signal transduction.

1.3.5 Hepatomegaly and Carcinogenesis

Exposure of rats to peroxisome proliferators leads to hepatomegaly (liver enlargement). Hepatomegaly results from two separable events: hyperplasia (cell proliferation) and hypertrophy (increased cell size) (Reddy and Lalwani, 1983; Lock *et al.*, 1989). The hyperplastic response is usually transient and is a result of increased DNA synthesis, increased mitosis, and decreased apoptosis (Rao and Reddy, 1991). Hypertrophy is mainly due to the increase in the volume occupied by peroxisomes, and a more modest increase in smooth endoplasmic reticulum (Meyer and Afzelius, 1989; Ganning *et al.*, 1983; Sharma *et al.*, 1988). Hepatomegaly is rapidly induced by peroxisome proliferators, in a dose-dependent manner, and is maintained as long as the chemical is administered. Longer-term effects of peroxisome proliferators include lipid deposition (lipofuscin), DNA damage, nodule formation, and finally, tumorigenesis (Lock *et al.*, 1989; Gibson, 1993).

Peroxisome proliferators are classified as non-genotoxic carcinogens, since they do not damage or interact with DNA (Warren *et al.*, 1980; Bentley *et al.*, 1987; Von

Däniken *et al.*, 1981; Goel *et al.*, 1985; Gupta *et al.*, 1985). The oxidative stress hypothesis proposes that peroxisome proliferators cause an imbalance in the H_2O_2-generating enzymes versus H_2O_2-reducing enzymes, leading to increased reactive oxygen species that damage DNA (reviewed in Ockner *et al.*, 1993). Accordingly, peroxisome proliferators induce a 10- to 30-fold increase in H_2O_2-producing β-oxidation enzymes, but only a 2-fold increase in H_2O_2-degrading catalase (Klucis *et al.*, 1991).

1.4 Mechanisms of Peroxisome Proliferation

Reddy and co-workers first proposed that the effects of peroxisome proliferators are mediated by a ligand-receptor mechanism (Reddy *et al.*, 1988) based on the following considerations: i) transcriptional induction and peroxisome proliferation are an inherent and tissue-specific property of hepatocytes; ii) structurally diverse peroxisome proliferators evoke similar biochemical and cellular effects; iii) there is a rapid and coordinated transcriptional induction of the genes encoding the peroxisomal β-oxidation enzymes and microsomal ω-hydroxylase enzymes, suggesting a common mechanism. There is now compelling evidence that the transcriptional effects of peroxisome proliferators are mediated through specific receptors belonging to the nuclear hormone receptor superfamily. Peroxisome proliferator-activated receptors (PPAR) activate transcription via peroxisome proliferator-responsive elements (PPRE) in the promoters of inducible genes.

In addition to structurally diverse chemicals, unsaturated free fatty acids, and physiological conditions such as a high fat diet and diabetes can induce peroxisome proliferation. Therefore, as an alternative mechanism, it was proposed that peroxisome

proliferation occurs as a direct consequence of substrate overload resulting from the accumulation of intracellular fatty acids, or as a result of the peroxisome proliferators or their metabolites perturbing lipid metabolism (Lock *et al.*, 1989). A disturbance in lipid metabolism by peroxisome proliferators is thought to result in the accumulation of medium- chain fatty acids (substrate overload), stimulating cytochrome P450IVA activity to generate long-chain dicarboxylic fatty acids (Lock *et al.*, 1989; Sharma *et al.*, 1988). In support of this model, endogenous fatty acids were shown to act as pretranslational regulators of P450IVA1 in primary rat hepatocytes. Dicarboxylic fatty acids, and other substituted fatty acids that cannot undergo β-oxidation, were significantly more active inducers (Tollet *et al.*, 1994). Dicarboxylic fatty acids also uncouple oxidative phosphorylation and are potent substrate inducers of peroxisomal β-oxidation. Moreover, they have been shown to stimulate DNA synthesis, which may account for the hyperplasia associated with peroxisome proliferation (Lock *et al.*, 1989). The substrate overload hypothesis and receptor-mediated mechanisms are not mutually exclusive; in fact the two can be linked by the actions of PPARs.

1.5 Peroxisome Proliferator-Activated Receptor

1.5.1 Discovery

Green and coworkers hypothesized that peroxisome proliferator action might be mediated by a member of the ligand-activated steroid/nuclear hormone receptor superfamily (Issemann and Green; 1990). These transcription factors are critical for cellular homeostasis, development, reproduction, and differentiation. Members of the family include receptors for thyroid hormone, adrenal steroids such as glucocorticoids,

vitamin D_3, and retinoic acids, as well as for ecdysone (Beato, 1989; Parker, 1993). The binding of a hormone to its receptor enables the receptor to bind a hormone response element (HRE) upstream of a target gene to activate transcription.

The first peroxisome proliferator-activated receptor was cloned from mouse (mPPAR) in a genetic screen for novel members of the steroid/nuclear hormone receptor superfamily (Issemann and Green, 1990). PPAR was shown to be activated by peroxisome proliferators in cell-based transfection assays. Since then, related PPAR subtypes have been identified in several species. Based on amino acid sequence conservation, the PPAR subfamily appears to consist of at least three distinct subtypes: PPARα, PPARβ or δ (also called NUCI or FAAR), and PPARγ. PPAR cDNAs have been cloned from rat (rPPARα; Göttlicher *et al.*, 1992), *Xenopus*, (xPPARα, β, and γ; Dreyer *et al.*, 1992), mouse (mPPARγ, mNUC1, and mPPARγ2; Zhu *et al.*, 1993; Chen *et al.*, 1993; Tontonoz *et al.*, 1994), hamster (haPPARγ; Aperlo *et al.*, 1995) and human (hNUC1, hPPARα, and hPPARγ; Schmidt *et al.*, 1992; Sher *et al.*, 1993; Greene *et al.*, 1995).

1.5.2 Structure and Classification

Like other members of the nuclear hormone receptor superfamily, PPARs display a modular structure consisting of at least 4 functional domains (A/B, C, D, E/F) (reviewed in Lemberger *et al.*, 1996b). The amino-terminal A/B domain shows the greatest sequence divergence and is required for transactivation. The highly conserved DNA-binding domain and an area involved in receptor dimerization are contained within the C region. Region D contains a connecting hinge and is less conserved. The carboxyl

terminal E/F region is required for ligand-binding (ligand-binding domain; LBD) and also includes most of the dimerization interface.

The DNA-binding domain contains 2 zinc finger DNA-binding motifs, each consisting of 4 cysteines coordinated with zinc. The zinc fingers are joined by amphipathic helices formed at the base of the two fingers. A crucial role in DNA-binding specificity is played by the P-box amino acids at the carboxy-terminal end of the first zinc finger. The P-box sequence of PPAR (CEGCKG) is identical to that of members of the subfamily of nuclear receptors including the retinoic acid (RAR), vitamin D (VDR), thyroid (TR) and retinoid X receptors (RXR), as well as of several orphan receptors. Thus, the P-box determines the subclassification of nuclear receptors, since it confers similar DNA-binding properties. The D-box, consisting of the amino acids between the first and second cysteines of the second zinc finger, is involved in contacts between dimerizing receptors. PPARs contain only 3 amino acids in the D-box, making them distinct from other members of the RAR/TR subfamily which have 5 or 6. As discussed below, this difference is thought to be significant with respect to the relative orientation of PPAR with its dimerizing partner.

1.5.3 Tissue Distribution

Clues to the function of the various PPAR subtypes can be provided by investigating their expression patterns, tissue distribution, and abundance (reviewed in Lemberger *et al.*, 1996b). In *Xenopus*, PPAR α and β are ubiquitously expressed, while PPARγ appears to be more restricted and most prevalent in fat body and kidney (Dreyer *et al.*, 1992; 1993). PPARα is more tissue-specific in rodents than in *Xenopus*. It is

mainly present in liver, kidney, heart, and the mucosa of the stomach and duodenum, with the highest levels in brown adipose tissue (Kliewer *et al.*, 1994; Braissant *et al.*, 1996; Lemberger *et al.*, 1996a). In rodents, PPARβ is present in all tissues examined, with some variation. Its abundance is weak in liver compared to kidney and lung. Similar to what was seen in *Xenopus*, PPARγ has a restricted expression pattern in rodents. Very high levels of PPARγ mRNA were seen in both white and brown adipose tissue, with much lower levels detected in spleen, the mucosa of duodenum, and the retina (Braissant *et al.*, 1996). mPPARγ2, an isoform of mPPARγ, was found exclusively in adipocytes (Tontonoz *et al.*, 1994).

1.6 Mechanisms of Action of PPARs

1.6.1 DNA-binding Properties of the RAR/TR Family of Nuclear Receptors

Nuclear hormone receptors bind to DNA by recognizing target sequences typically composed of six nucleotides (reviewed in Glass, 1994). PPAR and other nuclear receptors of the TR/RAR family recognize the consensus sequence TGACCT. These receptors generally bind to DNA as dimers. Accordingly, a functional hormone response element (HRE) is composed of two copies of the TGACCT motif. Members of the TR/RAR family bind preferentially as heterodimers with RXR (Kliewer *et al.*, 1992a). DNA-binding by dimeric nuclear receptors is generally ligand-independent, with the exception of RXRα which can bind as a homodimer in the presence of its ligand, 9-*cis* retinoic acid. As discussed below, PPARs strictly depend on dimerization with RXR because they do not function as homodimers or monomers.

Which set of receptors binds to a given HRE is controlled by the number, sequence, spacing, and relative orientation of the TGACCT half-sites (Umesono *et al.*, 1991). TR/RAR family members principally recognize half-sites that are present in direct repeats. Direct repeats with 3, 4, and 5 nucleotide spacing (DR3, DR4, DR5) are preferred targets for RXR heterodimers with VDR, TR, and RAR, respectively. DR1 motifs are the preferential targets for RXR homodimers and RXR/PPAR heterodimers (Kliewer *et al.*, 1992b). However, a considerable amount of degeneracy exists within this spacer rule, because different receptors can bind to a given response element and *vice versa* (Green, 1993). This phenomenon is critical for creating complexity, diversity, and cross-talk among the various receptor signalling pathways.

1.6.2 Peroxisome Proliferator-Response Elements

As described below, peroxisome proliferator-response elements (PPRE) have been identified and characterized in the promoters of many genes shown to be responsive to peroxisome proliferators. The demonstration that PPARs function through these elements confirms that these transcription factors are directly involved in the activation of peroxisome proliferator-responsive genes.

PPREs were first identified in both the AOx and HD genes by deletional and mutational analysis of their promoter regions, followed by transient transfections in the peroxisome proliferator-responsive Reuber rat hepatoma cell line H4IIEC3 (Osumi *et al.*, 1991; Zhang *et al.*, 1992; 1993). The AOx-PPRE is situated at position -565, with respect to the transcription start site, while the HD-PPRE is located at position -2930. Both PPREs have TGACCT-like repeats (Tugwood *et al.*, 1992; Dreyer *et al.*, 1992; Zhang *et al.*, 1993). The AOx-PPRE contains 2 direct repeats separated by 1 nucleotide

(DR1; 5' TGACCTtTGTCCT). Our laboratory showed that the HD-PPRE contains 3 direct repeats (TGACCTatTGAACTaTTACCT); the first and second separated by 2 nucleotides (DR2) and the second and third in a DR1 arrangement (Zhang *et al.*, 1993; Bardot *et al.*, 1993). The HD-PPRE was later shown to be extended, with an additional TCTCCT hexamer located 1 base pair upstream of the DR2 element to constitute a unified regulatory site (PPRE binding unit) (Chu *et al.*, 1995a). Related PPREs have since been identified in a number of other peroxisome proliferator-inducible genes, including those encoding ω-hydroxylases (CYP4A6, Muerhoff *et al.*, 1992; CYP4A1, Aldridge *et al.*, 1995), fatty acyl CoA synthetase (Schoonjans *et al.*, 1995) malic enzyme, a factor involved in lipid synthesis (Castelein *et al.*, 1994), liver fatty acid binding protein (Issemann *et al.*, 1992), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, an enzyme involved in ketogenesis (Rodríguez *et al.*, 1994), and lipoprotein lipase, an extracellular enzyme that hydrolyses triglycerides into glycerol and fatty acids (Schoonjans *et al.*, 1996).

PPREs display enhancer-like properties, since they function in a position- and orientation-independent manner, and can confer peroxisome proliferator-responsiveness to heterologous genes. The response can be further increased by multimerizing the elements. PPREs interact with a number of nuclear factors in a sequence-specific manner in DNA-binding assays. Competition, methylation interference, and mutational analysis show that both the AOx-and HD-PPREs interact with at least some common nuclear factors, and that interactions with one or more of these proteins are necessary for peroxisome proliferator-responsiveness (Zhang *et al.*, 1993).

As described in Chapter 2 of this thesis, various PPARs were examined for their ability to activate a luciferase reporter gene linked to the HD- or AOx-PPRE using transient transfection assays in the normally unresponsive monkey cell line COS-1. mPPARα, rPPARα, and xPPARα were able to mediate a response of the PPREs to ciprofibrate or Wy-14,643. Despite the fact that all of the PPARs could bind specifically to both PPREs *in vitro*, neither xPPARβ nor xPPARγ could transactivate the HD-PPRE. Interestingly, xPPARγ was effective with the AOx-PPRE, indicating the transactivating ability of PPARγ differs according to the nature of the PPRE. The above results directly demonstrate that PPARs mediate transcriptional activation by peroxisome proliferators.

1.6.3 9-cis Retinoic Acid Receptor (RXRα)

RXR family members (isoforms α, β, and γ) form DNA-binding heterodimers with several nuclear hormone receptors of the TR/RAR family, and thus play an important role in controlling multiple hormone response pathways (Kliewer *et al.*, 1992a). Chapter 2 of this thesis presents results demonstrating that PPAR binds cooperatively to both the HD-and AOx-PPREs through heterodimerization with human (h)RXRα. The affinity for the HD-PPRE is approximately 3-fold higher (Bardot *et al.*, 1993). Cotransfection of PPAR and RXRα results in synergistic transactivation via the AOx-PPRE in the presence of peroxisome proliferator or the RXR ligand 9-*cis* retinoic acid. Simultaneous exposure to both ligands results in additive (Keller *et al.*, 1993b) or synergistic effects (Kliewer *et al.* 1992b) on transcription. Unlike RAR and TR, which allosterically block the binding of ligands to RXR *in vitro* (Forman *et al.*, 1995b), PPAR is considered to be a permissive partner for RXR, since PPAR/RXR heterodimers

respond to RXR ligand. Moreover, the binding of RXR ligands to PPAR/RXR heterodimers stimulates interactions between RXR and the coactivator SRC-1 (DiRenzo *et al.*, 1997)

Further evidence that PPAR and RXR cooperate *in vivo* to activate transcription via PPREs comes from studies carried out in yeast, described in Chapter 3 of this thesis. This organism is devoid of endogenous nuclear receptors and retinoids. Co-expression of both mPPARα and hRXRα was necessary to activate the expression of a reporter gene linked to either the AOx-or HD-PPRE. Either receptor expressed alone was essentially inactive. The observed transactivation was independent of added ligand, suggesting that at least in yeast, a PPAR/RXR heterodimer functions as a constitutive transactivator or that yeast contain endogenous PPAR activators. PPAR and RXR interact in solution in the absence of target DNA, as shown by immunoprecipitation (Kliewer *et al.*, 1992b), and *in vivo* as shown by genetic assays detecting protein-protein interactions (Miyata *et al.*, 1994). PPAR/RXR heterodimers bind PPREs in the absence of peroxisome proliferators and 9-*cis* retinoic acid, suggesting that inactive PPAR/RXR heterodimers occupy PPREs until one or both ligands become available.

The integrity of the DR1 repeat in the AOx-PPRE is essential both for optimal PPAR/RXR binding and for peroxisome proliferator-responsiveness *in vivo* (Issemann *et al.*, 1993a). For the HD-PPRE, DR1 repeats are required for PPAR/RXR binding. (Miyata *et al.*, 1993; Chu *et al.*, 1995a). Mutational analysis showed that while some repeats are dispensable for PPAR/RXR heterodimer binding, the integrity of all 4 repeats is essential for full peroxisome proliferator-responsiveness *in vivo*. These results suggest

that PPAR/RXR binding to the HD-PPRE is necessary, but not sufficient, for transactivation.

Detailed analysis of the PPREs from the AOx (Osada *et al.*, 1997), CYP4A6 gene (Palmer *et al.*, 1995) and malic enzyme gene (IJpenberg *et al.*, 1997) has revealed additional sequence determinants of natural PPREs and binding properties of PPAR/RXR heterodimers. These PPREs contain DR1 elements; however, the actual PPAR/RXR-binding site appears to extend 3' of the DR-1. Moreover, PPAR/RXR heterodimers appear to display a strong preference for an A:T base pair as a spacer nucleotide in the malic enzyme PPRE. Thus, a comparison of the PPREs so far identified provides the consensus sequence 5'-TGACCT T TGNCCT AGTT (IJpenberg *et al.*, 1997). PPAR/RXR heterodimers display a binding polarity opposite to those of RXR/TR and RXR/RAR bound to DR4 and DR5 elements, respectively. Rather, PPAR binds to the 3' extended half-site of the response element, while RXR occupies the 5' half-site (IJpenberg *et al.*, 1997; Osada *et al.*, 1997). It has been speculated that this reversed binding polarity is a result of the unique PPAR D-box, which comprises only 3 amino acids. Moreover, the binding polarity of RXR versus its partner has been proposed to dictate whether the heterodimer responds to RXR ligands (*ie.* a response occurs primarily when RXR occupies the 5' half site).

The above findings clearly demonstrate a convergence of the peroxisome proliferator- and retinoid-dependent signaling pathways on PPRE-like elements. The relevance of this convergence is underscored by the fact that both RXRα and PPARα are most abundant in liver and kidney, and that 9-*cis* retinoic acid is present in both these tissues (Kliewer *et al.*, 1992b). Furthermore, retinoic acid is a weak peroxisome

proliferator, and has been shown to induce the transcription of the rat AOx gene in cultured rat hepatocytes (Hertz and Bar-Tana, 1992), presumably due to the metabolic conversion of all-*trans* retinoic acid to 9-*cis* retinoic acid. Therefore, it is likely due to the role of RXR as a co-regulator that places it at the centre of lipid metabolism (Kliewer *et al.*, 1992a; 1992b; Zhang *et al.*, 1992; Wolf and Phil, 1993).

1.7 Mechanisms of Transcriptional Activation

Nuclear receptors in the RAR/TR family can potentially occupy cognate HREs in the absence of ligand and repress transcription. Ligand binding induces a conformational change that generally converts the nuclear receptor dimer to a transcriptional activator (Mangelsdorf and Evans, 1995). Recent studies have begun to reveal the mechanisms by which nuclear receptors activate and/or repress transcription. Ligand-activated nuclear receptors bound to HREs appear to stabilize, or promote the formation of, a preinitiation complex consisting of basal transcription factors for RNA polymerase II on the downstream promoter (Tsai and O'Malley, 1994). Some evidence suggests that these effects may be transmitted by direct interactions between nuclear receptors and basal transcription factors (Baniahmad *et al.*, 1993; Schulman *et al.*, 1995). Nuclear receptors may also transmit signals to the basal transcription machinery via indirect interactions, mediated by bridging proteins called transcriptional coactivators and corepressors (Goodrich and Tjian, 1994; Horwitz *et al.*, 1996).

1.7.1 Coactivators and Corepressors

An intensive search for nuclear receptor coactivators has resulted in the identification of a number proteins that interact with multiple nuclear receptors in a

ligand-dependent manner, including SRC-1 (Õnate *et al.*, 1995), RIP140 (Cavaillès *et al.*, 1995), TIF1 (Le Douarin *et al.*, 1995), GRIP1 (Hong *et al.*, 1996). TRIP-1/Sug1 (Lee *et al.*, 1995b), CBP/p300 (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996), and p/CIP (Torchia *et al.*, 1997). Conversely, two proteins have been identified that interact with nuclear receptors in the absence of ligand: SMRT (silencing mediator for RARs and TRs) and N-CoR (nuclear receptor corepressor). These proteins interact with unliganded TR and RARs and are released upon ligand binding (Chen and Evans, 1995; Hörlein *et al.*, 1995).

Steroid receptor co-activator (SRC-1) was identified by a genetic screen detecting protein-protein interactions using the ligand-binding domain of the progesterone receptor (Õnate *et al.*, 1995). In transient transfections, cotransfected SRC-1 increased receptor and ligand-dependent transactivation for several steroid and nuclear hormone receptors, but did not affect basal promoter activity. These results indicate that SRC-1 is a *bona fide* transcriptional coactivator. As discussed below, SRC-1 also serves as a PPAR coactivator (DiRenzo *et al.*, 1997; Zhu *et al.*, 1997), a phenomenon that has aided in the identification of proximate PPAR ligands (Krey *et al.*, 1997). A partially homologous protein, GRIP1, was identified by its ability to interact with the glucocorticoid receptor (Hong *et al.*, 1996). GRIP1 has been shown to exhibit coactivator activity to a wide range of steroid and nuclear hormone receptors in several yeast systems, including VDR, TR, RAR, and RXR (Hong *et al.*, 1997; Walfish *et al.*, 1997). GRIP1 bound *in vitro* in a ligand-dependent manner with TR, RAR, and RXR, and markedly increased the ability of these full-length nuclear receptors to transactivate β-galactosidase reporter genes containing cognate HREs *in vivo* (Walfish *et al.*, 1997). SRC-1, GRIP-1 and its human homologue TIF2 (Voegel *et al.*, 1996), and p/CIP share extensive sequence homology

and form a new family of nuclear proteins designated the NCoA family (Torchia *et al.*, 1997).

1.7.2 p/CIP and CBP/p300: Coactivators and Cointegrators

It has been proposed that recruitment of nuclear receptor coactivators helps to destabilize nucleosomes locally, allowing transcription factors to access recognition elements. In particular, nucleosomes can be destabilized by the acetylation of their histones. Conversely, histone deacetylation stabilizes the repressed state (Wolffe, 1997). Certain transcriptional coactivators have been shown to possess histone acetylase activity (Brownell *et al.*, 1996; Yang *et al.*, 1996). The CREB-binding protein (CBP) and the functionally related protein p300 are coactivators that harbour intrinsic histone acetylase activity (Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996). These proteins are essential for transcriptional activation by a large number of regulated transcription factors, including CREB (Kwok *et al.*, 1994), mitogen-activated transcription factors (Arias *et al.*, 1994), as well as steroid/nuclear receptors (Kamei *et al.*, 1996; Chakravarti *et al.*, 1996). CBP has been found to be part of a multiprotein complex including the ligand-binding domain of nuclear receptors and a variant of SRC-1. Microinjection of fibroblasts with anti-CBP antibodies abrogates RAR- or glucocorticoid receptor (GR)-dependent transcription, demonstrating the involvement of CBP in nuclear receptor signaling *in vivo* (Chakravarti *et al.*, 1996). Furthermore, p300 has been shown to function as a mPPARα coactivator (Dowell *et al.*, 1997). Thus, SRC proteins are thought to mediate activation of nuclear hormone receptors via association with CBP/p300 (Smith *et al.*, 1996).

Another recently identified member of the NCoA family, p/CIP, has been found to be associated with a significant fraction of the cellular CBP/p300 (Torchia *et al.*, 1997). Antibody microinjection studies indicated that p/CIP is required for regulated transcription by nuclear receptors as well as by other CBP-dependent factors, including STAT and AP-1; however, SRC-1 appears to be a specific requirement for transactivation by nuclear receptors. Therefore, it is suggested that p/CIP and CBP are components of a larger complex critical for the integration of several signal transduction pathways (Torchia *et al.*, 1997). CBP/p300, p/CIP, and other NCoA family members contain a leucine-rich interaction motif (LXXLL; where L denotes leucine and X denotes any amino acid) that has been shown to mediate binding to the ligand-dependent activation function 2 (AF2) of liganded nuclear receptors (Torchia *et al.*, 1997; Heery *et al.*, 1997). The residues within the LXXLL motifs seem to determine the specificity of nuclear receptors for the various coactivators. Interestingly, microinjection of peptides containing certain versions of the LXXLL motif can allow selective inhibition of distinct signal transduction pathways (Torchia *et al.*, 1997).

1.8 PPAR Activators and Ligands

PPARs can be activated by a wide spectrum of peroxisome proliferators, as observed in cell-based transfection assays using PPRE-linked reporter genes (Issemann and Green, 1990; Dreyer *et al.*, 1992; 1993; Marcus *et al.*, 1993; Keller *et al.*, 1993a; 1993b; Tugwood *et al.*, 1992; Issemann *et al.*, 1993b). PPARs are also activated by a number of naturally occurring and synthetic fatty acids such as linoleic, docosahexaenoic, or arachidonic acids, and various arachidonic acid metabolites (Auwerx, 1992; Göttlicher *et al.*, 1992; Keller *et al.*, 1993a; 1993b; Dreyer *et al.*, 1993; Yu *et al.*, 1995; Issemann *et*

al., 1993b). The synthetic arachidonic acid analogue 5,8,11,14-eicosatetraynoic acid (ETYA) is 100-fold more potent than Wy-14,643 (Keller *et al.*, 1993b). Significantly, there is a good correlation between the ability of peroxisome proliferators to activate PPAR and with the potency of various peroxisome proliferators to induce peroxisome proliferation or hepatocarcinogenesis in rats (Issemann *et al.*, 1993b). Furthermore, mice with a targeted disruption of the gene encoding PPARα are viable and fertile, but do not display the characteristic pleiotropic response when challenged with peroxisome proliferators (Lee *et al.*, 1995a). These results demonstrate that PPARα is the major isoform required for mediating the pleiotropic cellular effects of peroxisome proliferators. The finding that fatty acids and their metabolites are strong inducers of PPAR transcriptional activity may link the receptor and substrate overload hypotheses of peroxisome proliferation (Auwerx, 1992). It has been proposed that peroxisome proliferators perturb lipid metabolism, resulting in an accumulation of fatty acids. These fatty acids and the peroxisome proliferators themselves are then thought to alter target gene expression via PPARs. The finding that fatty acids are PPAR activators also explains how a high fat diet can induce peroxisome proliferation.

Until recently, none of the PPAR activators described above were shown to be true PPAR ligands, since binding of radiolabeled compounds to PPAR could not be detected. Furthermore, these compounds are structurally diverse and relatively high concentrations are required for receptor activation. It was these observations that led to the premise that the effects of PPAR activators are exerted indirectly through their metabolism to an active form. Chapter 3 of this thesis presents the reconstitution of mPPARα and hRXRα activity in yeast, and explores the requirements for PPAR

activation. PPAR/RXR heterodimers proved to be constitutive activators of transcription in yeast via PPREs. Several peroxisome proliferators were tested but, these failed to stimulate PPAR/RXR activity above background levels. Of the fatty acids tested only one, petroselinic acid (C18:ω12), was able to potentiate PPAR/RXR function. It increased reporter gene activity approximately two- to three-fold over levels observed in the absence of fatty acid. Similar experiments were carried out in a yeast strain lacking peroxisomes, and in a strain lacking a β-oxidation pathway. While both strains supported constitutive PPAR/RXR activity, peroxisomes, but not an intact β-oxidation system seemed to be required for potentiation by petroselinic acid.

PPARs appear to modulate many metabolic reactions, particularly those involved in lipid metabolism. To gain a clearer understanding of such complex functions, it is essential to identify true PPAR ligands. From the abundance of PPAR activators, classical ligand-binding studies using radiolabeled ligands have only identified a few ligands. Antidiabetic compounds of the thiazolidinedione class and an arachidonic acid metabolite, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, are ligands of PPARγ (Lehmann *et al.*, 1995; Kliewer *et al.*, 1995; Forman *et al.*, 1995a). The hypolipidemic drug Wy-14,643 and the natural inflammation mediator leukotriene B_4 (LTB4) are PPARα ligands (Devchand *et al.*, 1996). Several non-steroidal anti-inflammatory drugs, including indomethacin, fenoprofen, and ibuprofen have been shown to activate and bind both PPARα and PPARγ (Lehmann *et al.*, 1997b).

Krey and coworkers (1997) presented a novel assay to investigate whether a broad range of compounds could interact directly with PPARs. This assay is based on the hypothesis that the binding of ligand to PPAR would induce interactions of the receptor

with transcriptional coactivators, and is termed coactivator-dependent receptor ligand assay (CARLA). This study provides evidence that many of the known PPAR activators are also ligands, and identifies natural and synthetic ligands for all three PPAR subtypes. All of the compounds previously shown to be PPAR ligands by Scatchard analysis specifically induced PPAR/SRC-1 interactions *in vitro*. The PPAR subtypes bound to a wide variety of compounds including fatty acids, arachidonate metabolites and hypolipidemic drugs. Some of the compounds showed a partial overlap, while others showed a strict subtype specificity. The most potent synthetic ligands were the fatty acid analogue ETYA, bezafibrate, and the antidiabetic thiazolidinedione BRL 49653 for the xPPARα, -β, and -γ subtypes, respectively. Dose-response curves for the various compounds obtained by the CARLA assay generally correlate with transcriptional activation profiles (Keller *et al.*, 1993a; Krey *et al.*, 1997). One interesting example is an arachidonic acid metabolite, 8(S) hydroxyeicosatatraenoic acid [8(S)-HETE], a potent activator of the human PPARα. This receptor has been shown to be stereoselective for this compound over its 8(R) enantiomer (Yu *et al.*, 1995). Accordingly, [8(S)HETE] had a much higher (about 50-fold) affinity for PPARα than [8(R)HETE] (Krey *et al.*, 1997). Some compounds, such as nafenopin, were negative in the CARLA assay but had been previously shown to be potent PPAR activators. It has been suggested that these compounds may activate PPAR through their metabolites or by releasing endogenous ligands.

1.9 Modulation of PPAR Function by Other Cellular Factors

DNA-binding assays have revealed that PPAR and RXR are only minor components of the PPRE-binding proteins present in rat hepatoma cells (Chapter 2, this

thesis). COS cells also appear to possess factors distinct from RXR that generate protein/DNA complexes. Furthermore, mutations in the HD-PPRE, which do not affect binding of PPAR/RXR heterodimers, still abrogate the ability of the HD-PPRE to respond to peroxisome proliferators *in vivo* (Miyata *et al.*, 1993). These findings raise the possibility that other factors besides RXR are involved in PPAR activation of target genes through direct or cooperative binding to PPREs. Several other PPRE-binding proteins, with differing affinities for the AOx- and HD-PPREs, and which can differentially affect PPAR function, have been identified. These include TR and two orphan members of the nuclear hormone receptor superfamily: chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and hepatocyte nuclear factor-4 (HNF-4). The diversity of PPAR signaling is further enhanced by the findings that PPAR can heterodimerize with partners other than RXR, such as TR and the orphan nuclear receptor LXRα.

1.9.1 Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF)

COUP-TFs bind as homodimers to a wide spectrum of TGACCT-like response elements (Cooney *et al.*, 1992), allowing COUP-TF to bind to many hormone response elements recognized by VDR, RAR, RXR, TR, PPAR, and the orphan receptor HNF-4. A significant consequence of this promiscuous DNA-binding activity is the repression of the transcriptional activities of these receptors (reviewed in Qiu *et al.*, 1994). COUP-TF has been shown to inhibit target gene transcription by a number of mechanisms. First, COUP-TFs can compete with other nuclear receptors for response element binding (Cooney *et al.*, 1993; Tran *et al.*, 1992). Second, COUP-TFs heterodimerize with RXR to reduce the concentration of RXR available for heterodimerization with TR, VDR,

RAR, and PPAR (Cooney *et al.*, 1993). Third, COUP-TFs can transrepress the activities of TR, RAR, and RXR via LBD-LBD interactions (Leng *et al.*, 1996). Finally, COUP-TF can actively repress basal and activator-dependent transcriptional activities via binding sites upstream or downstream of the promoter (Leng *et al.*, 1996). This transcriptional repression has been shown to result from interaction with SMRT and NCoR (Shibata *et al.*, 1997).

COUP-TF is present among the HD-PPRE binding proteins in rat hepatoma extracts, and is the most abundant HD-PPRE binding factor in HeLa cell extracts (Miyata *et al.*, 1993). Furthermore, human COUP-TFI synthesized *in vitro* binds as a homodimer with high affinity to the HD-PPRE, and has been shown to antagonize PPAR-mediated activation of reporter genes linked to the HD-PPRE *in vivo*. DNA-bound PPAR/COUP-TF1 heterodimers could not be detected, therefore inhibition of PPAR transactivation most likely occurs through competition for target binding sites *in vivo*. Thus, members of the COUP-TF family may play a physiological role in modulating PPAR-mediated activation of peroxisome proliferator-responsive genes.

1.9.2 Hepatocyte Nuclear Factor 4 (HNF-4)

HNF-4 is another orphan member of the nuclear receptor superfamily that binds to TGACCT-like elements (Sladek *et al.*, 1990). HNF-4 is a liver-enriched factor that plays an important role in the transcriptional regulation of several genes encoding products involved in diverse metabolic pathways including lipid metabolism (reviewed in Winrow *et al.*, 1994). HNF-4 binds *in vitro* with differing affinities to the AOx-and HD-PPREs. In cotransfection assays, HNF-4 repressed PPAR-dependent activation of an

AOx-PPRE-linked reporter gene in both the presence and absence of the ligand Wy-14,643. Interestingly, when the HD-PPRE was used, HNF-4 repressed PPAR-dependent transcription in the absence of ligand but functioned cooperatively with PPAR in the presence of ligand (Winrow *et al.*, 1994). Therefore, the AOx and HD genes are subject to differential regulation by the interplay of nuclear receptors, depending on the PPRE structure and the presence of PPAR activators.

1.9.3 Other PPAR Dimerization Partners: TR and LXRα

Peroxisome proliferators and thyroid hormones have been shown to have overlapping metabolic effects. Furthermore, thyroid hormone attenuates peroxisome proliferator-mediated transcriptional induction of genes encoding peroxisomal β-oxidation enzymes, implying the existence of crosstalk between the respective signaling pathways (Pacot *et al.*, 1993; Takeda *et al.*, 1992). Accordingly, TR has been shown to bind to the AOx-PPRE (Chu *et al.*, 1995b; Hunter *et al.*, 1996) and the HD-PPRE (Chu *et al.*, 1995b) through heterodimerization with RXR. These receptors have little effect on transcription of PPRE-linked genes on their own, but can differentially modulate activation by PPAR/RXR heterodimers in a response element-dependent manner. Conversely, rPPARα has been shown to negatively regulate the expression of certain genes classically considered to be thyroid hormone responsive, either by forming non-DNA-binding heterodimers with particular TR subtypes (Bogazzi *et al.*, 1994; Jow and Mukherjee, 1995), or by competing with TR for the common heterodimerizing partner RXR (Juge-Aubry *et al.*, 1995). Thus, peroxisome proliferator and thyroid hormone signaling pathways converge at the level of their respective nuclear hormone receptors.

Furthermore, it appears that gene regulation by PPARs is a net transcriptional response subject to a dynamic balance between at least three nuclear hormone receptors (RXR, PPAR, and TR).

The recently described orphan receptor LXRα has been shown to bind DR4 response elements by heterodimerizing with RXR (Willy *et al.*, 1995; Lehmann *et al.*, 1997a). LXR has been found to be differentially regulated by multiple products of mevalonic acid metabolism (Lehmann *et al.*, 1997a; Forman *et al.*, 1997), suggesting that LXRs play a critical role in the regulation of cholesterol metabolism. LXRα forms non-DNA-binding heterodimers with PPARα *in vitro* (Miyata *et al.*, 1996). Furthermore, LXRα antagonizes PPAR-mediated transactivation *in vivo*, probably by sequestering PPAR. These results suggest that fatty acid metabolism is linked to cholesterol homeostasis by the actions of PPARs and LXRs.

As discussed in the preceding sections, it appears that there are multiple regulatory strategies that converge via PPREs. Understanding these mechanisms of transcriptional regulation requires the identification of the full spectrum of cellular factors that bind to PPREs and/or interact with PPAR. Chapter 4 of this thesis describes a genetic screen in yeast for novel PPRE-binding proteins. We identified COUP-TFII as a PPRE-interacting protein. Surprisingly, COUP-TFII activated the transcription of a PPRE-linked reporter gene in yeast, but antagonized PPAR-mediated transactivation in mammalian cells.

We hypothesized that the repression by COUP-TFII seen in mammalian cells is due to the absence of one or more corepressors in yeast. Chapter 5 describes the identification of a COUP-TFII-interacting protein that apparently can convert COUP-TFII from a transcriptional repressor into an activator. This protein, which we call ORCA (for Orphan Receptor CoActivator) is identical to a ligand for the tyrosine kinase signaling molecule $p56^{lck}$.

1.10 This Project

This project was designed to obtain a more complete molecular picture of the mechanisms of transcriptional activation by PPARs as well as the interplay of nuclear hormone receptors with PPREs. Chapters 2 and 3 are a study of PPARs and their interactions with various response elements. Chapter 4 describes a genetic screen for other positively acting PPRE-binding factors and describes one such factor, the orphan nuclear hormone receptor COUP-TFII. Chapter 5 describes the identification of ORCA as a COUP-TFII-interacting protein. Chapter 6 describes an additional function of ORCA, an ability to stimulate transcription from the SV40 enhancer/promoter.

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CHAPTER 2

DIVERSE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS BIND TO THE PEROXISOME PROLIFERATOR-RESPONSIVE ELEMENTS OF THE RAT HYDRATASE/DEHYDROGENASE AND FATTY ACYL-COA OXIDASE GENES BUT DIFFERENTIALLY INDUCE EXPRESSION[1]

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[1] A version of this chapter has been published. Marcus, S.L., Miyata, K.S., Zhang, B., Subramani, S., Rachubinski, R.A., and Capone, J.P. 1993. Proc. Natl. Acad.Sci. USA **90**:5723-5727. Used with permission from the National Academy of Sciences, USA.

2.1 Introduction

Peroxisomes are essential for lipid metabolism (1). Many xenobiotics, including amphipathic carboxylates used as hypolipidemic agents, induce peroxisome proliferation and ultimately hepatocarcinogenesis in rodents (2). These peroxisome proliferators are nongenotoxic carcinogens that apparently act as tumor promoters by modulating the expression of cellular genes involved in growth and differentiation (3, 4).

Administration of peroxisome proliferators leads to the rapid and coordinated transcriptional induction of the nuclear genes encoding the enzymes of the peroxisomal β-oxidation pathway: fatty acyl-CoA oxidase (AOx; acyl-CoA:oxygen 2-oxidoreductase, [EC 1.3.3.6]), enoyl-CoA hydratase (EC 4.2.1.17)/3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (HD), and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) (5, 6). *Cis*-acting peroxisome proliferator-responsive elements (PPREs) have been identified in the 5' flanking regions of both the AOx (7, 8) and HD (9) genes. Both PPREs contain direct repeats of the sequence TGACCT, the consensus binding site for several members of the nuclear hormone receptor superfamily. Signal transduction by peroxisome proliferators is apparently mediated through distinct ligand-activated receptors, collectively known as peroxisome proliferator-activated receptors (PPARs), that belong to this family of transcription factors (10-13). Recently, the mouse PPAR (mPPAR) has been shown to bind cooperatively to the AOx-PPRE through heteromerization with the 9-*cis*-retinoic acid receptor, RXRα (14).

Here we demonstrate that homologous and heterologous PPARs mediate peroxisome proliferator-dependent transcriptional induction of reporter genes linked to

either the AOx- or HD-PPRE through cooperative protein-DNA interactions between the different PPARs and other cellular factors, including RXRα. However, PPAR-cofactor-DNA interaction is not necessarily sufficient to confer this induction, since we have found that with at least one type of PPAR, induction is differentially accorded by the nature of the PPRE.

2.2 Materials and Methods

2.2.1 Cells

Rat hepatoma H4IIEC3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) horse serum and 5% (v/v) fetal bovine serum. COS-1 cells were maintained in DMEM plus 10% calf serum.

2.2.2 Plasmids and Antibody

pCPS*luc* contains the minimal promoter from the rat liver carbamoyl-phosphate synthetase (CPS) gene (9). pHD(×3)*luc* contains three tandem copies of the HD-PPRE cloned into pCPS*luc*. It was constructed by inserting the oligonucleotide 5'-gatCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA and its complement, 5'-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAG (nucleotides –2956 to –2919 of the rat HD promoter), into the *Bam*HI site of pCPS*luc*. pAOx(×2)*luc* contains two tandem copies of the rat AOx-PPRE generated by inserting the oligonucleotide 5'-gatCCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTa and its complement,

5'-gatctAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGGAAAG (nucleotides −583 to −544 of the rat AOx promoter), into the *Bam*HI site of pCPS*luc*. Nucleotides designated in lowercase letters were added to provide *Bam*HI-*Bgl*II ends. Plasmids expressing *Xenopus* PPARs, xPPARα, xPPARβ, and xPPARγ, were kindly provided by W. Wahli (Lausanne, Switzerland). A plasmid expressing mPPAR was kindly provided by S. Green (Macclesfield, U.K.). cDNAs encoding these PPARs were all originally cloned into the expression vector pSG5 (15). Rat PPAR (rPPAR) cloned into pBluescript II SK(+)(Stratagene) was provided by D. Noonan (Ligand Pharmaceuticals, San Diego). The rPPAR cDNA was excised from this plasmid with *SpeI*/*Eco*RV, and the 2.6-kilobase pair fragment was cloned into appropriately modified sites of the expression vector pRc/CMV (Invitrogen, San Diego).

2.2.3 Transfections

Transfections of H4IIEC3 cells (10-cm dishes at 50% confluence) were done by the calcium phosphate method followed by a dimethyl sulfoxide shock (9). COS-1 cells were transfected similarly except that the cells were incubated for 24 h before and during transfection in medium without phenol red and containing 5% charcoal-stripped fetal bovine serum. Transfections typically contained 5 μg of a reporter gene construct [pHD(×3)*luc* or pAOx(×2)*luc*] and 2 μg of a PPAR expression plasmid. Promoter dosage was normalized for each transfection with pSG5 or pRc/CMV, as appropriate, and the total amount of DNA was maintained at 15 μg with sonicated salmon sperm DNA. Ciprofibrate or Wy-14,643 (each from a ×100 stock in dimethyl sulfoxide) was added to fresh medium to final concentrations of 0.5 mM and 0.1 mM, respectively, at 4 h and

again at 24 h after transfection (control cells received an equal amount of dimethyl sulfoxide). Cell extracts were prepared 48 h after transfection. Luciferase activity from equivalent numbers of cells was measured with a luminometer.

2.2.4 In Vitro Transcription/Translation

Transcription of cDNAs for different PPARs and RXRα and subsequent translation in rabbit reticulocyte lysate were performed by using a commercially available kit (Promega). Translations of proteins for use in gel retardation assays were done with unlabeled methionine.

2.2.5 Gel Retardation Analysis

Nuclear extracts were prepared from monolayer cultures of H4IIEC3, COS-1, and COS-1 cells transfected with various PPAR expression plasmids (16, 17). Gel retardation analysis was performed as described (9). All reactions were normalized for protein content. HD-PPRE and AOx-PPRE double-stranded probes consisting of the oligonucleotides described above were end-labeled with [α-^{32}P]dATP and Klenow fragment of DNA polymerase I. Binding reactions were analyzed by electrophoresis at 4°C on pre-run 3.5% polyacrylamide gels (30:1 acrylamide/*N,N'* - methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer. For binding reactions done with *in vitro* synthesized protein, 2 to 4 μl of translation mixture was incubated with labeled probe. Protein concentrations were normalized with unprogrammed rabbit reticulocyte lysate as necessary.

2.3 Results

2.3.1 Diverse PPARs Differentially Mediate Peroxisome Proliferator-Dependent Transcriptinal Activation via PPREs

Cotransfections of reporter plasmids containing either the HD-PPRE or AOx-PPRE and expression plasmids encoding various PPARs were carried out with COS cells, which are unresponsive to peroxisome proliferators. Expression of pHD(×3)*luc* was induced 3- to 5- fold after cotransfection with either xPPARα or mPPAR in the presence of ciprofibrate or Wy-14,643 (Fig. 2-1B). Induction was dependent upon both receptor plasmid and drug, as shown by control transfections done with pSG5. Drug-dependent induction was not observed with genes encoding either xPPARβ or xPPARγ.

mPPAR accorded a 2- to 3-fold drug-independent induction, which was further increased by either drug. This suggests that COS cells may contain endogenous factors that activate mPPAR (and also rPPAR, see below) but that are unable to activate xPPARs.

Drug and receptor-dependent induction of pAOx(×2)*luc* expression was observed with xPPARα (3- to 6-fold) and mPPAR(2-fold). Similarly, mPPAR also conferred a 3-fold drug-independent induction, which was further increased by either drug. Interestingly, pAOx(×2)*luc* expression was also induced by xPPARγ in the presence of ciprofibrate or Wy-14,643, in contrast to the results obtained with pHD(×3)*luc*. Thus, the ability of xPPARγ to mediate peroxisome proliferator-dependent induction is conditional upon the PPRE used. No drug-dependent induction of expression by the AOx-PPRE was

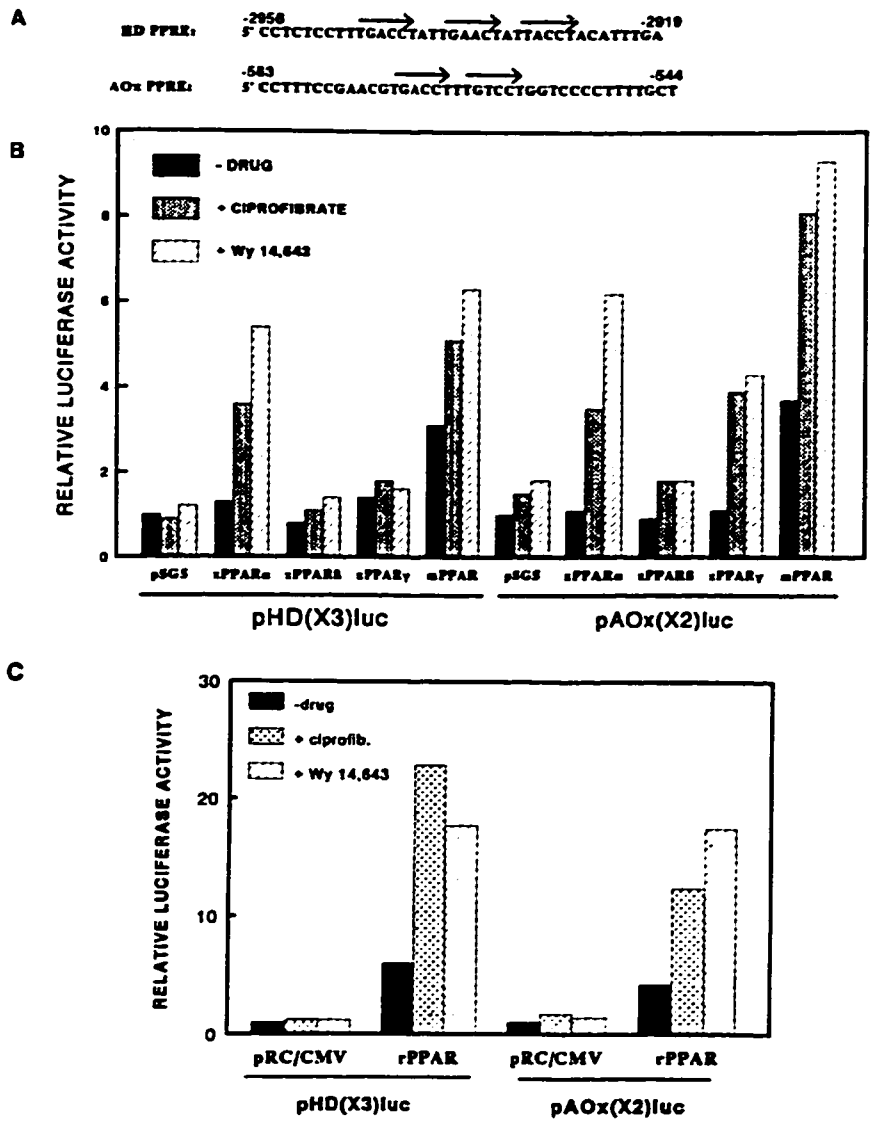

Figure 2-1. Activation of a luciferase reporter gene linked to the HD- or AOx-PPRE by PPARs. (A) Sequences of the HD- and AOx-PPREs. Promoter coordinates are numbered with respect to the transriptional start site of each gene. Arrows indicate TGACCT-like motifs. (B) Luciferase reporter plasmids pHD(×3)*luc* and pAOx(×2)*luc* were cotransfected into COS-1 cells in the presence of control vector pSG5 or pSG5 vectors expressing xPPARα, xPPARβ, PPARγ, or mPPAR, as indicated. An appropriate amount of pSG5 was included in all transfections to normalize promoter dosage. Transfections were done in the absence or presence of the peroxisome proliferators ciprofibrate and Wy-14,643, as indicated. Luciferase activity was measured from cell lysates corresponding to equal numbers of cells. Values are averaged from at least two independent transfections with duplicate samples and were normalized to the activity of control transfections done with pSG5 in the absence of drug, which was taken as 1. (C) Transfections were performed as above except that the luciferase reporter plasmids were transfected with either a pRc/CMV plasmid expressing rPPAR or with the control plasmid pRc/CMV, as indicated.

observed with xPPARβ. This is in contrast to the results of Dreyer *et al.* (11). In their experiments, the AOx-PPRE was placed upstream of the basal thymidine kinase promoter, and transfection were carried out in HeLa cells. The differences in experimental conditions may explain our inability to detect a xPPARβ-dependent induction. Taken together, the results suggest that promoter context or specific cellular coregulators can modulate the induction mediated by particular PPARs.

Both pHD(×3)*luc* and pAOx(×2)*luc* responded in a similar fashion to the drugs in transfections performed with rPPAR (Fig. 2-1C). rPPAR was the most effective receptor at mediating peroxisome proliferator signaling (15- to 25-fold induction). There was also a drug-independent but rPPAR-dependent induction of expression observed with both pHD(×3)*luc* (5-fold) and pAOx(×2)*luc* (3-fold). This finding further supports the suggestion that COS cells contain specific endogenous PPAR-activated ligands.

2.3.2 PPARs Bind to the HD- and AOx-PPREs

To determine if the differential response of the HD- and AOx-PPREs to specific PPARs was due to differences in DNA-protein interactions, gel retardation analyses were performed with nuclear extracts from COS cells transfected with expression plasmids encoding different PPARs (Fig. 2-2). A protein-DNA complex was observed with both the HD-PPRE probe (Fig. 2-2, lanes i-l) and AOx-PPRE probe (Fig. 2-2, lanes c-f) when using extracts of transfected cells but not of untransfected cells (Fig. 2-2, lanes b and h).

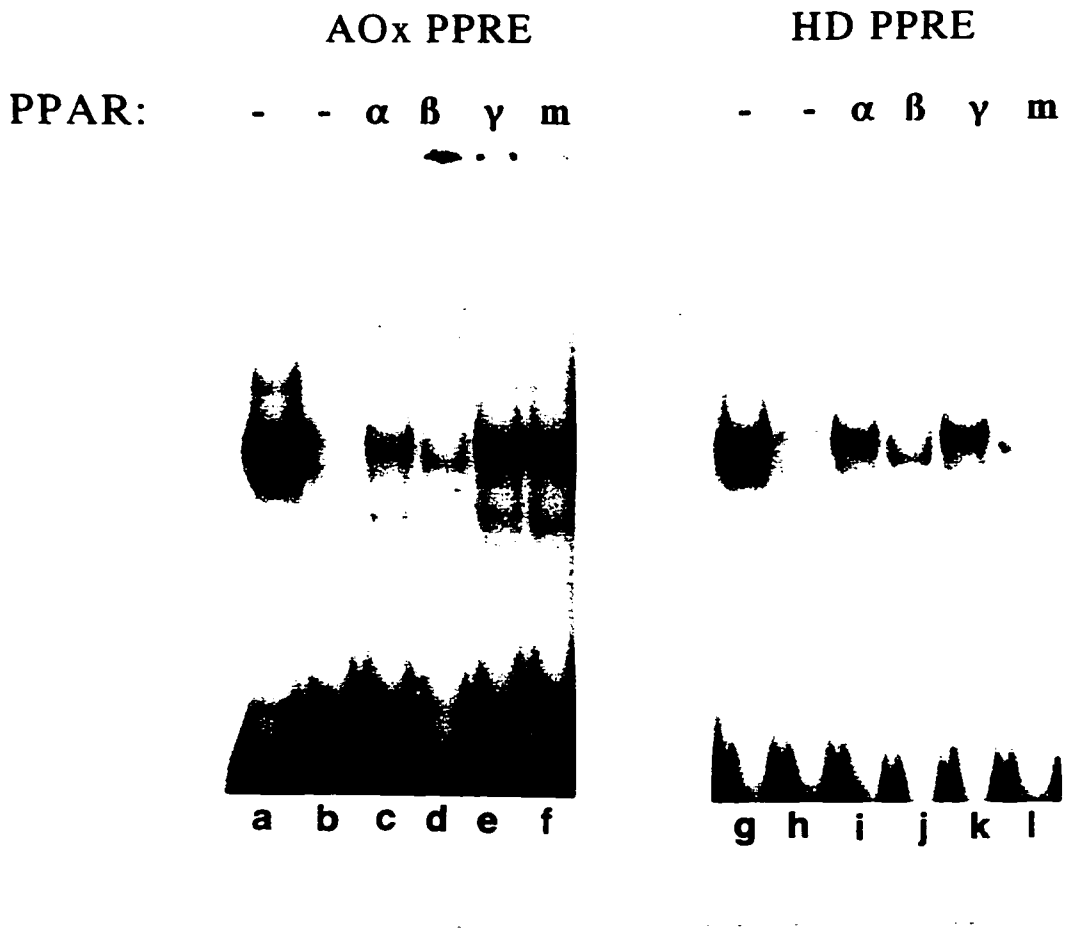

Figure 2-2. PPARs expressed *in vivo* bind to the AOx- and HD-PPREs. Nuclear extracts prepared from COS-1 cells transfected with pSG5 (lanes b and h) or transfected with various PPAR-expressing plasmids (lanes: α, xPPARα; β, xPPARβ; m, mPPAR) were incubated with labeled AOx-PPRE probe (lanes b-f) or HD-PPRE probe (lanes h-l) and analyzed by gel retardation. Lanes a and g are reactions carried out with the AOx- or HD-PPREs, respectively, and extract prepared from H4IIEC3 cells. The PPRE probes used in this and all subsequent binding reactions are described in Materials and Methods.

There was a correspondence in the mobilities of the complexes formed between the HD and AOx probes and a particular PPAR, indicating that the same or similar factors bound to both the HD- and AOx-PPREs. Assays done with extracts from peroxisome proliferator-responsive H4IIEC3 cells generated complexes of similar mobility with both the HD- and AOx-PPREs (Fig. 2-2, lanes g and a, respectively).

Therefore, the failure of xPPARβ to induce expression via either the AOx- or HD-PPRE is not due an inability of this receptor to bind these elements *in vitro* or to the possibility that this particular receptor was unstable and rapidly degraded *in vivo*. Similarly, the differential effects observed with xPPARγ-mediated induction via the AOx-PPRE *vis-à-vis* the HD-PPRE cannot be ascribed to differences in the ability of xPPARγ to bind to the HD-PPRE as opposed to the AOx-PPRE.

2.3.3 A Cellular Cofactor Stimulates PPAR Interaction with PPREs

PPARs belong to the nuclear steroid hormone receptor superfamily. The DNA-binding activity of this class of receptors is stimulated in several cases by cooperative interactions with other cellular factors, including the RXR family of receptors (18-20). Gel retardation analyses were done with *in vitro* translated PPARs to determine if they could interact cooperatively with the HD-PPRE in the presence of cellular factors. Fig. 2-3A shows the [^{35}S]methionine-labeled PPARs. Each PPAR bound to the HD-PPRE (Fig. 2-3B, lanes c-g), and binding was enhanced by the addition of COS cell nuclear extract (Fig. 2-3B, lanes h-l). Complexes were not generated with unprogrammed rabbit reticulocyte lysate or lysate supplemented with COS cell extract (Fig. 2-3B, lanes a and b, respectively). Similar results were obtained with the AOx-PPRE (data not presented).

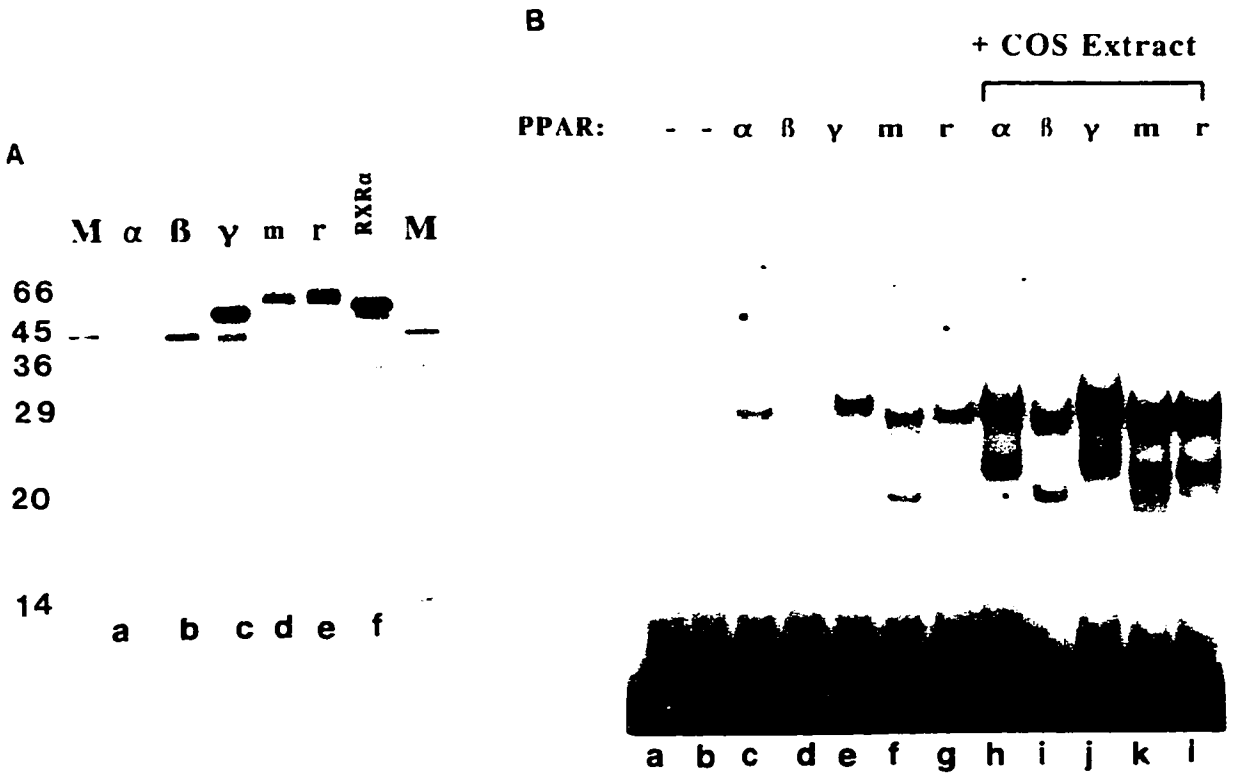

Figure 2-3. A cellular factor stimulates PPAR-DNA binding. The cDNAs encoding the various PPARs were transcribed and translated *in vitro*, and the proteins were used for gel retardation assays with the HD-PPRE probe. (A) SDS/polyacrylamide gel of [^{35}S]methionine-labeled translation products from rabbit reticulocyte lysates programmed with mRNA transcribed *in vitro* from plasmids encoding the various PPARs or RXRα. Lanes: r, rPPAR; M, molecular weight standards (in kDa); others, as in Fig. 2-2. (B) The different PPARs were synthesized *in vitro* as above but with unlabeled methionine. Each translation mixture (2 μl) was incubated with labeled HD-PPRE probe in the absence (lanes c-g) or the presence (lanes h-l) of 0.2 μg of nuclear extract from COS-1 cells. Control lanes include probe incubated with 2 μl of unprogrammed reticulocyte lysate (lane a) or unprogrammed reticulocyte lysate and 0.2 μg of COS extract (lane b). All reactions were normalized as to protein content with bovine serum albumin.

2.3.4 Cooperative DNA Binding with RXRα

The spacing of the two proximal TGACCT-like repeats in the HD-PPRE conforms to that of RXR elements (21,22). Consistent with this, all PPARs were shown to bind cooperatively to the HD-PPRE in the presence of *in vitro* translated RXRα (Fig. 2-4A, compare lanes b-f with lanes i-l). RXRα-dependent stimulation of PPAR binding was most pronounced with xPPARγ, mPPAR, and rPPAR (Fig. 2-4A, compare lanes j-l to lanes d-f) and to a lesser extent with xPPARα and xPPARβ (compare lanes h and i to lanes b and c); RXRα alone had no binding activity (lane g). Similar results were obtained with the AOx-PPRE probe (Fig. 2-4B). These results indicate that all the PPARs are capable of interacting cooperatively with RXRα on either the AOx- or HD-PPRE.

Anti-RXRα antibody decreased the amount of complex formed between *in vitro* translated rPPAR and RXRα and resulted in the concomitant appearance of a supershifted complex, demonstrating the presence of RXRα in this complex (Fig. 2-5, compare lanes c and g, respectively). Similar results were obtained with *in vitro* translated rPPAR incubated with COS extract (Fig. 2-5, compare lanes d and h). The amount of protein-DNA complex formed with *in vitro* translated rPPAR alone also decreased in the presence of anti-RXRα antibody; however, a supershifted complex was not readily seen, perhaps because of the small amount of complex originally formed and to the interference of the antibody with complex stability (Fig. 2-5, compare lanes b and f). These results suggest that one of the cofactors supplied by the COS extract is indeed RXRα. Preimmune serum had no effect on complex stability or electrophoretic

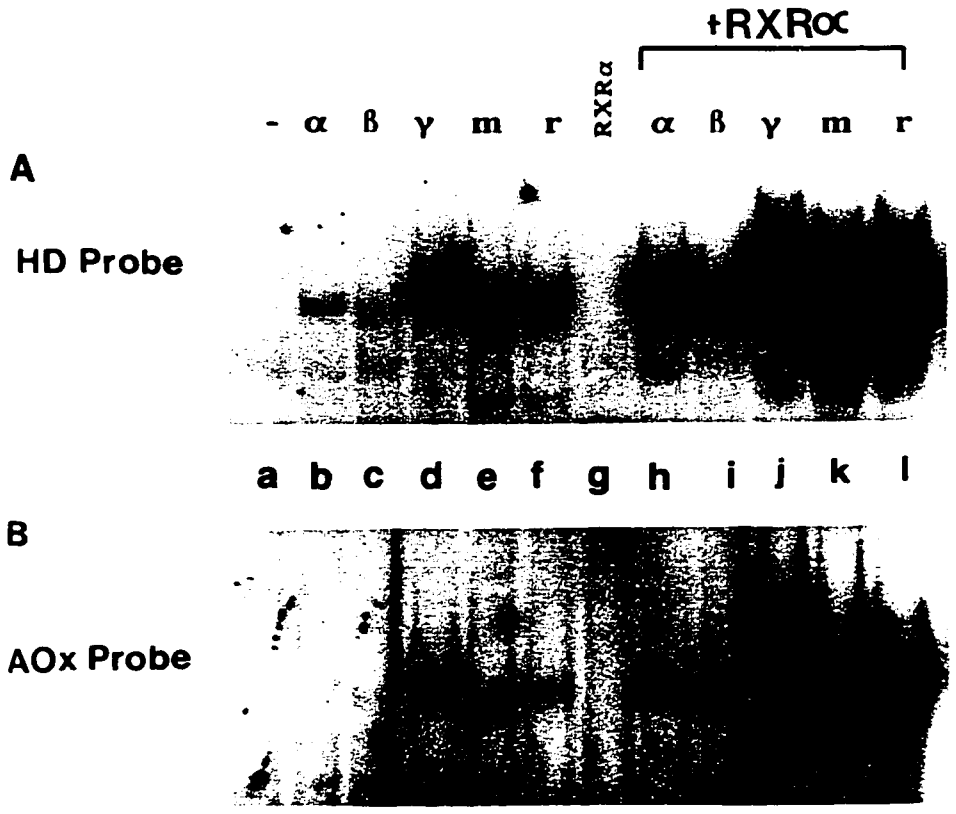

Figure 2-4. PPARs bind cooperatively with RXRα to both the HD- and AOx-PPREs. Unlabeled *in vitro* translated PPARs were incubated with either labeled HD-PPRE (A) or labeled AOx-PPRE (B) in the absence (lanes b-f) or presence (lanes h-l) of unlabeled *in vitro* translated RXRα. Two microliters of each translation mixture was used. Unprogrammed reticulocyte lysate (2 μl) was added to reactions a-g to normalize for total protein. Lanes: a, unprogrammed reticulocyte lysate incubated with each probe; g, RXRα alone incubated with each probe.

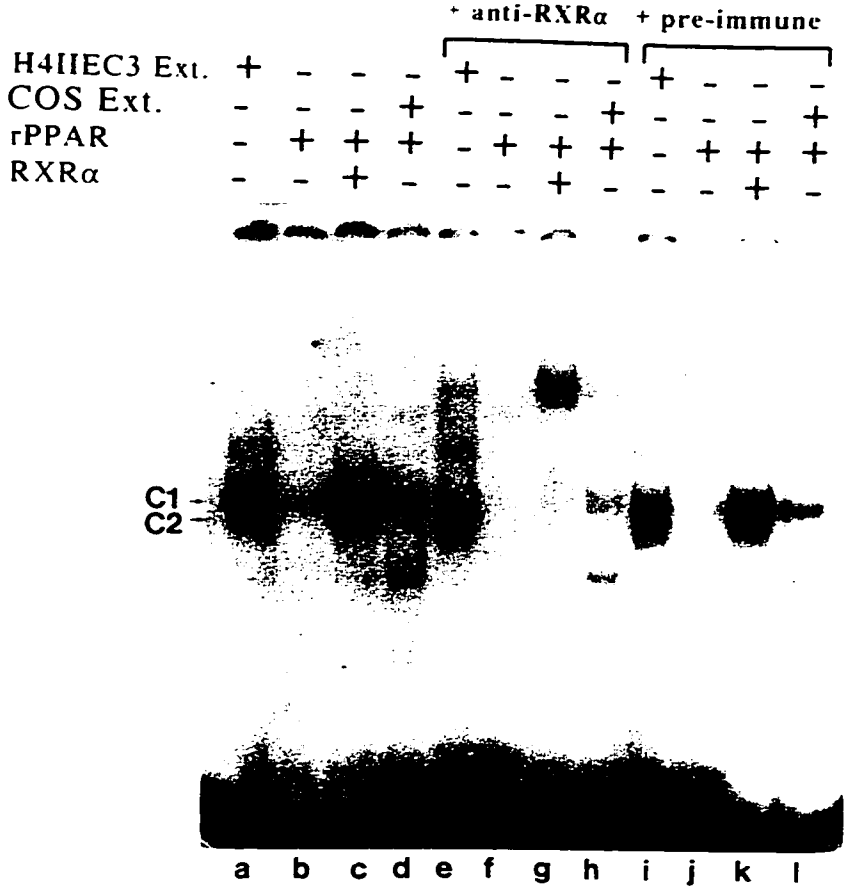

Figure 2-5. RXRα is present in protein-DNA complexes formed between H4IIEC3 nuclear extract or *in vitro* translated rPPAR and HD PPRE. Labeled HD-PPRE was incubated with extract from HR4IIEC3 cells or with *in vitro* translated rPPAR supplemented with RXRα or COS-1 cell extract, as indicated, and analyzed by gel retardation (lanes a-d). C1 and C2 correspond to the two protein-DNA complexes formed between HD-PPRE and H4IIEC3 cell extract. The arrowhead corresponds to the supershifted complex observed in reactions carried out in the presence of anti-RXRα serum.

mobility (Fig. 5, lanes i-l). RXRα was shown to be present in the protein-DNA complexes generated by the other PPARs (data not presented).

Incubation of the HD probe with extracts from H4IIEC3 cells generated two protein-DNA complexes (C1 and C2; Fig. 2-5, lane a), of which only the upper was supershifted with anti-RXRα antibody (lane e). This supershifted complex comigrated with the supershifted complex observed with *in vitro* translated rPPAR and RXRα (Fig. 2-5, lane g). Therefore, these results suggest that at least one of the complexes formed on the HD-PPRE in peroxisome proliferator-responsive H4IIEC3 cells contains RXRα.

2.3.5 xPPARγ Interferes with the Functional Activity of PPARs in Vivo

The *in vitro* DNA binding results suggest that the ability of xPPARγ to stimulate drug-dependent transcription from the AOx-PPRE but not from the HD-PPRE *in vivo* is not due to an intrinsic inability of this receptor to bind to the HD element or to interact cooperatively with cellular factors such as RXRα. To test whether this receptor interferes with signaling by other functional isoforms of PPARs, cells were cotransfected in the presence or absence of ciprofibrate with pHD(×3)*luc*, a constant amount of rPPAR or xPPARα, and an increasing amount of xPPARγ. Cotransfection of rPPAR and xPPARα with increasing amounts of xPPARγ reduced the luciferase activities mediated by rPPAR and xPPARα in a dose-dependent manner (Fig. 2-6). xPPARγ had no effect on transfections carried out in the absence of ciprofibrate, indicating that the inhibition was specific and not the result of squelching. Therefore, xPPARγ can interfere with the activity of rPPAR and xPPARα, presumably because xPPARγ is capable of competing

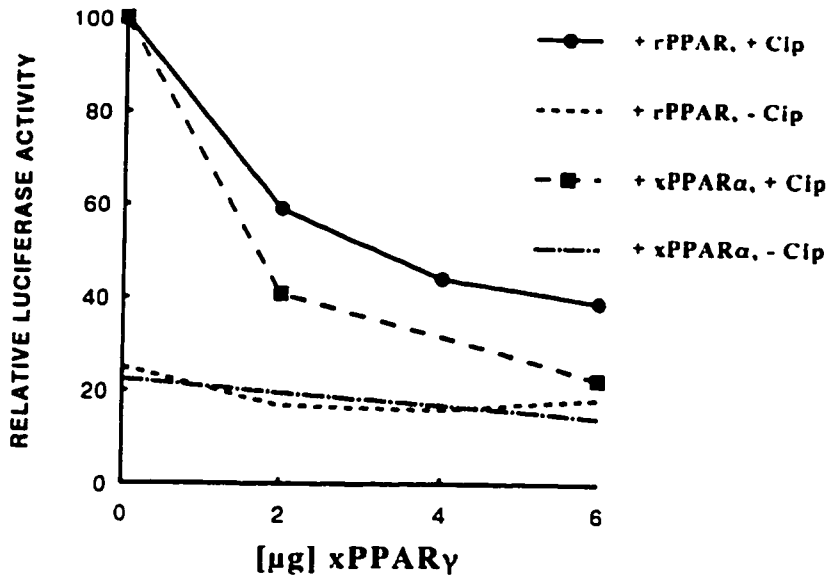

Figure 2-6. xPPARγ *trans*-dominantly inhibits transcription induction mediated by rPPAR and xPPARα. pHD(×3)*luc* (5 μg) was cotransfected into COS-1 cells with either 2 μg of rPPAR or 2 μg of xPPARα expression plasmid in the absence or the presence of increasing amounts of plasmid expressing xPPARγ, as indicated at the bottom of the figure. Transfections were done in duplicate in the presence (+) or absence (–) of ciprofibrate (Cip). The amount of DNA in each transfection was normalized with pSG5. The values shown are normalized to the activity obtained from the respective reactions carried out in the absence of competitor plasmid, which was taken as 100%.

in vivo with functional receptors for the cognate PPRE binding site.

2.4 Discussion

The discovery of a number of related PPARs (10-13) raises the possibility that members of this family of ligand-activated receptors may be involved in distinct and specific regulatory signaling pathways. In this report, we demonstrate that peroxisome proliferator-mediated activation of the HD gene can be elicited by diverse PPARs via direct interaction of these, and possibly other, peroxisome proliferator-responsive genes.

Our findings demonstrate that each PPAR, and isoforms of xPPAR, can bind to the AOx- and HD-PPREs and can do so synergistically through interaction with RXRα. These results are consistent with the structural homologies among the PPARs (10-13) and with the similarities between the AOx- and HD-PPREs (8,9). Both PPREs contain two imperfect direct copies of a TGACCT-like motif separated by a single nucleotide (see Fig. 2-1A) and thereby conform to retinoid X response elements (21,22).

Interestingly, the expression of the luciferase reporter gene linked to the HD- or AOx-PPRE was not induced, or was differentially activated, by particular PPARs. For instance, xPPARβ failed to stimulate pHD(×3)*luc* or pAOx(×2)*luc* expression in the presence of either ciprofibrate or Wy-14,643. More significantly, xPPARγ stimulated expression of the luciferase reporter linked to the AOx-PPRE but not to the HD-PPRE. These results show that PPAR-DNA binding or cooperative interactions with cellular factors, including RXRα, are required but not necessarily sufficient to elicit peroxisome proliferator-mediated activation. Importantly, as demonstrated with xPPARγ, activity can depend on the nature of the PPRE. The AOx- and HD-PPREs are fairly divergent.

There are differences both in the sequences of the TGACCT-like repeats as well as in the flanking nucleotides (8,9). Moreover, the HD-PPRE contains a third TGACCT motif two nucleotides farther upstream, which is not present in the AOx-PPRE (see Fig. 2-1A). It is likely that some or all of these differences underlie the target gene specificity observed with xPPARγ and perhaps other PPAR isoforms. It is intriguing to speculate that ligand activation or requisite protein-protein interactions (for instance with basal transcription factors or coactivators) may be influenced by differences in receptor-coregulator-DNA interactions or conformation of protein-DNA complexes imparted by different target PPREs.

Finally, the ability of xPPARγ to interfere with the *in vivo* induction of transcription mediated by rPPAR or xPPARα implies that PPAR isoforms may act as both repressors and activators of specific target genes. Our findings reveal a complex interactive network of both positive and negative control pathways underlying the regulation of genes involved in lipid homeostasis and drug detoxification.

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CHAPTER 3

TRANSACTIVATION BY PPAR/RXR HETERODIMERS IN YEAST IS POTENTIATED BY EXOGENOUS FATTY ACID VIA A PATHWAY REQUIRING INTACT PEROXISOMES[2]

[2] A version of this chapter has been published. Marcus, S.L., Miyata, K.S., Rachubinski, R.A., and Capone, J.P. 1995. Gene Expression 4:227-239. Used with permission from Cognizant Communication Corporation.

3.1 Introduction

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 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 ß-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.*, 1992; 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, while 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 ß-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 ß-oxidation pathway or some enzymatic step prior to ß-oxidation (Göttlicher *et al.*, 1993; Bentley *et al.*, 1993; Tomaszewski and Melnick, 1994). However, the role of the peroxisome in general, and the peroxisomal ß-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 ß-oxidation, are mechanistically similar to their mammalian counterparts. Indeed, fatty acid ß-oxidation in yeast is carried out exclusively in peroxisomes, while 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.

3.2 Materials and Methods

3.2.1 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-kilobase 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 vector *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-kilobase 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, PP-RXR425, 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 3' 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 ß-galactosidase activity was assayed (Ausubel *et al.*, 1989; Himmelfarb *et al.*, 1990).

3.2.2 Electrophoretic Mobility Shift Analysis

Electrophoretic mobility shift analysis using *in vitro* translated mPPAR and hRXRα and radiolabeled 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 (pH7.5)/0.1 mM EDTA/20% glycerol/2 mM dithiothreitol/pepstatin (1 $\mu g \cdot ml^{-1}$)/chymostatin (0.1 $\mu g \cdot ml^{-1}$)/antipain (2.5 $\mu g \cdot ml^{-1}$)/leupeptin (0.5$\mu g \cdot ml^{-1}$)/aprotinin (5 $\mu g \cdot ml^{-1}$)/0.2 mM phenylmethylsulfonyl fluoride) in a final volume of 15 μl. Where indicated in Fig. 3-2, 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*.

3.3 Results

3.3.1 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 hRXR (yhRXRα) or mPPAR (ymPPAR) alone had little effect on the basal activity of the reporter gene construct (Table 3-1). 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

Table 3-1. 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* strain DL-1 containing the plasmids indicated were grown, harvested, lysed, and assayed for ß-galactosidase activity (Ausubel *et al.*, 1989; Himmelfarb *et al.*, 1990). Units are given as the $A_{420}X10^3$/min. The values reported are the averages of at least two independent assays done in duplicate. Values did not vary by more than 10%.

Table 3-2. 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

**ß-galactosidase activity was measured as described in Table 3-1. The reporter plasmid was 2HDΔL1. Plasmid constructs are defined in Materials and Methods.

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 3-1). 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 3-2).

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. Disruption of either the first or second TGACCT

repeat in the AOx-PPRE (1AOxM1ΔL1 and 1AOxM2ΔL1, respectively) abolished transactivation by mPPAR/hRXRα (Fig. 3-1A). 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. 3-1B). 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.

3.3.2 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. 3-2A,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. 3-2B, lanes d and e, with Fig. 3-2C, lane c) and on the AOx-PPRE (Fig. 3-2A, 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

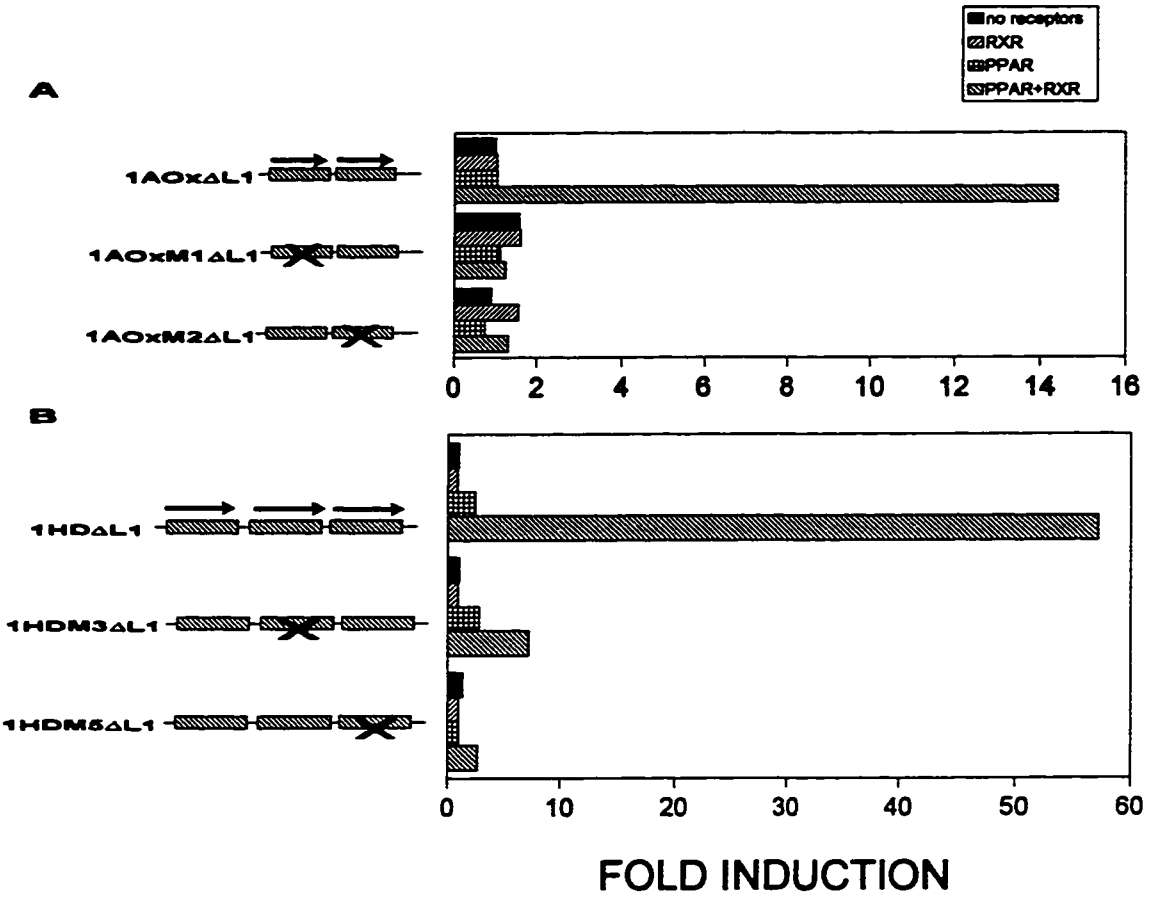
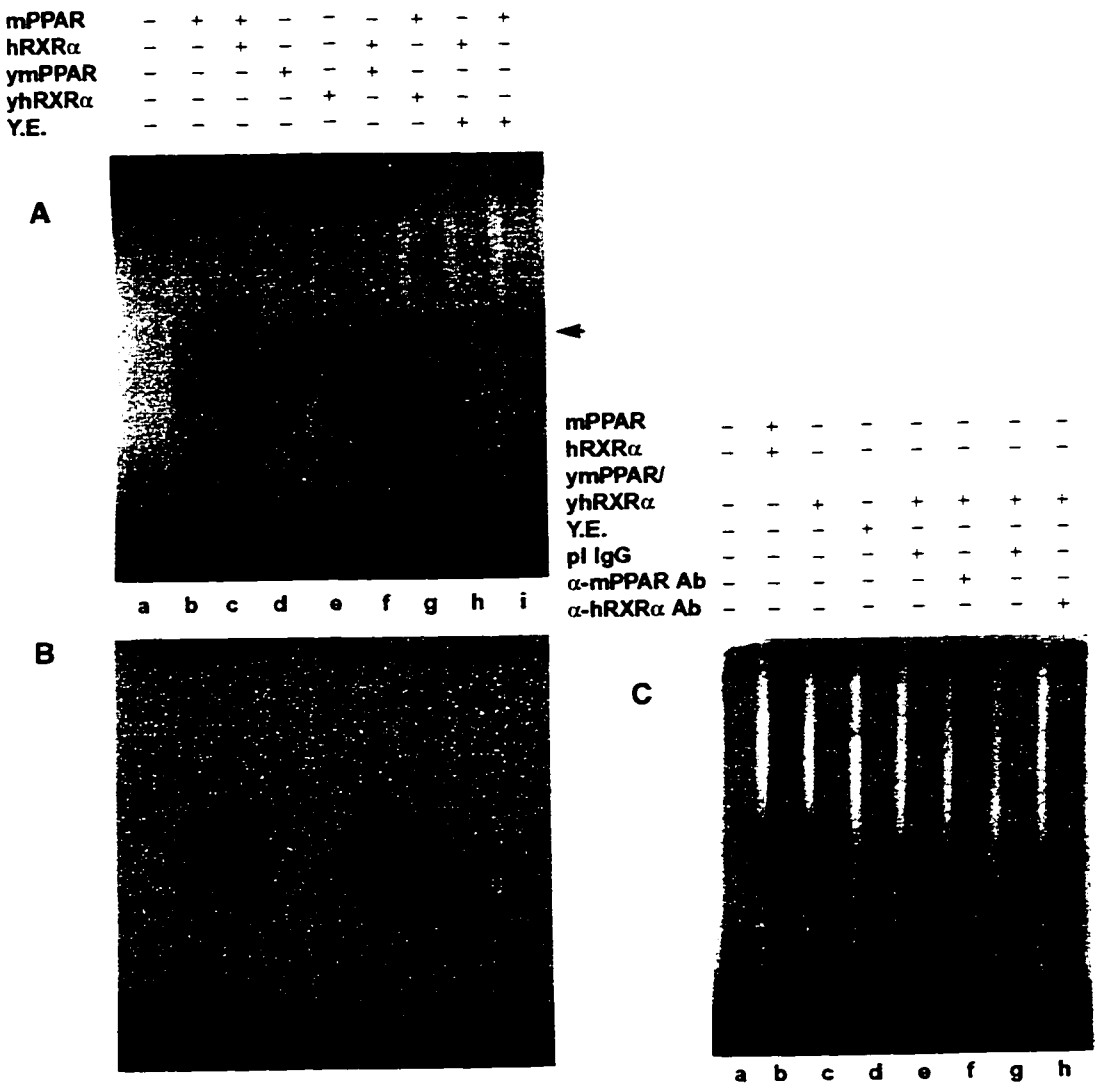


Figure 3-1. The integrity of repeats in the AOx-PPRE (A) and HD-PPRE (B) is essential for activation by mPPAR/hRXRα in yeast. Reporter gene constructs containing one copy of either the wild-type AOx- or HD-PPRE or mutant PPREs in which individual TGACCT repeats were mutated, as indicated, were introduced 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 ß-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 the Materials and Methods.

Figure 3-2. 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. 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. 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).


mPPAR
hRXRα
ymPPAR
yhRXRα
Y.E.
A
a b c d e f g h i
mPPAR
hRXRα
ymPPAR/
yhRXRα
Y.E.
pI IgG
α-mPPAR Ab
α-hRXRα Ab
B
C
a b c d e f g h

(Fig. 3-2C, lanes f and h, respectively). mPPAR synthesized in yeast cooperated with *in vitro* translated hRXRα to bind DNA (Fig. 3-2A, B, lanes f) and *vice versa* (lanes g). The small amount of complex seen with *in vitro*-translated mPPAR alone (Figs. 3-2A, B, lanes b) or when mixed with untransformed yeast extract (Fig. 3-2A, 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. 3-2A, 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 ß-galactosidase activity observed with the AOx-PPRE reporter construct *vis-à-vis* the HD-PPRE reporter construct (see Table 3-1). 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 mPPAR and hRXRα physically interact *in vivo* in yeast in the absence of a cognate target site using the two-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.

3.3.3 Exogenously Added Fatty Acid Potentiates PPAR Transactivation in Yeast

Because a large number of hypolipidemic agents as well as polyunsaturated and monounsaturated 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

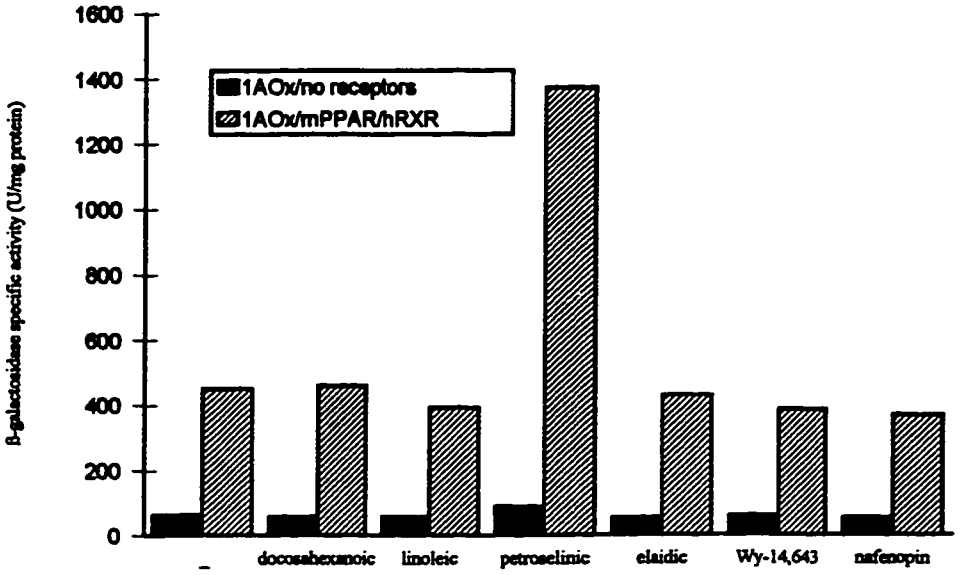
yeast. The potent peroxisome proliferators Wy-14,643 and nafenopin (Fig. 3-3A) or the fibrate drugs clofibrate and ciprofibrate (not presented) had no effect on transcription of the reporter gene construct containing a single copy of the AOx-PPRE. The inclusion of 9-*cis*-retinoic acid (0.1 μM) along 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. 3-3A). Growth of yeast expressing mPPAR and hRXRα in medium containing 0.01% (w/v) petroselinic acid resulted in an approximately 3-fold 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. 3-3B) and was dose-dependent (Fig. 3-3C). 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.

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

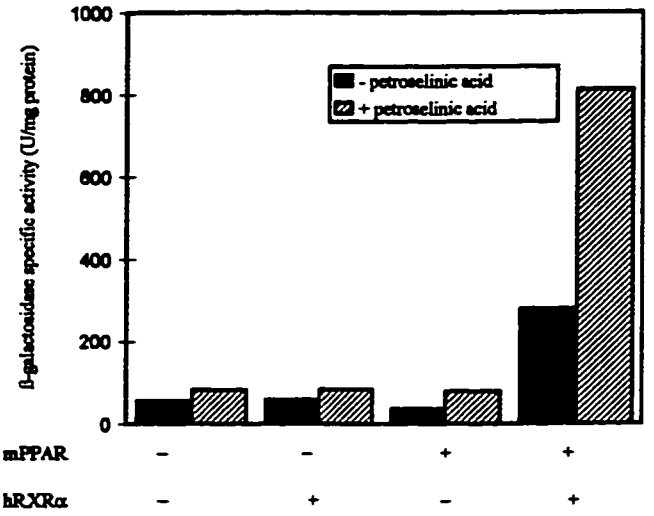
The relaxed structural specificity of PPAR activators is paradoxical with the concept of high-specificity ligand interactions for nuclear receptors. It has been

Figure 3-3. mPPAR/hRXRα-dependent transactivation in yeast is potentiated by petroselenic 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.

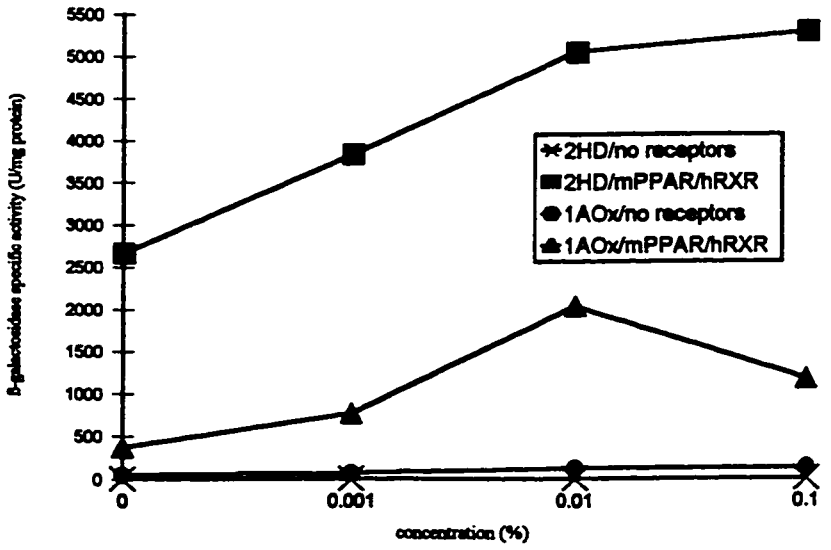
A


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

B


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

C


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

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 ß-oxidation or some step prior to ß-oxidation such as thioesterification (Göttlicher *et al.*, 1993, Bentley *et al.*, 1993). The results given in Tables 3-1 and 3-2 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, 2μm) 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 3-3, 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 ß-oxidation pathway. Similar results were obtained using the AOx-PPRE reporter gene (not shown).

Table 3-3. Activation of mPPAR by petroselinic acid requires intact peroxisomes but not an intact β-oxidation pathway.

		β-Galatosidase Activity (U/mg Protein)	
Yeast Strain	mPPAR/hRXRα	– 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. 3-3. Transformants were assayed for β-galactosidase activity as in Table 3-1. Control transformants contained the corresponding empty vectors.

In contrast, intact peroxisomes appear to be necessary for stimulation of mPPAR/RXR activity by petroselinic acid. Thus, as shown in Table 3-3, 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. 3-3. In contrast, petroselinic 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 since peroxisomal assembly mutants fall into at least nine complementation groups. To explore the requirement for ß-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 ß-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 3-3, 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 ß-oxidation pathway.

3.4 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 since 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 whose 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, while maximal PPAR/RXR-dependent activation of AOx-PPRE-linked reporter genes in mammalian 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 the 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 ß-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 ß-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 ß-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 ß-oxidation pathway in modulating PPAR function. Our findings show that neither intact peroxisomes nor ß-oxidation is necessary for constitutive activity of PPAR/RXR heterodimers. However, intact peroxisomes, but not necessarily the integrity of the peroxisomal ß-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 ß-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[3]. Our findings are consistent with the observation that poorly metabolized fatty acids or

[3] S. L. Marcus, R. A. Rachubinski, and J. P. Capone, unpublished observations

derivatized fatty acids that cannot undergo ß-oxidation are more potent substrate inducers of PPARs in mammalian cells. Indeed, Göttlicher *et al.* (1993) have shown that blocking ß-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 ß-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 ß-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 activity 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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CHAPTER 4

IDENTIFICATION OF COUP-TFII AS A PEROXISOME PROLIFERATOR RESPONSE ELEMENT BINDING FACTOR USING GENETIC SELECTION IN YEAST: COUP-TFII ACTIVATES TRANSCRIPTION IN YEAST BUT ANTAGONIZES PPAR SIGNALING IN MAMMALIAN CELLS[4]

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4.1 Introduction

Peroxisome proliferator-activated receptors (PPAR) are recently described ligand-activated members of the nuclear hormone receptor superfamily that regulate the transcription of a large number of genes important for lipid and metabolic homeostasis. PPARs were originally identified on the basis of their ability to mediate transcriptional induction by peroxisome proliferators (Issemann and Green, 1990; Dreyer *et al.*, 1992), a diverse group of xenobiotic agents that include hypolipidemic drugs, herbicides, and plasticizers, which have been shown to act as non-genotoxic rodent hepatocarcinogens (Rao and Reddy, 1991). PPARs are also activated by fatty acids and lipid-like compounds, suggesting that these compounds, or derivatives thereof, may be endogenous regulators of PPAR (Göttlicher *et al.*, 1992; Keller *et al.*, 1993; Forman *et al.*, 1995; Kliewer *et al.*, 1995). PPARs exist in a variety of subtypes and isoforms (Dreyer *et al.*, 1992; Schmidt *et al.*, 1992; Chen *et al.*, 1993; Kliewer *et al.*, 1994). Some are ubiquitously expressed, while others are expressed in a tissue- and cell-specific manner and display distinct pharmacological properties. It is becoming increasingly apparent that members of this receptor subfamily play central roles in a wide spectrum of cellular functions that control growth, development, and differentiation (Chawla and Lazar, 1994; Tontonoz *et al.*, 1994). Accordingly, there is a great deal of interest in elucidating the pleiotropic functions of PPARs and understanding how these transcription factors mediate appropriate cellular responses to multiple nutritional and hormonal stimuli.

PPARs activate transcription by binding to peroxisome proliferator-response elements (PPRE) located upstream of target genes through heterodimerization with retinoid X receptors (RXR) (Kliewer *et al.*, 1992; Marcus *et al.*, 1993). PPREs were first

identified in the promoter regions of the genes encoding the peroxisomal ß-oxidation enzymes fatty acyl-CoA oxidase (AOx) (Osumi *et al.*, 1991; Tugwood *et al.*, 1992) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) (Zhang *et al.*, 1992; Bardot *et al.*, 1993). PPREs have subsequently been identified in many genes important for the regulation of lipid homeostasis, as well as in genes involved in cellular differentiation and proliferation (Chawla and Lazar, 1994; Tontonoz *et al.*, 1994). The core PPRE consists of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (TGACCT) separated by one nucleotide (DR1) (Tsai and O'Malley, 1994). However, PPREs are not exclusive targets of PPAR/RXR heterodimers. Rather, PPREs appear to be composite response elements that can interact with other members of the nuclear hormone receptor family, consistent with the relaxed DNA binding specificity of many nuclear hormone receptors that allows promiscuous binding to degenerate hormone response elements (Tsai and O'Malley, 1994). Thus, the orphan nuclear hormone receptors chicken ovalbumin upstream promoter transcription factor-I (COUP-TFI) and HNF-4 have been shown to interact with both the AOx- and HD-PPREs (Miyata *et al.*, 1993; Winrow *et al.*, 1994). Recently, thyroid hormone receptor (TR) has been shown to bind to the AOx-PPRE (Chu *et al.*, 1995b; Hunter *et al.*, 1996) and the HD-PPRE (Chu *et al.*, 1995b) through heterodimerization with RXR. These receptors have little effect on transcription of PPRE-linked genes on their own but have been shown to differentially modulate activation by PPAR/RXR heterodimers in a response element-dependent manner.

PPAR function is therefore subject to differential modulation by multiple nuclear receptors that can recognize the PPAR cognate response element. The diversity of PPAR

signaling is further enhanced by the findings that PPAR can heterodimerize with partners other than RXR, such as TR (Bogazzi *et al.*, 1994) and the recently described orphan receptor LXRα (Willy *et al.*, 1995; Miyata *et al.*, 1996) Unraveling the multiplicity of regulatory strategies that converge via PPREs requires the identification of the full spectrum of cellular factors that bind to PPREs and/or interact with PPAR. Towards this goal, we present a genetic screening system in yeast for the direct identification of positively acting PPRE-binding proteins. Using this strategy, we identified the orphan nuclear hormone receptor COUP-TFII (ARP1) as a PPRE-interacting transcription factor. Significantly, while COUP-TFII is a potent activator of PPRE-linked genes in yeast, it represses PPAR/RXR-mediated transactivation in mammalian cells.

4.2 Materials and Methods

4.2.1 Yeast Strains and Plasmids

pmPPAR/HDHIS305 is an integrative yeast vector that contains the histidine gene under the transcriptional control of the minimal *CYC1* TATA-box and two tandem copies of the HD-PPRE, and also constitutively expresses the mouse PPARα under control of the phosphoglycerate kinase (PGK) promoter. It was constructed through a series of subcloning steps (Fig. 4-1). The *CYC1* promoter was first amplified from *S. cerevisiae*genomic DNA using the oligonucleotides 5'-ATTCCCGGGCAGATCCGCCAGGC (forward primer) and 5'-ATTGAATTCAGTCATTATTAATTTAGTG (reverse primer) (McNeil and Smith, 1986). The resulting product, containing the minimal *CYC1* TATA-box and the codons for the first four amino acids of the *CYC1* gene product, was subcloned into p2HD314

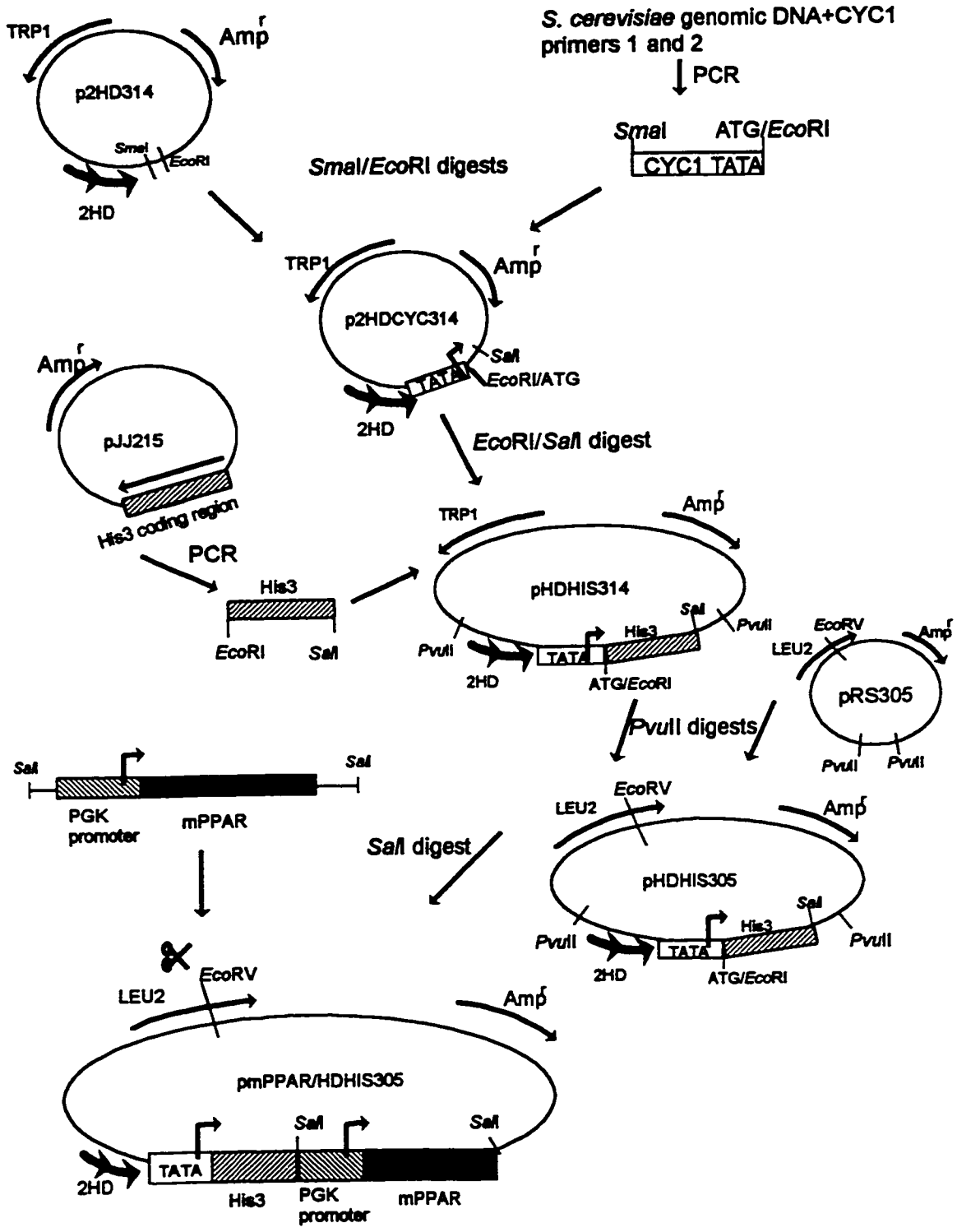

Figure 4-1. Schematic of the construction of the vector pmPPAR/HDHIS305.

(Marcus *et al.*, 1995), a derivative of the shuttle vector pRS314 that contains two copies of the HD-PPRE synthetic oligonucleotide (5'-CCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA; the underlined nucleotides correspond to the TGACCT-like direct repeats). The *HIS* gene was amplified by polymerase chain reaction (PCR) from pJJ215 (Jones and Prakash, 1990) and cloned into the above plasmid in frame with the *CYC1* codons coding for the amino-terminal amino acids. The entire HD-PPRE-*CYC1-HIS* cassette was then inserted as a *Pvu*II fragment into the integrative vector pRS305 (Marcus *et al.*, 1995) to generate pHDHIS305. A mouse PPARα/PGK expression cassette (Marcus *et al.*, 1995) was cloned into the *Sal*I site of pHDHIS305 to generate pmPPAR/HDHIS305.

pmPPAR/HDHIS305 was linearized at its unique *Eco*RV site in the *LEU2* gene and integrated into the yeast genome by transformation into *S. cerevisiae* YPH500 (*Matα, ura3, lys2, ade2, trp1, his3, leu2*) (Sikorski and Hieter, 1989). Cells were grown in 0.67% yeast nitrogen base without amino acids/2% glucose supplemented with uracil, adenine, lysine, tryptophan, histidine, and leucine each at 20 $\mu g \cdot ml^{-1}$, as required. Integrant W18-2 containing a single copy of the vector at the *LEU2* locus, as confirmed by Southern blot analysis, was isolated and used for library screening.

4.2.2 Library Screening

A rat liver cDNA 5'-stretch library constructed in the *Eco*RI site of the vector YEUra3 was obtained from Clontech (Palo Alto, CA). Plasmid DNA from the amplified library was purified on a CsCl gradient (Ausubel *et al.*, 1989). W18-2 was transformed with the amplified library by the polyethylene glycol-lithium acetate method (Ausubel *et*

al., 1989), except that dimethylsulfoxide was added to a final concentration of 10% (v/v) during a 15 min heat shock at 42°C. The yeast were then plated onto 150 mm agar plates containing 0.67% yeast nitrogen base without amino acids/2% galactose/L-tryptophan at 30 μg ml^{-1}/20 mM 3-aminotriazole, and his$^+$/ura$^-$ colonies were selected. Plasmids were rescued into *Escherichia coli*. Sequence analysis showed that one plasmid contained the entire open reading frame for rat COUP-TFII and was used for further analysis.

4.2.3 Expression of COUP-TFII in Yeast and Assay of β-galactosidase Activity

pCOUP-TFII.314, a low copy COUP-TFII yeast expression vector, was constructed by subcloning the COUP-TFII open reading frame including the *gal*1-10 promoter from the YEUra3 library plasmid into pRS314 (trp$^-$). A plasmid containing only the *gal*1-10 promoter (pGal10.314) was also constructed to serve as a control. cmPPAR is a *CEN*-vector expressing mouse PPARα under the control of the PGK promoter (Marcus *et al.*, 1995). β-galactosidase reporter plasmids containing one or two copies of wild-type HD-PPRE, 1HDΔL1 and 2HDΔL1, respectively or mutant PPREs, 1HDM3ΔL1 and 1HDM5ΔL1, have been described (Marcus *et al.*, 1995). YPH500 was transformed as above with various expression plasmids and reporter genes as indicated in the figure legends and selected as appropriate. Transformants were grown overnight to saturation in 5 ml of medium (0.67% yeast nitrogen base without amino acids/2% glucose), pelleted, washed in sterile water, and resuspended in 5 ml of induction medium (0.67% yeast nitrogen base without amino acids/2% galactose). 3.5 ml of the resuspended yeast was then added to 6.5 ml of induction medium and grown for an

additional 12 to 16 h to an OD_{600} of 0.5 to 1. Cells were harvested, lysed by three freeze/thaw cycles and β-galactosidase activity was measured (Ausubel *et al.*, 1989).

4.2.4 Electrophoretic Mobility Shift Analysis

In vitro transcription/translation vectors for rat PPARα and human RXRα have been described (Marcus *et al.*, 1993; Miyata *et al.*, 1993). The *in vitro* expression vector for COUP-TFII was constructed by cloning the 1.4-kilobase pair cDNA into the plasmid pSG5 (Green *et al.*, 1988). Electrophoretic mobility shift analysis using *in vitro* translated COUP-TFII, PPARα and RXRα proteins with radiolabeled wild type or mutant PPRE oligonucleotide probes was performed as described (Marcus *et al.*, 1993; Miyata *et al.*, 1993). Yeast extracts for binding reactions were prepared from transformants harboring a high-copy expression plasmid for COUP-TFII, constructed by cloning the 1.4 kilobase pair COUP-TFII cDNA downstream of the glycerol phosphate dehydrogenase promoter in the high-copy vector p2UGPD (ura^-) (Bitter and Egan, 1984) (kindly provided by S. Lindquist, University of Chicago). Preparation of yeast extracts and binding reactions were performed as described (Marcus *et al.*, 1995).

4.2.5 Transfections and Measurement of Luciferase Activity

A COUP-TFII mammalian expression vector was constructed by first removing the 5'-untranslated region of the rat COUP-TFII cDNA by site-directed deletion mutagenesis (using the mutagenic oligonucleotide 5'-CTCACTATAGGGCGAATTCGATATGGCAATGGTAGTCAG) and cloning the modified cDNA into the *Eco*RI site of pSG5 (Green *et al.*, 1988). Expression vectors for PPARα and RXRα, luciferase reporter constructs containing one or three copies of the

HD-PPRE (pHD(×1)*luc* and pHD(×3)*luc*, respectively), and the control parental reporter plasmid pCPS*luc* have been described (Marcus *et al.*, 1993; Miyata *et al.*, 1993). Transfections of BSC40 cells were carried out by the calcium phosphate method as described (Zhang *et al.*, 1992; Marcus *et al.*, 1993). Briefly, cells were incubated during transfection in medium without phenol red and containing 10% charcoal-stripped fetal bovine serum. Transfections typically contained 5 μg of the HD-PPRE luciferase reporter gene construct and, where indicated, 2 μg of PPARα, 2 μg of RXRα, and 0-2 μg of COUP-TFII expression plasmids. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the corresponding empty vector, and total DNA was kept at 20 µg with sonicated salmon sperm DNA. Wy-14,643 (in dimethylsulfoxide) was added to fresh medium to a final concentration of 0.1 mM. Extracts were prepared 48 h post-transfection, and luciferase activity was measured as before (Zhang *et al.*, 1992).

4.3 Results

4.3.1 Isolation of Rat COUP-TFII by Genetic Selection in Yeast

We have recently demonstrated that expression of PPARα and RXRα synergistically stimulates the expression of PPRE-linked reporter genes in *S. cerevisiae* (Marcus *et al.*, 1995). Efficient transactivation required the presence of both receptors but was independent of added ligands. In order to develop a genetic strategy for the direct identification of other PPRE-binding transcription factors that might exist, we engineered a yeast strain, W18-2, that contained a single integrated copy of the HIS3 coding region linked to the *CYC1* TATA-box and two tandem copies of the HD-PPRE. W18-2 also contained an expression cassette for PPARα in the eventuality that novel

factors require cooperativity with PPARα for function. W18-2 was unable to grow in the absence of histidine as expected (data not shown). Transformation of an expression vector for RXRα (Marcus *et al.*, 1995) into W18-2 allowed for growth in the absence of histidine, confirming the functional integrity of both the resident *HIS* reporter gene and the PPARα expression cassette.

W18-2 was transformed with a rat liver cDNA library constructed in YEUra3 (ura^-) and his^-/ura^- transformants were selected. Plasmids were rescued in *E. coli* and characterized by partial 5' and 3' sequence analysis. One plasmid (hereafter called COUP-TFII/YEUra3), which contained a 1.4-kilobase pair insert encoding the complete rat homologue of mouse and human COUP-TFII, was selected and used for further analysis.

To determine if COUP-TFII transactivation required the presence of PPARα, COUP-TFII/YEUra3 was cotransformed into *S. cerevisiae* YPH500 with a *lacZ* reporter gene containing two copies of the HD-PPRE (p2HD.314) in the presence or absence of a PPARα expression plasmid, and β-galactosidase activity was monitored. As shown in Table 4-1, COUP-TFII on its own induced activation of this reporter gene 75-fold over basal levels. PPARα on its own had no effect on the basal level of β-galactosidase activity, and did not significantly affect COUP-TFII-mediated activation. These results indicate that COUP-TFII-mediated activation does not require PPARα.

COUP-TFII activates transcription through the PPRE, since a reporter gene that lacked a PPRE (ΔL1) was not activated by cotransformed COUP-TFII expression vector, whereas β-galactosidase activity of reporter constructs that contained either one or two

Table 4-1. Transactivation by COUP-TFII in yeast.

cmPPAR (his+)	COUP-TFII/YEUra3 (ura+)	β-galactosidase acitvity (units)*
–	–	0.2 ± 0.01
+	–	0.3 ± 0.1
–	+	14 ± 2.5
+	+	18 ± 2.0

*Expression vectors for mouse PPARα (cmPPAR(his$^-$)) and rat COUP-TFII (COUP-TFII/YEUra3 (ura$^-$)) were cotransformed with the reporter plasmid 2HD.314 (trp$^-$) into *S. cerevisiae* strain YPH500 as indicated, and β-galactosidase activity was measured. Plasmid dosage was kept constant in controls by the addition of the corresponding "empty" vector. Units are given as the $A_{420} \times 10^3$/min/ml culture, normalized to the A_{600} of the culture at the start of the assay. Values reported are the averages of at least 3 independent transformants (± standard deviation).

Table 4-2. Transactivation by COUP-TFII requires a PPRE.

Reporter Construct	COUP-TFII	β-galactosidase activity (units)*
ΔL1	–	0.3 ± 0.03
	+	0.7 ± 0.06
1HDΔL1	–	0.7 ± 0.1
	+	67 ± 9.5
1HDM3ΔL1	–	0.6 ± 0.2
	+	10 ± 0.4
1HDM5ΔL1	–	0.8 ± 0.2
	+	57 ± 8.7
2HDΔL1	–	0.2 ± 0.01
	+	75 ± 4.3

*Reporter constructs (ura$^-$) that contained one or two copies of the HD-PPRE or the parental plasmid ΔL1 were transformed in *S. cerevisiae* strain YPH500 in the absence or presence of a plasmid expressing COUP-TFII (COUP-TFII.314 (trp$^-$)), as indicated, and β-galactosidase activity was measured as in Table 4-1. Plasmid dosage was kept constant by the addition of "empty" vector in controls. Values reported are the averages of at least 3 independent transformants (± standard deviation).

copies of the HD-PPRE (1HDΔL1 and 2HDΔL1, respectively) was induced 100- to 150-fold over basal levels of the respective reporter genes (Table 4-2). The higher level of induction under these conditions as compared to the results presented in Table 4-1 was due to the fact that the reporter gene was expressed from a high copy vector. The relative

level of induction is similar to that which was observed with cotransfected PPARα and RXRα expression plasmids (Marcus *et al.*, 1995). These results indicate that COUP-TFII activates transcription autonomously via the PPRE and does so with a potency comparable to that of PPARα/RXRα heterodimers.

The HD-PPRE contains four TGACCT-like direct repeats consisting of two DR1 elements overlapping a DR2 element (Chu *et al.*, 1995a). PPAR/RXR heterodimers have been shown to bind independently to the DR1 motifs, as well as to the DR2 element (Chu *et al.*, 1995a). To determine whether the structure of the PPRE is important for COUP-TFII-mediated activation, we carried out experiments with derivatives of 1HDΔL1 in which the third or fourth repeat was altered. As shown in Table 4-2, disruption of the third repeat (1HDM3ΔL1) but not the fourth repeat (1HDM5ΔL1) abolished responsiveness to COUP-TFII. Therefore, transactivation by COUP-TFII in yeast requires the integrity of the PPRE.

4.3.2 COUP-TFII Binds to the HD-PPRE In Vitro

The above results suggest that COUP-TFII stimulates transcription in yeast by binding to the HD-PPRE. To examine this directly, yeast extracts were prepared from COUP-TFII-expressing cells (using a high copy vector) and used for mobility shift analysis with labeled wild-type and mutant HD-PPRE probes. As shown in Fig. 4-2, extracts from yeast transformed with a high copy COUP-TFII expression plasmid formed a specific protein/DNA complex with the wild-type HD-PPRE probe (lane d), whereas extracts from yeast transformed with the corresponding empty vector did not generate

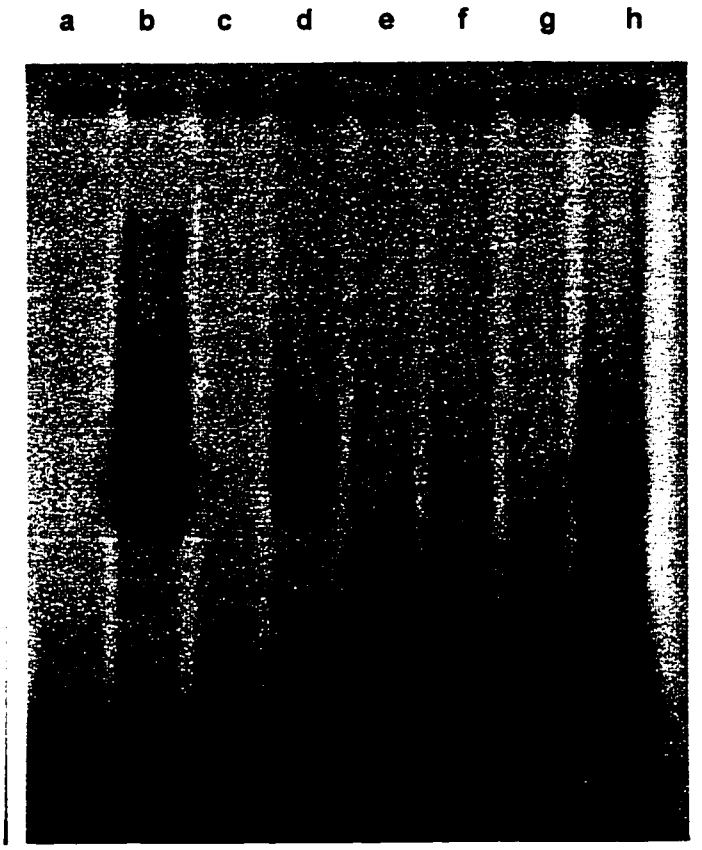

Figure 4-2. Rat COUP-TFII synthesized in yeast or *in vitro* binds to the HD-PPRE. Extracts prepared from *S. cerevisiae* synthesizing rat COUP-TFII were used in mobility shift assays with a labelled oligonucleotide probe corresponding to the wild-type HD-PPRE (lane d), or with mutant oligonucleotides in which the second (M3; lane f) or third (M5; lane h) TGACCT repeats were individually mutated, as indicated. Lanes c, e, and g are yeast extracts prepared from a transformant harboring the corresponding 'empty' vector and incubated with the above probes, respectively. In lane b, the wild-type HD-PPRE probe was incubated with rat COUP-TFII synthesized *in vitro* in rabbit reticulocyte lysate, while lane a is the probe incubated with unprogrammed lysate.

any complex (lane c). Similar experiments were carried out with the HD-PPRE probes containing mutations in the third and fourth repeats (M3 and M5, respectively). COUP-TFII expressed in yeast interacted strongly with the M5 probe (lane h) but weakly with the M3 probe (lane f), whereas control yeast extracts did not generate any complexes on these probes (lanes e and g). These findings are in agreement with the *in vivo* transactivation results presented in Table 4-2. To confirm that COUP-TFII isolated from the rat cDNA library binds directly to the HD-PPRE, COUP-TFII was synthesized *in vitro* by transcription/translation and incubated with the HD-PPRE. As shown in lane b. COUP-TFII binds avidly to the probe, generating several complexes. The most abundant complex comigrated with the complex generated with COUP-TFII synthesized in yeast. COUP-TFII did not generate DNA-binding heterodimers with PPARα or with RXRα (data not shown).

4.3.3 COUP-TFII Interferes with PPARα/RXRα-Mediated Transactivation in Mammalian Cells

To examine the properties of COUP-TFII in mammalian cells, we carried out cotransfections with HD-PPRE-linked luciferase reporter genes. In contrast to what was observed in yeast, expression of COUP-TFII had no specific effect on expression of the HD-PPRE-linked reporter genes compared to the parental reporter plasmid pCPS*luc* (data not shown). However, COUP-TFII was able to inhibit transactivation mediated by PPARα/RXRα. Cotransfection of PPARα and RXRα expression plasmids led to an 8- to 10-fold increase in the activity of pHD(×3)*luc*, a luciferase reporter gene that contains

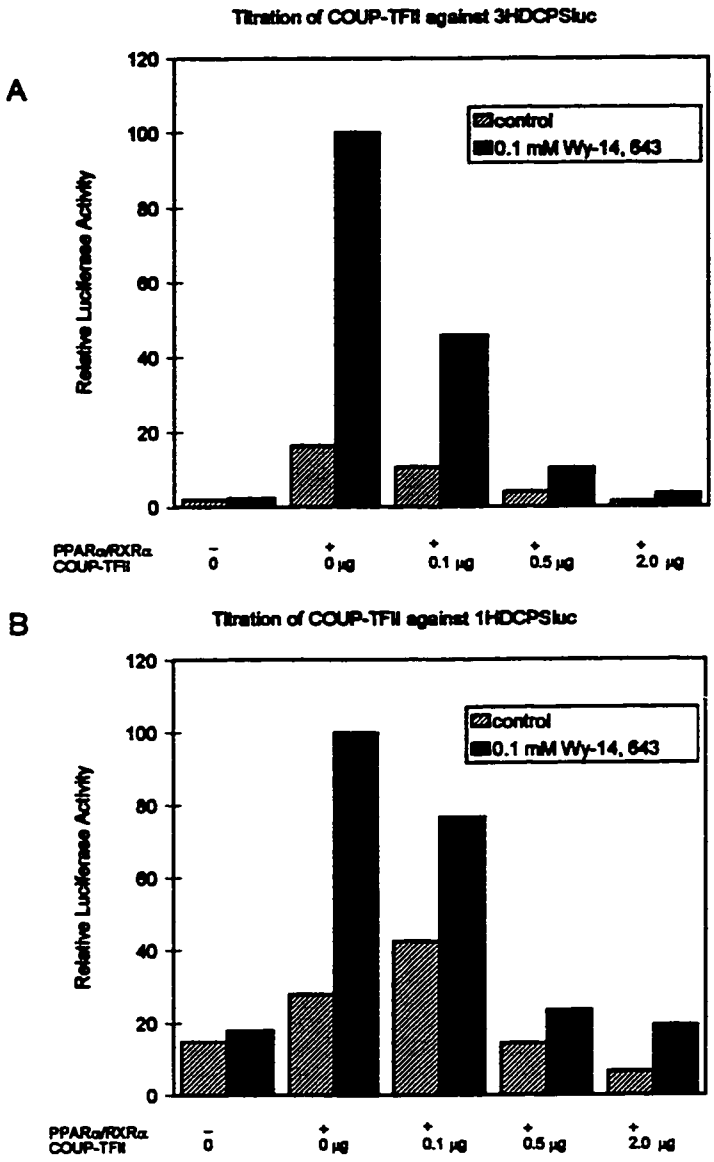

Figure 4-3. Rat COUP-TFII antagonizes peroxisome proliferator-mediated signaling. pHD(×3)*luc* (A) and pHD(×1)*luc* (B) were were transfected into BSC40 cells along with effector plasmids expressing PPARα, RXRα, and various amounts (in μg) of the rat COUP-TFII expression plasmid, as indicated. Cells were incubated in the presence of the peroxisome proliferator Wy-14,643 (0.1 mM) or with the equivalent amount of vehicle (0.05% dimethylsulfoxide). The results represent the average of three separate transfections carried out in duplicate. Values were normalized to parallel transfections carried out with the parental pCPS*luc* reporter gene and equivalent amounts of the COUP-TFII expression plasmid, and to the value obtained for Wy-14,643-treated cells cotransfected with PPARα and RXRα expression plasmids, which was taken as 100%. Values of individual measurements did not vary by more than 15%.

three tandem copies of the HD-PPRE (Fig. 4-3A). The presence of the strong peroxisome proliferator Wy-14,643 resulted in a 50-fold stimulation of activity. Addition of increasing amounts of the COUP-TFII expression vector led to nearly complete inhibition of both the proliferator-dependent and proliferator-independent transactivtion mediated by PPARα/RXRα. Similar results were obtained using a reporter gene that contained a single copy of the HD-PPRE (pHD(×1)*luc*, Fig. 4-3B). These findings demonstrate that COUP-TFII does not activate transcription in mammalian cells via a PPRE, but can potently antagonize activation mediated by PPARα/RXRα.

4.4 Discussion

We report the development and exploitation of a genetic selection strategy in yeast to identify a HD-PPRE-binding factor, COUP-TFII. The genetic screen was designed to identify factors that might cooperate with PPAR to activate transcription, since the yeast strain used in these studies was engineered to also express the gene encoding mouse PPARα . The demonstration that COUP-TFII acts as a positive activator via the HD-PPRE in yeast was unexpected, as we had previously shown that the related nuclear hormone receptor COUP-TFI, which also binds to the AOx- and HD-PPREs *in vitro*, had no activation potential via the HD-PPRE in mammalian cells (Miyata *et al.*, 1993). Similarly, we show here that COUP-TFII does not activate transcription via the HD-PPRE in mammalian cells but does intefere with activation mediated by PPAR/RXR heterodimers, as we have reported with COUP-TFI. Therefore, our results demonstrate that at least two known members of the COUP subfamily of nuclear receptors bind to PPREs and have analogous effects on PPAR-mediated transactivation *in*

vivo. Our findings are consistent with and extend a recent report by Palmer *et al.* (1995) showing that ARP-1 (human COUP-TFII) binds to both the AOx-PPRE and the HD-PPRE. However, in their report only a very weak binding of ARP-1 to the HD-PPRE was observed, and no functional assays in mammalian cells on the effects of COUP-TFII expression on transactivation from the HD-PPRE were performed. The weak binding of ARP-1 to the HD-PPRE observed by Palmer *et al.* (1995) is likely due to the fact that they used a truncated version of the HD-PPRE containing only the downstream DR1 repeat element in their binding assays. As we demonstrate here, when the complete HD-PPRE is used, efficient binding of COUP-TFII is observed.

COUP-TFs are ubiquitously expressed and seem to play complex and multilevel roles in the regulation of genes important for cell differentiation, embryonic development, and metabolic homeostasis (Tsai and O'Malley, 1994). COUP-TFI and COUP-TFII are highly homologous, but their amino-terminal regions are not conserved and they likely play distinct roles in the cell (Wang *et al.*, 1991). COUP-TFs generally seem to function as transcriptional repressors but have also been shown to positively regulate gene expression in some cases (Hall *et al.*, 1995). Therefore, COUP-TFs can have diverse and complex effects on gene regulatory networks. COUP-TFs have been shown to be antagonistic to several nuclear hormone response pathways, including the vitamin D, thyroid hormone, and retinoic acid pathways (Cooney *et al.*, 1993). Repression is mediated principally by competition for cognate binding sites by COUP-TF homodimers, but COUP-TFs may also act by sequestering common heterodimerization partners such as RXR or by forming non-DNA binding heterodimers with other receptors. Competition for PPRE binding sites by COUP-TFII homodimers likely

explains the inhibition of PPAR signaling by COUP-TFII reported here, since COUP-TFII binds strongly to the HD-PPRE and recognizes overlapping determinants in this element. The physiological relevance of COUP-TFs in PPAR-mediated signaling remains to be determined, but it is interesting to note that a growing number of genes involved in lipid and metabolic homeostasis that are targets for COUP-TF-mediated modulation also seem to be responsive to peroxisome proliferators. Indeed, ARP-I was originally described as a factor that bound to the apolipoprotein AI enhancer (Ladias and Karanthanasis, 1991), a gene which is also subject to regulation by PPARs (Vu-Dac *et al.*, 1994). The potential relevance of crosstalk between PPARs and COUP-TFs is further underscored by the recent finding that a cryptic PPRE close to the transcription start site of the CYP4A6 gene overlaps a COUP-TFII binding site (Palmer *et al.*, 1994). Thus, distinct PPREs from at least three separate genes (AOx, HD, and CYP4A6) are recognized and subject to negative regulation by COUP-TFs.

Interestingly, while COUP-TFII did not activate transcription via the HD-PPRE in mammalian cells, it functioned as a potent transcriptional activator from the HD-PPRE in yeast. ARP-I (human COUP-TFII) does possess intrinsic activation potential *in vitro*, and has been shown to interact with the basal transcription factor TFIIB (Malik and Karathanasis, 1995). However, whether these properties of COUP-TFII are responsible for the activation seen in yeast is not known. Possible explanations for the differences in the results in yeast *vis-à-vis* mammalian cells is that transcriptional activation pathways for COUP-TFII differ between these cells or that there is a more relaxed specificity in the recognition of downstream effector targets in yeast. Several nuclear hormone receptors have been shown to function in yeast in the absence of exogenously added cognate

ligands (Hall *et al.*, 1993; Heery *et al.*, 1993). Moreover, activation by COUP-TFII in mammalian cells may be attenuated by endogenous ligands and/or auxiliary cofactors that do not exist in yeast. This may be similar to what is observed with TR, which acts as a constitutive silencer in the absence of ligand but activates transcription in its presence (Baniahmad *et al.*, 1992). Corepressors that bind to both TR and retinoic acid receptor in a ligand- and response element-dependent manner have been identified and shown to mediate repression by these receptors (Chen and Evans, 1995; Hörlein *et al.*, 1995). Recently, Power and Cereghini (1996) have shown that COUP-TFII can positively regulate the vHNF1 promoter and that the activity of COUP-TFII can be modulated by direct interaction with the Oct family of DNA-binding proteins. It is therefore possible that, in a particular context, COUP-TFII can act as both a positive and negative regulator of peroxisome proliferator-responsive genes. The genetic system we describe affords a facile strategy to determine whether COUP-TFII interacts with identical or similar mammalian factors that interact with COUP-TFII to modulate its activity.

In summary, we have used a yeast-based functional assay to identify COUP-TFII as a PPRE-binding protein that functions positively in yeast but antagonizes PPAR signaling in mammalian cells. The approach described here should be generally applicable for the isolation of any sequence-specific DNA-binding transcription factor by incorporating a suitable target site upstream of the *HIS3* gene. Moreover, as discussed above, it should also be possible to isolate potentially novel heterodimerization partners for PPAR, since the engineered yeast constitutively express PPARα which is able to cooperate with ectopically expressed RXRα to activate transcription.

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CHAPTER 5

A P56^{LCK} LIGAND SERVES AS A COACTIVATOR OF AN ORPHAN NUCLEAR HORMONE RECEPTOR[5]

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5.1 Introduction

The nuclear hormone receptor superfamily comprises a large group of ligand-activated transcription factors important for the normal development and functioning of an organism. These receptors mediate transcriptional responses to steroids, retinoids, vitamin D, thyroid hormone, and fatty acids/peroxisome proliferators by binding as homodimers or heterodimers to characteristic DNA hormone response elements in target genes (1, 2). The largest subgroup within this superfamily are the orphan receptors, so called because their cognate ligands have not been identified or may not exist (3). Accordingly, the mechanisms of action and physiological roles of orphan receptors remain poorly characterized.

Among the most studied of the orphan receptors is the chicken ovalbumin upstream promoter transcription factor (COUP-TF) subfamily, which includes COUP-TFI (Ear3) and COUP-TFII (Arp1) (4). COUP-TFs are spatially and temporally regulated transcription factors implicated in many fundamental biological processes, including myogenesis, neurogenesis, organogenesis, determination of cell fate and metabolic homeostasis. The importance of the COUP-TFs is underscored by their evolutionary conservation and by the observation that ablation of either COUP-TFI or COUP-TFII in the mouse is lethal (5).

COUP-TFs act principally as repressors of ligand-mediated hormone receptor signaling pathways via both protein-protein and protein-DNA interactions (5-8). For example, COUP-TFs bind promiscuously to hormone response elements recognized by other nuclear receptors, thereby competing with them for their target sites. COUP-TFs can also titrate the common heterodimerization partner, retinoid X receptor (RXR), that is

required for high-affinity DNA binding of most members of the thyroid hormone/retinoic acid receptor subfamily (9-11). In addition to these indirect mechanisms of repression, COUP-TFs can also actively silence basal and activated transcription (5), likely through direct interactions with TFIIB or other general transcription factors (12). COUP-TFs therefore antagonize cellular responses to multiple hormone signaling pathways and can have profound effects on numerous biological processes.

Paradoxically, both COUP-TFI and COUP-TFII can activate transcription in certain cell types and promoter contexts (13-18). Moreover, COUP-TFII has been shown to function as a constitutive transactivator *in vitro* (19) and in yeast (20), suggesting that it possesses intrinsic activation potential. The mechanisms underlying this functional duality is unknown. Evidence suggests that the function of many nuclear hormone receptors is dependent upon, or modulated by, the actions of an increasing number of both common and distinct receptor-binding cofactors that differentially recognize liganded and unliganded receptors (21-28). Most of the auxiliary factors so far identified act as corepressors or negative modulators of receptor function (*e.g.* NCoR, SMRT, TRUP, and TRIP1). However, in a few cases, receptor-selective, positively acting coactivators (*e.g.* RIP140, SRC-1 and CBP/p300) have been identified. To determine if differential COUP-TF activity is mediated through the actions of auxiliary proteins, we used the yeast two-hybrid interaction cloning system to identify novel COUP-TFII interacting proteins. We identified a factor that bound COUP-TFII *in vitro* and allowed COUP-TFII to act as a transcriptional activator in mammalian cells. This factor is a recently reported ligand of the tyrosine kinase signaling molecule $p56^{lck}$. Our results

suggest that this factor mediates cross-talk between mitogenic and nuclear hormone receptor signal transduction pathways.

5.2 Materials and Methods

5.2.1 Two-hybrid Library Screening

Two-hybrid analysis was carried out using the Matchmaker System (Clontech) as described (29). pGBD-COUP-TFII contains the full-length cDNA for rat COUP-TFII (20) in the Gal4 DNA binding domain expression plasmid pGBT9 (Clontech). This bait plasmid was transformed into yeast HF7c cells along with a human liver cDNA library constructed in the Gal4 activation domain vector pGAD10 (Clontech). Transformants (1×10^6) were plated onto synthetic complete plates lacking histidine, leucine, and tryptophan, and $His^-/Leu^-/Trp^-$ colonies were recovered and assayed for β-galactosidase activity using filter assays. Library plasmids were rescued by electroporation into *Escherichia coli*, retransformed into yeast strain SFY526 and tested for specificity against pGBD-COUP-TFII, pGBT9, and several irrelevant Gal4 DNA binding domain fusion expression plasmids. Of the positive clones recovered, six independent isolates contained the same 2.1-kilobase pair insert based on restriction enzyme analysis. Two of these clones were sequenced and shown to encode a 440 amino acid long protein, which we refer to as ORCA (orphan receptor coactivator). The predicted amino acid sequence of ORCA is identical to the published sequence of the $p56^{lck}$-interacting protein p62 (30)(GenBank Accession No. U46751).

5.2.2 Protein Binding Assays

The COUP-TFII cDNA was cloned as an *Eco*RI fragment into the *Eco*RI site of pMal-c2 (New England Biolabs), and the maltose binding protein (MBP) chimera was purified from induced cultures of *E. coli* according to the manufacturer's instructions. Control MBP was purified under identical conditions. Protein binding assays were carried out as previously described (29) using proteins synthesized *in vitro* with a coupled transcription/translation system (InVitrogen). Full-length ORCA cDNA was cloned into the *Eco*RI site of pSG5 (Stratagene) to generate pORCA/SG5, which is suitable for *in vitro* and *in vivo* expression. pORCA Δ258-440 (numbers refer to amino acid residues) was constructed by inserting a double-stranded oligonucleotide (5'- GCGTAATTAATTAATTACGC) containing termination codons in all three reading frames into the blunt-ended *Cla*I site of pORCA/SG5. pORCA Δ128-163 was constructed by site-directed deletion mutagenesis using 5'-GTGCACCCCAATGTGATCACCAAGCTCGCATTCCCC and single-stranded DNA prepared from pORCA/SG5. Mutagenesis was carried out following standard procedures (31), and accuracy was confirmed by DNA sequencing in each case.

5.2.3 Transient Transfections and Measurement of Luciferase Activity

The luciferase reporter plasmid pHD(×3)*luc*, containing the rat hydratase-dehydrogenase PPRE, and effector plasmids expressing full-length cDNAs for rat PPARα, human RXRα, human COUP-TFI and rat COUP-TFII nuclear receptors have been described (20, 32-34). pORCA/SG5 is described above. BSC40 cells (10-cm subconfluent dishes) were transfected, and luciferase activity was measured as described

previously (32) using conditions described in the figure legends. Plasmid and promoter dosage was kept constant by addition of the appropriate amount of corresponding empty vector. Where indicated, the peroxisome proliferator Wy-14,643 was added to a final concentration of 0.1 mM from a 100× concentrated stock solution in dimethylsulfoxide.

5.3 Results and Discussion

5.3.1 ORCA Interacts with COUP-TFII in Yeast and in Vitro

Using rat COUP-TFII fused to the Gal4 DNA binding domain (Gal4 DBD) as bait to screen a human liver cDNA library fused to the Gal4-acidic activation domain, we isolated several clones that specifically interacted with COUP-TFII but not with the Gal4 DBD itself or with various control Gal4 DBD fusion proteins. Sequence analysis of the 2.1-kilobase pair insert of two of these clones showed that they encoded a predicted 440-amino acid protein unrelated to other known nuclear receptor-interacting factors. However, the deduced protein was identical to a recently described human phosphoprotein (p62) originally identified by its ability to interact with the SH2 domain of the tyrosine kinase signaling protooncogene molecule $p56^{lck}$ (30). We refer to this protein as ORCA.

To determine if ORCA bound directly to COUP-TFII, we made use of pull-down assays with *in vitro* synthesized ORCA (Fig. 5-1B, *top panel*) and a MBP-COUP-TFII fusion protein. ORCA showed binding to MBP-COUP-TFII (Fig. 5-1B, *middle panel*), with little or no binding to MBP itself (Fig. 5-1B, *bottom panel*). A luciferase control did not bind to MBP-COUP-TFII. ORCA contains a cysteine-rich, zinc finger-like motif (residues 128-163), which could serve as a protein interaction motif, and a Ser-rich

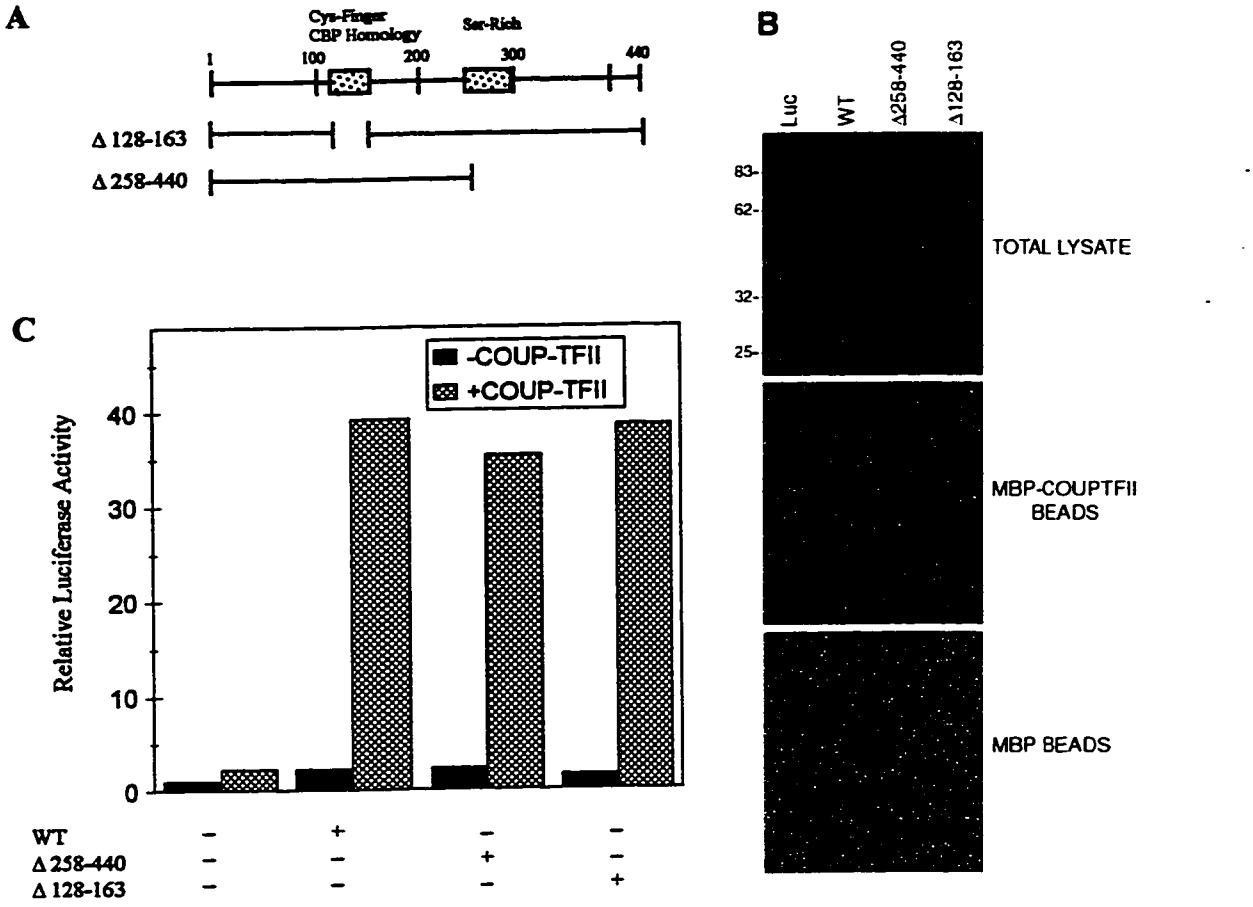

Figure 5-1. ORCA binds to COUP-TFII *in vitro* and functions as a coactivator *in vitro*. *Panel A*, the full-length cDNA encoding ORCA was obtained from the yeast two-hybrid screen and encodes a 440 amino acid long protein identical to the $p56^{lck}$ interacting protein, p62 (30). The positions of a putative Cys-finger that shares homology with the coactivators CBP and p300, and a Ser-rich domain are indicated. Derivatives lacking amino acid residues 128-163 or lacking amino acid residues carboxyl to position 258 were constructed as described under "Materials and Methods". *Panel B*, [^{35}S]-methionine labeled wild-type ORCA (*WT*) and deletion derivatives, as well as a luciferase (*Luc*) control, were synthesized *in vitro* (*top panel*) and incubated with beads complexed with MBP-COUP-TFII fusion protein (*middle panel*) or MBP alone (*bottom panel*). Beads were washed extensively, and bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. *Panel C*, ORCA converts COUP-TFII into a transcriptional activator in mammalian cells. BSC40 cells were transfected with 5 μg of pHD(x3)*luc* reporter plasmid alone or cotransfected in the presence of 0.5 μg of the plasmid expressing COUP-TFII and 4 μg of the plasmids expressing ORCA or the mutant derivatives, as indicated. Luciferase activity was measured 48 h posttransfection. The values shown are averages from at least two transfections carried out in duplicate and normalized to the value obtained with the reporter plasmid alone (taken as 1). Values from individual transfections did not vary by more than 15%.

domain (downstream of residue 266) that may be a target for protein kinases (Fig. 5-1A) (30). To determine the importance of these regions in mediating interaction with COUP-TFII, the domains were altered by site-directed mutagenesis and tested for activity in protein interaction experiments. Derivatives truncated at amino acid 258 or missing residues 128-163 interacted with COUP-TFII, indicating that these regions are not necessary for binding. Interestingly, the protein truncated at amino acid 258 bound to COUP-TFII much more avidly than wild-type ORCA. Therefore, interaction determinants are contained within the amino terminal 258 amino acids of ORCA, but downstream elements appear to modulate its binding activity.

5.3.2 ORCA is a Selective Coactivator of COUP-TFII in Mammalian Cells

To determine what effects ORCA might have on COUP-TFII activity *in vivo*, we carried out cotransfection experiments in mammalian cells with a luciferase reporter gene that contained the peroxisome proliferator-response element (PPRE) from the rat hydratase-dehydrogenase gene (33). COUP-TFII has been shown to bind avidly to this PPRE as a homodimer, but it has little effect on basal transcriptional activity of a linked reporter gene (Fig. 5-1C) (20). Cotransfection of ORCA on its own had no effect on basal level expression. However, coexpression of ORCA and COUP-TFII resulted in a 30- to 40-fold induction in luciferase expression. The 128-163 deletion and the carboxyl terminal truncation derivatives also stimulated transactivation by COUP-TFII. Stimulation of COUP-TFII-mediated transactivation by ORCA was also observed with a reporter construct that contained a COUP-TFII binding response element from the rat ornithine transcarbamylase gene (35) (data not shown).

To examine the specificity of ORCA, we examined its effects on COUP-TFI, a highly related receptor that also binds strongly to the hydratase-dehydrogenase PPRE (32). COUP-TFI and COUP-TFII are nearly identical in their DNA binding and putative ligand binding domains but diverge in their respective amino termini. Transfections were carried out in parallel with COUP-TFI and COUP-TFII in the presence of various amounts of ORCA expression plasmid (Fig. 5-2A). ORCA had a stimulatory effect on COUP-TFI activity, but the effect was much less pronounced than that observed with COUP-TFII and was seen only with relatively high concentrations of ORCA (Fig. 5-2A).

To further investigate the *in vivo* selectivity of ORCA, we examined its effect on transactivation by the peroxisome proliferator-activated receptor (PPAR)/RXR heterodimer. ORCA had no effect on transcriptional activation mediated by PPAR/RXR heterodimers, either in the absence or presence of the PPAR activator, Wy-14,643 (Fig. 5-2B). As we have previously shown (20), COUP-TFII antagonized transactivation mediated by PPAR/RXR and decreased Wy-14,643-dependent, PPAR/RXR-mediated activation by 50-60% (Fig. 5-2B). However, in the presence of coexpressed ORCA, repression by COUP-TFII was completely relieved. Indeed, transactivation by PPAR/RXR in the presence both COUP-TFII and ORCA was approximately twice that observed with PPAR/RXR alone. This additive effect is expected if the transcriptional response is the combination of PPAR/RXR- and COUP-TFII/ORCA-mediated positive effects. Our findings indicate that ORCA is a selective coactivator of COUP-TFII and allows COUP-TFII to function as a positive transcriptional activator in mammalian cells.

The central role of nuclear hormone receptors in cell proliferation, differentiation, and development implies intuitively that their function must somehow be integrated with

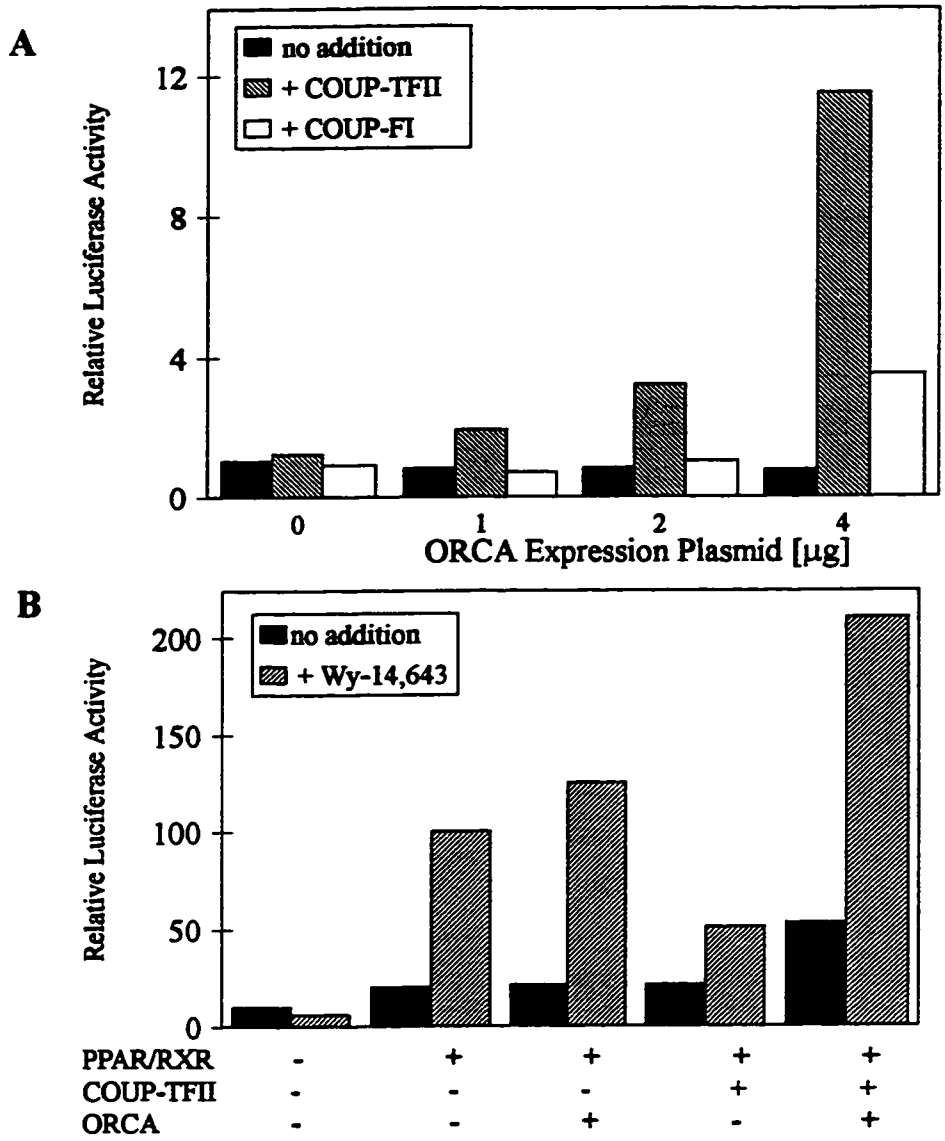

Figure 5-2. ORCA is a selective coactivator. *Panel A*, transfections were carried out as in Fig. 5-1C with either COUP-TFI or COUP-TFII expression plasmids (0.5 μg each) and increasing amounts of ORCA expression plasmid, as indicated. Luciferase activity was normalized to the value obtained with the reporter gene alone, which was taken as 1. The values shown are averages from three transfections carried out in duplicate. Values from individual transfections did not vary by more than 15%. *Panel B*, ORCA relieves COUP-TFII-mediated repression of transactivation by PPAR/RXR. BSC40 cells were transfected with 5 μg of pHD(×3)*luc* reporter plasmid, along with expression plasmids for rat PPAR and human RXRα (2 μg each), COUP-TFII (0.5 μg) and ORCA (4 μg), as indicated. Wy-14,643 was added to a final concentration of 0.1 mM. Luciferase activity was measured as above and normalized to the value obtained with PPAR/RXR in the presence of Wy-14,643, which was taken as 100%. The values shown are averages from three transfections carried out in duplicate. Values from individual transfections did not vary by more than 15%.

other signal transduction pathways important in the multifactorial regulation of these processes. Indeed, several lines of evidence indicate that COUP-TF activity is regulated by, or linked with, cell surface signaling pathways and second messenger activation. For example, the *Drosophila* COUP-TFII homologue *seven-up*, which is necessary for photoreceptor cell determination, requires an active *ras* signal transduction pathway for its activity (36, 37). Moreover, a fusion between the COUP-TFI ligand binding domain and the progesterone receptor DNA binding domain was activated by the catecholamine neurotransmitter dopamine, suggesting that COUP-TFI can be regulated by cAMP-dependent protein kinase A phosphorylation cascades (38). Our finding that a $p56^{lck}$-interacting partner also serves as a COUP-TFII transcriptional coactivator suggests that this factor may link COUP-TFII and cell surface signal transduction pathways. This integrating role may be similar to what is observed with the cAMP response element binding protein CBP and the related protein p300, which functions as a coactivator of AP-1 and cAMP response element binding transcription factor families (39). CBP has recently been shown to be a constituent of a multicomponent coactivator complex that is necessary for activation of several ligand-dependent nuclear hormone receptors, including the retinoic acid and thyroid hormone receptors (40). Therefore, CBP family proteins play a role in integrating cAMP second messenger and nuclear hormone receptor signal transduction pathways. Interestingly, ORCA shares a small region of homology with CBP (residues 136-154 of ORCA and residues 1715-1722 of CBP/p300), suggesting a potential similarity in their mechanism of action.

A putative role for ORCA in linking distinct signaling pathways remains to be established, since its function in cell surface signal transduction is not yet known.

ORCA/p62 was originally isolated based on its interaction with the $p56^{lck}$, a T-cell specific *src* family tyrosine kinase required for T-cell signal transduction. However, it is unlikely that ORCA/p62 function is restricted to $p56^{lck}$-mediated events, since ORCA/p62 is ubiquitously expressed with at least two known isoforms in humans (30). Moreover, a homologue of unknown function has been identified in mouse (GenBank Accession No. U40930), and related proteins exist in *Drosophila* (41). This suggests that ORCA/p62 may be part of a larger family of factors that play a more general role in signal transduction in the cell. Indeed, p62 has been reported to also bind the Ras-GTPase activating protein (42) and a novel cytokine receptor induced in Epstein-Barr virus-infected B lymphocytes (43).

The mechanism by which ORCA enhances COUP-TFII activity is unknown at present, but several possible scenarios, which are not necessarily mutually exclusive, can be proposed. ORCA may function by binding directly to COUP-TFII to generate a DNA-bound multicomponent activating complex, similar to CBP and retinoic acid receptor (40). However, ORCA does not bind directly to COUP-TFII binding sites, and we have been unable to detect a COUP-TFII/ORCA supercomplex in gel retardation experiments, suggesting that if such a ternary complex forms, the COUP-TFII/ORCA/DNA interaction is weak or transient. Alternatively, ORCA may function directly or indirectly by phosphorylating COUP-TFII. This would be consistent with evidence implicating phosphorylation in activation of COUP-TF (38). It is interesting to note in this regard that ORCA/p62 is a phosphoprotein that possesses a tightly associated or intrinsic Ser/Thr protein kinase activity (42) and that both COUP-TFI and COUP-TFII contain a conserved consensus mitogen-activated protein kinase site (P*X*(S/T)P) in their

amino-terminal domains. Finally, ORCA may override the function of a specific COUP-TFII-associated corepressor. This would be consistent with the observation that COUP-TFII constitutively activates transcription in yeast and *in vitro*, where presumably such a corepressor is not present or is limiting, respectively. In agreement with this possibility, expression of ORCA in yeast did not further potentiate transactivation mediated by COUP-TFII (data not shown).

In summary, we have identified a novel cellular factor that is known to interact with components of cell surface signal transduction pathways and which converts COUP-TFII from a transcriptional repressor into a transcriptional activator in mammalian cells. Our findings illustrate a novel mechanism by which an orphan nuclear hormone receptor can differentially regulate gene expression in an apparently ligand-independent manner. Moreover, our findings point to a role for ORCA and related factors in mediating cross-talk among distinct signal transduction pathways important for cellular growth and differentiation.

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CHAPTER 6

A P56^{LCK} LIGAND ACTIVATES TRANSCRIPTION OF THE SV40 EARLY ENHANCER/PROMOTER

6.1 Introduction

Studies of eukaryotic gene expression have revealed that RNA polymerase II promoters and their upstream activators consist of functional modules whose synergistic action regulates gene transcription. Enhancers are promoter elements that can activate transcription over large distances, irrespective of orientation. The SV40 enhancer was the first to be described and has been extensively characterized by mutagenesis, which has revealed sequences required for full activity (reviewed in Atchison, 1988; Jones *et al.*, 1988; McKnight and Tjian; 1986). These sequences contain a modular arrangement of short DNA motifs that have little or no enhancing activity on their own, but which act synergistically to give high levels of activity.

The SV40 early promoter consists of three copies of a 21-base pair repeat followed by a TATA box (Fig. 6-1). Each repeat has two copies of a GC-hexanucleotide motif that has been shown to bind the transcription factor Sp1. The prototype SV40 enhancer is derived from SV40 strain 776 and contains two tandem copies of a 72-base pair repeat. The "minimal" enhancer has been localized to the distal 72-base pair repeat and 5'-flanking region. In one study, viral revertants of mutants harboring mutations within the enhancer sequence were characterized, revealing three separate domains (Herr and Clarke, 1986). The mutation of one domain could be compensated for by the duplication of another. Another study involved transfection assays of a reporter gene in HeLa cells (Zenke *et al.*, 1986). Mutational analysis showed that the SV40 enhancer is composed of at least two domains, A and B. These domains have very little enhancing activity on their own, but their association results in a dramatic increase (about 400-fold) in the transcription of a reporter gene. Domains A and B contain multiple sequence

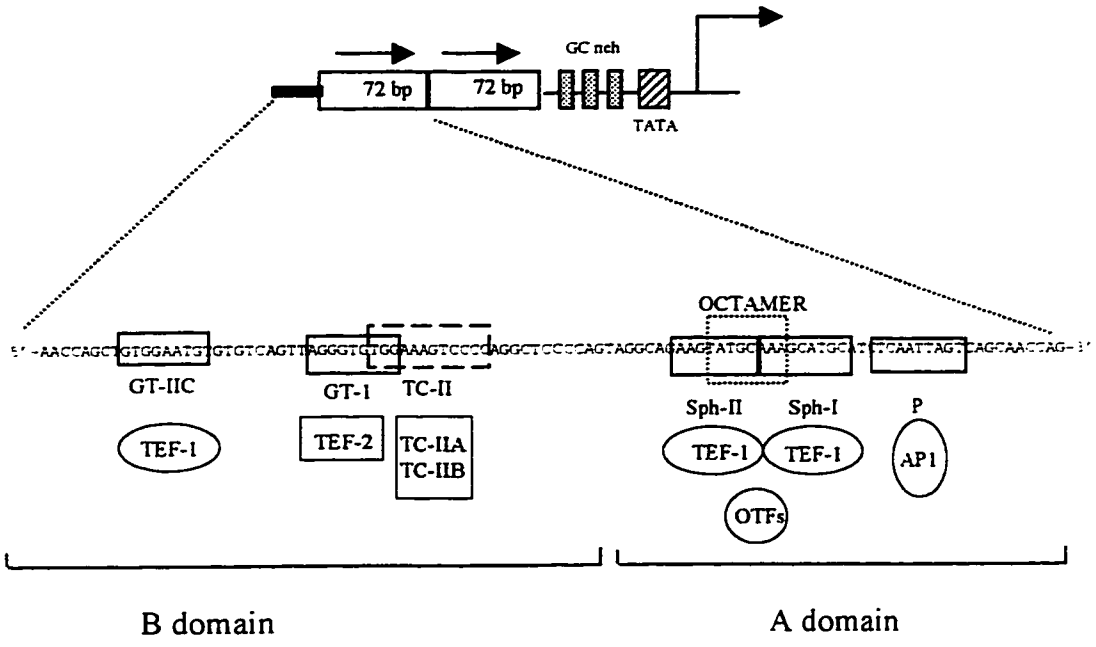

Figure 6-1. Organization of the SV40 early promoter (adapted from Xiao et al., 1991; Zenke et al., 1986; Jones et al., 1988). A schematic diagram of the SV40 early promoter and enhancer is shown. The GC-rich area denotes the 21-base pair repeats that bind Sp1. Also shown is the sequence of the distal 72 base pair repeat and 5' flanking sequence (indicated by the broken lines). The brackets show the limits of the A and B domains. Below the sequence are some of the DNA-binding proteins identified for each enhanson (see text for references). OTFs are octamer binding transcription factors whose action appears to be restricted to lymphoid cells (Rosales *et al.*, 1987).

motifs GT-IIC, GT-I, TC-II, Sph-II, Sph-I, octamer, and P. These so called enhansons function synergistically in a cell-specific manner and have been shown to bind a variety of ubiquitous and cell-specific factors. These findings explain how the SV40 enhancer can be active in such a wide range of cell types (see Fig. 6-1; Jones *et al.*, 1988; Atchison, 1988; Xiao *et al.*, 1991). Thus, enhancer activity depends not only on the assortment of sequence motifs, but also on the presence of *trans*-acting factors. Some of these factors may be present in some tissues in an inactive or masked form that can be converted to an active form by post-translational modification. For example, TC-IIA is a protein found in several cell types. It is similar to NF-κB, a B cell-specific factor that binds to the κB enhanson of the immunoglobulin κ chain enhancer and can be induced by phorbol esters (Sen and Baltimore, 1986a; 1986b; Kanno *et al.*, 1989; Macchi *et al.*, 1989)

Here, we describe a novel protein that activates transcription via the SV40 enhancer. We originally identified this protein based on its ability to interact with the orphan nuclear hormone receptor COUP-TFII (Marcus *et al.*, 1996). This protein, which we called ORCA/p62 (Orphan Receptor CoActivator) is identical to the $p56^{lck}$-interacting protein p62 (Joung *et al.*, 1996). We show that ORCA/p62 activates the transcription of an SV40 enhancer-linked reporter gene. ORCA/p62 contains an SH2-binding domain at its N-terminus that mediates interaction with $p56^{lck}$ (Joung *et al.*, 1996). At least part of this domain is critical for ORCA/p62 transactivating ability. Shortening the enhancer element to a single 72-base pair repeat reduced the activity of ORCA/p62, suggesting that ORCA/p62-mediated transactivation requires the cooperation of multiple *cis*-acting elements of the SV40 enhancer. Removal of the B domain completely abolished the response to ORCA/p62, suggesting that this domain is essential.

6.2 Materials and Methods

6.2.1 Cells

BSC40 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum.

6.2.2 Plasmid Constructions

Expression vectors for PPARα and RXRα (Marcus *et al.*, 1993), and the luciferase reporter plasmid pSV2*luc* (de Wet *et al.*, 1987) have been described elsewhere. pCMVL was a kind gift of Scott Bunnell (University of Alberta).

The SV40 enhancer was amplified by PCR from pSV-SPORT (GIBCO-BRL) using the oligonucleotides 5′-ATTGGATCCGAATGTGTGTCAGTTAGC (forward primer) and 5′-ATTGGATCCTGGTTGCTGACTAATTGAGA (reverse primer). Two products resulted from this amplification, one of 160 base pairs and containing the entire SV40 enhancer, the other of 100 base pairs and containing the distal 72-base pair repeat and 5'-flank. The products were digested with *Bam*HI, gel purified, and inserted into the *Bgl*II site of luciferase reporter vector pGL2-promoter (Promega). The resulting plasmids pENH.FOR/GL2 and pENH.REV/GL2 contain the 160-base pair insert in the forward and reverse orientation, respectively. pENH.MIN/GL2 contains the distal 72-base pair repeat and 5'-flanking region in the forward orientation.

A double-stranded oligonucleotide containing *Xho*I overhangs (TCGAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGAAC) encompassing the A domain of the SV40 enhancer (Zenke *et al.*, 1986) was inserted into

the *Xho*I site of the pGL2-promoter. The resulting plasmids pA(×1)/GL2 and pA(×3)/GL2 contain 1 and 3 copies of the A domain, respectively.

The B domain of the SV40 enhancer (Zenke *et al.*, 1986) was amplified by PCR with the primer pairs 5'-ATTCTCGAGCAGCTGTGGAATGTG (forward) and 5'-ATTCTCGAGCTGGGGAGCCTGG (reverse). The product was digested with *Xho*I and inserted into the *Xho*I site of pGL2-promoter to generate pB(×1)/GL2 and pB(×2)/GL2, containing 1 and 2 copies of the B domain, respectively.

The thymidine kinase (TK) promoter was amplified by PCR using the primer pairs 5'-ATTAGATCTCAAACCCCGCCCAGCG (forward) and 5'-ATTAAGCTTGATCTGCGGCACGCTG (reverse). The resulting product was digested with *Bgl*II and *Hind*III and inserted into the corresponding sites of the promoterless pGL2 luciferase vector.

The 21-base pair repeats were amplified by PCR using 5'-ATTAGATCTGCATCTCAATTAGTCAG (forward primer) and 5'-ATTAGATCTGGGGCGGAGAATGGGC (reverse primer). The product was digested with *Bgl*II and inserted into the *Bgl*II site of TK*luc* to generate pGC/TK*luc*. The entire natural SV40 enhancer was amplified by PCR using the primer pairs 5'-ATTCTCGAGCTGTGGAATGTGTGTCAG (forward) and 5'-ATTCTCGAGTGGTTGCTGACTAATTGAG (reverse). The product was digested with *Xho*I and inserted into the *Xho*I site of TK*luc* to generate pENH/TK*luc*.

Construction of the expression vector pORCA/SG5 and derivatives pORCAΔ128-163 and pORCAΔ258-440 have been described (Marcus *et al.*, 1996). pORCAΔ1-187

was constructed as follows: An *Eco*RI site followed by an initiator ATG codon (lower case letters below) was inserted just upstream of codon 188 by site-directed mutagenesis using the primer 5'-CAGCCGCTGGCTCCGGAAGgaattcatgGTGAAACACGGACACTTC, and single-stranded DNA prepared from pORCA/SG5. The resulting plasmid was digested with *Eco*RI, and the fragment corresponding to amino acid residues 188-440 was inserted into the *Eco*RI site of pSG5.

The first 29 amino acid residues of ORCA/p62 were deleted by site-directed mutagenesis using 5'-CACTATAAGGCGAATTCGCCATGGAGCCTGAGGCGGAAGC, and single-stranded DNA prepared from pORCA/SG5 to generate pORCAΔ1-29. pORCAΔ29-50 was constructed similarly using 5'-CTTCAGCTTCTGCTGCAGCGTGGCCGCCCTGTTCCCC. Correct mutagenesis was confirmed by sequencing in all cases.

6.2.3 Transfections and Measurement of Luciferase Activity

Three days before transfection, BSC40 or COS cells (~380, 000) were seeded onto 10-cm dishes. Transfections were done by the calcium phosphate method, as described (Zhang *et al.* 1992), followed by a glycerol shock after 16 h. Transfections typically contained 5 μg of a luciferase reporter gene construct and, where indicated, 4 μg of ORCA/p62 expression plasmid. Effector plasmid dosage was kept constant by the addition of pSG5. Total DNA was kept at 20 μg with sonicated salmon sperm DNA.

Extracts were prepared 48 h post-transfection, and luciferase activity was measured as described previously (Zhang *et al.*, 1992).

6.2.4 Epitope Tagging of ORCA/p62

The stop codon of ORCA/p62 was changed to a codon for glutamine followed by a *Bgl*II site by site-directed mutagenesis using 5'-GCATCCCCCGCCGTTGcagatctTTTTGCCCACCTCTTCTG and pORCA/SG5 single-stranded DNA. A fragment with *Bgl*II termini, encoding the peptide DEDPLAMYPYDVPDYAAMYPYDVPDYAAMGKGES, which contains two repeats of the 9-amino acid influenza virus hemagglutinin (HA) epitope (underlined residues) (Kolodziej and Young, 1991), was ligated into the *Bgl*II site at the ORCA/p62 stop codon to generate pORCA-HA/SG5.

6.2.5 Northern Blot Analysis

Total RNA was isolated from transfected COS-1 cells by guanidine isothiocyanate extraction using a commercially available kit (Tri-Reagent; Molecular Research Centre Inc.). RNA was quantified by spectrophotometry. Northern blot analysis was carried out according to established methods (Ausubel *et al.*, 1989). 10 μg of RNA was loaded per lane. Specific mRNA levels were quantified by densitometry with an Ultroscan XL laser densitometer (LKB Instruments, Bromma, Sweden).

6.2.6 Antibodies

Antisera to full-length mPPARα and hRXRα were raised in rabbits by injection of affinity-purified maltose binding protein fusions expressed in *Escherichia coli*. The

12CA5 monoclonal antibody, which recognizes the 9-amino acid HA epitope, was purchased from the Berkeley Antibody Company (Richmond, CA). Antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Life Sciences).

6.2.7 Gel Retardation Analysis

Nuclear extracts were prepared from COS-1 cells transfected with pORCA/SG5 or with control vector (Dignam et al, 1983; Andrews and Faller, 1991). Gel retardation analysis was performed as described previously (Zhang *et al.*, 1992). All reactions were normalized for protein content. The entire SV40 enhancer plus 21-base pair repeats was amplified by PCR using 5'-ATTGGATCCGAATGTGTGTCAGTTAGC (forward primer) and 5'-ATTGGATCCGGGGCGGAGAATGGGC (reverse primer). The resulting 200-base pair product was digested with *Bam*HI and end-labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. Binding reactions were analyzed by electrophoresis at 4°C on pre-run 3.5% polyacrylamide gels (30:1 acrylamide/*N,N'* -methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer. Recombinant ORCA/p62 was produced in bacteria as a fusion to glutathione S-transferase (a kind gift from C. Winrow). The fusion protein was affinity purified from bacterial lysates on glutathione Sepharose 4B resin (Pharmacia), and the glutathione S-transferase moiety was cleaved with bovine thrombin according to the manufacturer's instructions.

6.3 Results and Discussion

6.3.1 ORCA/p62 Activates the Transcription of an SV40 Enhancer-Linked Reporter Gene

Luciferase reporter construct pSV2*luc* containing the SV40 early promoter and enhancer was transfected into BSC40 cells. Cotransfection of an expression vector for ORCA/p62 (pORCA/SG5) resulted in a 6-fold stimulation of luciferase activity as compared to the empty expression vector (Fig. 6-2). This increase is specific to the SV40 enhancer, as cotransfection of pORCA/SG5 with a luciferase reporter construct containing the cytomegalovirus enhancer/promoter (pCMVL) or the minimal SV40 promoter (pGL2-promoter) did not result in stimulation of luciferase activity.

6.3.2 Part of the SH2-Binding Domain of ORCA/p62 is Essential for Transactivation of the SV40 Enhancer/Promoter

ORCA/p62 contains a cysteine-rich zinc finger-like motif (residues 128-163) which could serve as a protein interaction motif, and a Ser-rich domain (downstream of residue 266) that may be a target for protein kinases (Fig. 6-3). Furthermore, the first 50 residues of ORCA/p62 were shown to bind the $p56^{lck}$ SH2 domain (Joung *et al.*, 1996). To determine the importance of these regions in mediating transcriptional activation from the SV40 enhancer, domains were altered by site-directed mutagenesis and tested for activity in transfection experiments (Fig. 6-3). Derivatives truncated at amino acid 257 or missing residues 128-163 still activated transcription, indicating that these regions are not essential for activity. However, a derivative missing the first 187 residues failed to function in the transfection assay. To determine if the SH2-binding domain is essential in

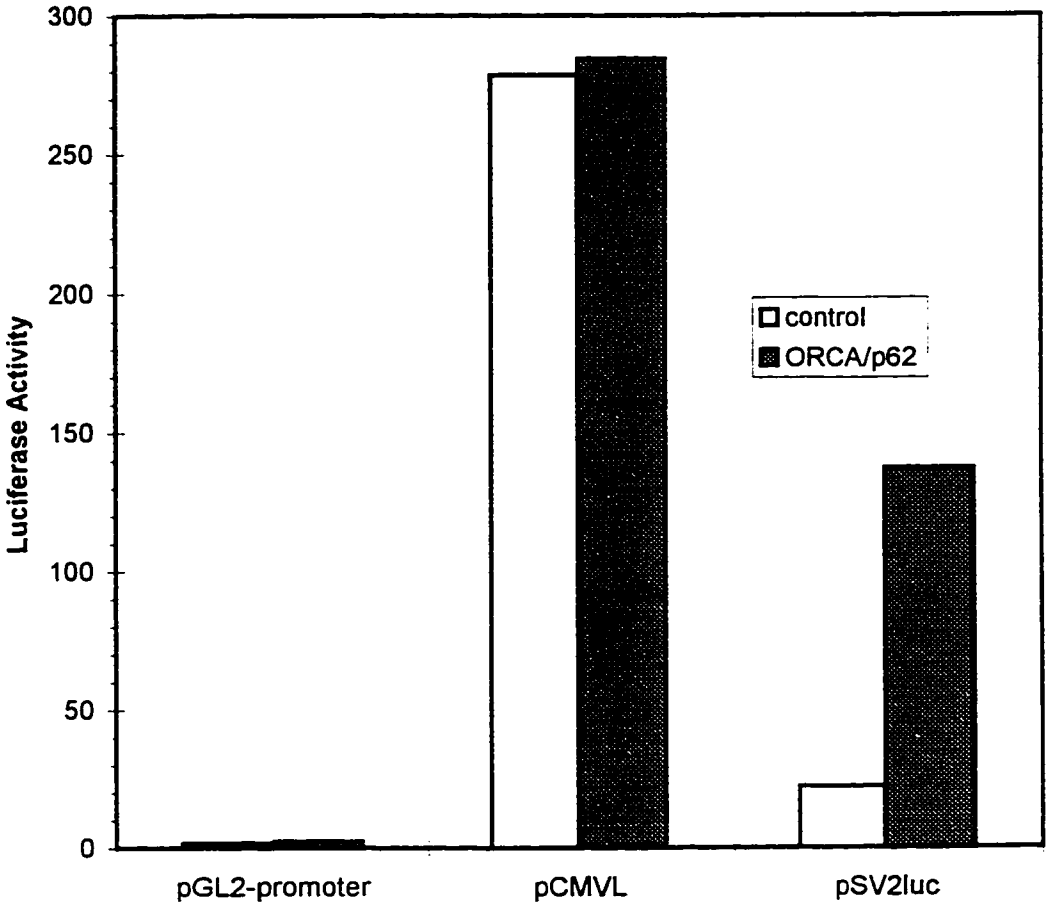

Figure 6-2. ORCA/p62 activates the transcription of an SV40 enhancer-linked reporter gene. The indicated luciferase reporter plasmids were cotransfected into BSC40 cells in the presence or absence of an ORCA/p62 expression vector as described in Materials and Methods. Values shown are luciferase activities in light units, and are the averages of at least two independent transfections done in duplicate.

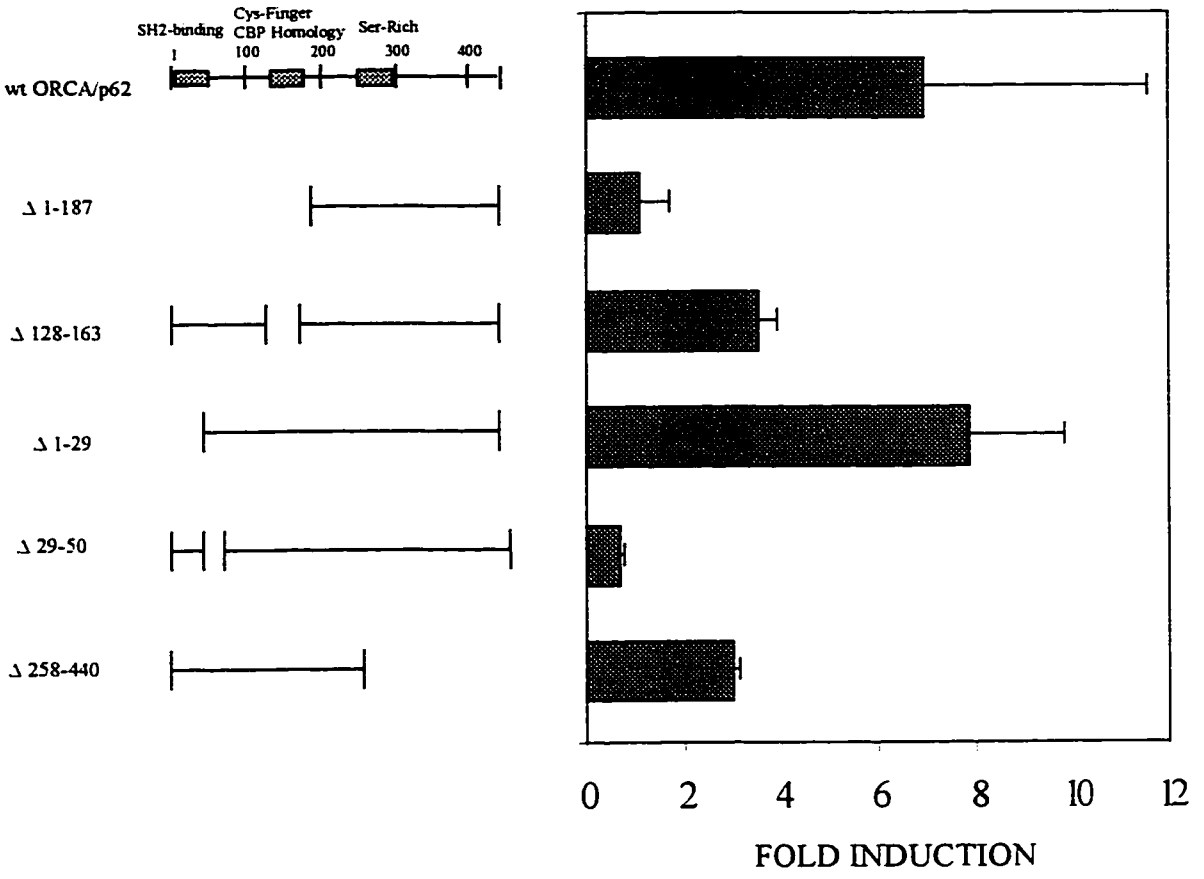

Figure 6-3. The SH2-binding domain of ORCA/p62 is essential for transactivating the SV40 enhancer/promoter. The luciferase reporter plasmid pSV2*luc* was cotransfected into BSC40 cells in the presence or absence of expression vectors for either wild-type (wt) ORCA/p62 or mutant ORCAs lacking the indicated amino acid residues. Values shown represent the fold induction of luciferase activity as compared to the empty vector pSG5, and represent the averages (± SEM) of at least 2 independent transfections done in duplicate.

mediating transactivation, derivatives were made missing residues 1-29 and residues 29-50. As shown in Fig. 6-3, Δ1-29 is active in the transfection assay, while Δ29-50 is not. These results indicate that at least part of the SH2-binding domain of ORCA/p62 is essential for transactivation of the SV40 enhancer.

6.3.3 ORCA/p62 Stimulates Transcription of cDNAs from the SV40 Enhancer/Promoter

We wanted to determine if the ORCA/p62-dependent increase in luciferase activity from the SV40 enhancer/promoter is due to an increase in transcription. Plasmids containing the SV40 promoter/enhancer and cDNAs for several nuclear hormone receptors were cotransfected into COS-1 cells in the presence or absence of pORCA-HA/SG5, which encodes a C-terminal epitope-tagged ORCA/p62. Cells were harvested 48 h post-transfection and divided into two aliquots. Total RNA was isolated from one aliquot and subjected to Northern blot analysis. Protein extracts were prepared from the other aliquot and subjected to immunoblot analysis. mRNA levels for hRXRα and mPPARα were increased in the presence of ORCA-HA (2.9- and 1.8-fold respectively; Fig. 6-4, top panels, compare lanes 2 to lanes 3). There was also an increase in the levels of the corresponding proteins (7.3- and 2.4-fold respectively; Fig. 6-4, bottom panels compare lanes 2 to lanes 3). The presence of full-length ORCA-HA is seen in the bottom panel, lane b.

6.3.4 The B site of the SV40 Enhancer is Required for Responsiveness to ORCA/p62

To characterize the ORCA/p62-responsive *cis*-acting elements of the SV40 enhancer, we made several plasmid constructs based on pGL2-promoter, which contains

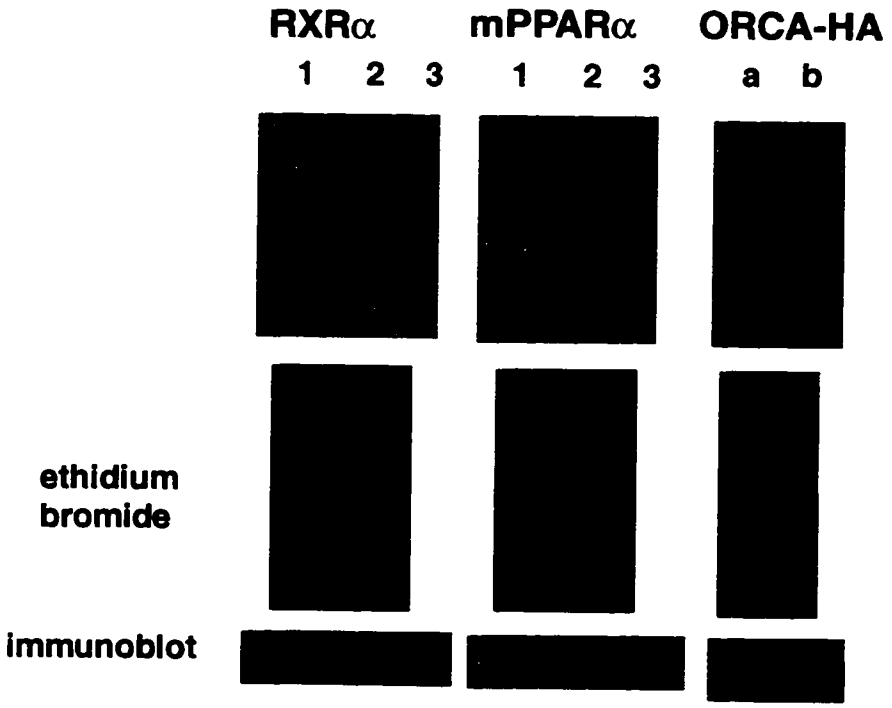

Figure 6-4. ORCA/p62 stimulates transcription of cDNAs under the control of the SV40 promoter/enhancer. Northern blot analysis (top panels) was carried out using total RNA isolated from COS-1 cells transfected with the appropriate empty expression vector (lanes 1), expression vectors for the indicated nuclear hormone receptors (lanes 2 and 3), in the absence (lanes 2) or presence (lanes 3) of pORCA-HA/SG5. Lanes a and b represent RNA isolated from cells transfected in the absence or presence of pORCA-HA/SG5, respectively. Cells were transfected with 2 μg of each plasmid, with the total plasmid dosage kept constant at 4 μg. Northern blot analysis was carried out as described in Materials and Methods. The middle panels show gels stained with ethidium bromide before transfer to nylon membranes. The top panels show nylon membranes containing the transferred RNA probed with full-length cDNAs, as indicated. In each case, a corresponding immunoblot is shown in the bottom panels. After harvesting, a portion of the transfected cells was retained for the preparation of protein extracts. Extracts were prepared in 50 mM Tris-Cl pH 8.0/0.1% Nonidet P-40. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as a standard. 50 μg of each protein extract was subjected to electrophoresis on an SDS-10% polyacrylamide gel, as described (Laemmli, 1970). Proteins were transferred to nitrocellulose for immunoblot analysis.

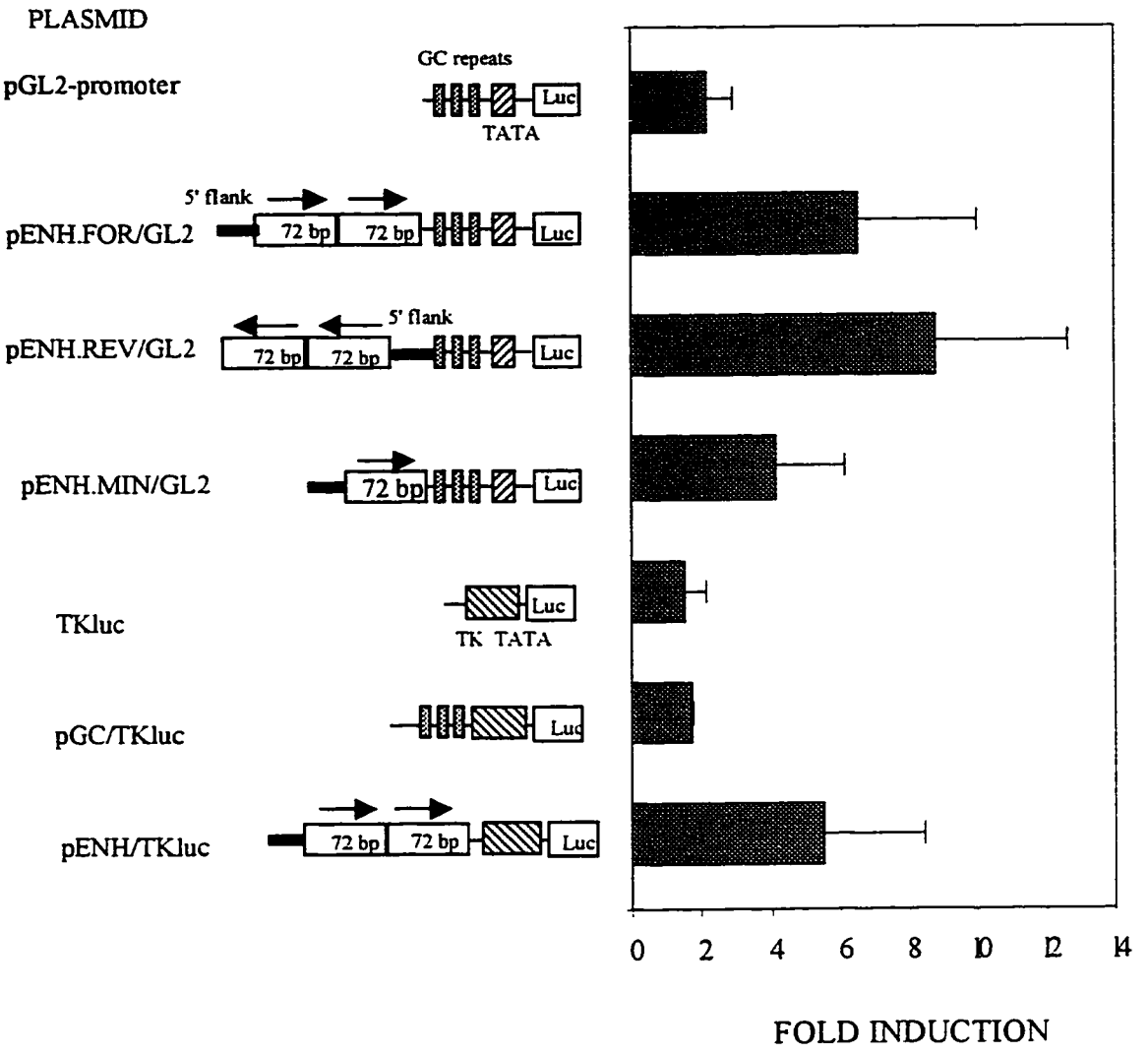

Figure 6-5. Both 72-base pair repeats are required for a full transcriptional response to ORCA/p62. The indicated luciferase reporter plasmids were cotransfected in the presence or absence of an ORCA/p62 expression vector as described in Materials and Methods. Values shown are the fold induction of luciferase activity in the presence of ORCA/p62, representing the averages (± SEM) of at least three independent transfections done in duplicate.

a luciferase reporter gene linked to the SV40 promoter (Fig. 6-5). Cotransfection of this plasmid in the presence of pORCA/SG5 resulted in little change (2-fold) in luciferase activity. Adding the natural SV40 enhancer in the forward (pENH.FOR/GL2) or reverse (pENH.REV/GL2) orientation resulted in an ORCA/p62-dependent 6.5-fold and 8.8-fold increase in luciferase activity, respectively. Reducing the enhancer to only the distal 72-base pair repeat and 5'-flanking region reduced ORCA/p62-dependent transactivation to 4-fold, indicating that the entire SV40 enhancer is required for full activity. To examine whether the GC-rich 21-base pair repeats are required for transactivation by ORCA/p62, we made use of a reporter construct linked to the thymidine kinase promoter (TK*luc*). As expected, cotransfection of either TK*luc* or pGC/TK*luc* with pORCA/SG5 did not result in significant stimulation of luciferase activity. However, linking the natural SV40 enhancer to TK*luc* resulted in a more than 5-fold ORCA/p62-dependent stimulation of luciferase activity. Therefore, the GC-rich 21 base pair repeats are not required for transcriptional activation by ORCA/p62.

The SV40 enhancer is one of the most extensively characterized enhancers. Its full function has been shown to depend on the cooperation of multiple sequence motifs (Zenke *et al.*, 1986; Herr and Clark, 1986). It has been demonstrated that the SV40 enhancer encompasses ~100 nucleotides, containing the 72-base pair repeat and its 5' flanking region (Zenke *et al.*, 1986; see Fig. 6-1). It is composed of at least two distinct domains, A and B, which possess very little enhancing activity on their own. However, their association results in a strong enhancement of transcription which is independent of orientation, and to some extent, of the distance between them. Furthermore, enhancer activity can be generated by duplication of either domain. We made luciferase reporter

gene constructs based on pGL2-promoter. The transcriptional activation of ORCA/p62 via the B site alone (pB(×1)/GL2) or multimerized (pB(×2)/GL2) is relatively weak and variable (3- to 4-fold), but is above the fold induction seen with the A site. Indeed, a reporter construct with 3 copies of the A site (pA(×3)/GL2) was not stimulated by ORCA/p62 over controls (Fig. 6-6).

6.3.5 The Mobility of Protein/DNA Complexes on the SV40 Enhancer/Promoter Does Not Change in the Presence of ORCA/p62

To determine if the transactivating ability of ORCA/p62 is due to a direct or indirect interaction with the SV40 enhancer, we performed gel mobility shift analysis using a labeled DNA fragment containing the natural SV40 enhancer and the 21-base pair repeats. The probe was incubated with extracts prepared from COS-1 cells transfected with pORCA/SG5 or pSG5. The mobility of the protein/DNA complexes did not change in the presence of ORCA/p62 (Fig. 6-7A; compare lane b to lane c). Furthermore, purified ORCA/p62 does not bind directly to the SV40 enhancer under our assay conditions (Fig. 6-7B; compare lane a to lane e). Adding increasing amounts of cell extracts to the binding reactions did not promote interaction of purified ORCA/p62 with probe DNA (Fig. 6-7B; compare lanes b-d to lanes f-h, respectively).

ORCA/p62 was originally identified based on its interaction with $p56^{lck}$, a T-cell-specific *src* family tyrosine kinase required for T-cell signal transduction. However, ORCA/p62 function is probably not restricted to T-cell-mediated events, since it is ubiuitously expressed and at least two isoforms are known to exist in humans (Joung *et al.*, 1996). While the function of ORCA/p62 in cell surface signal transduction is not yet

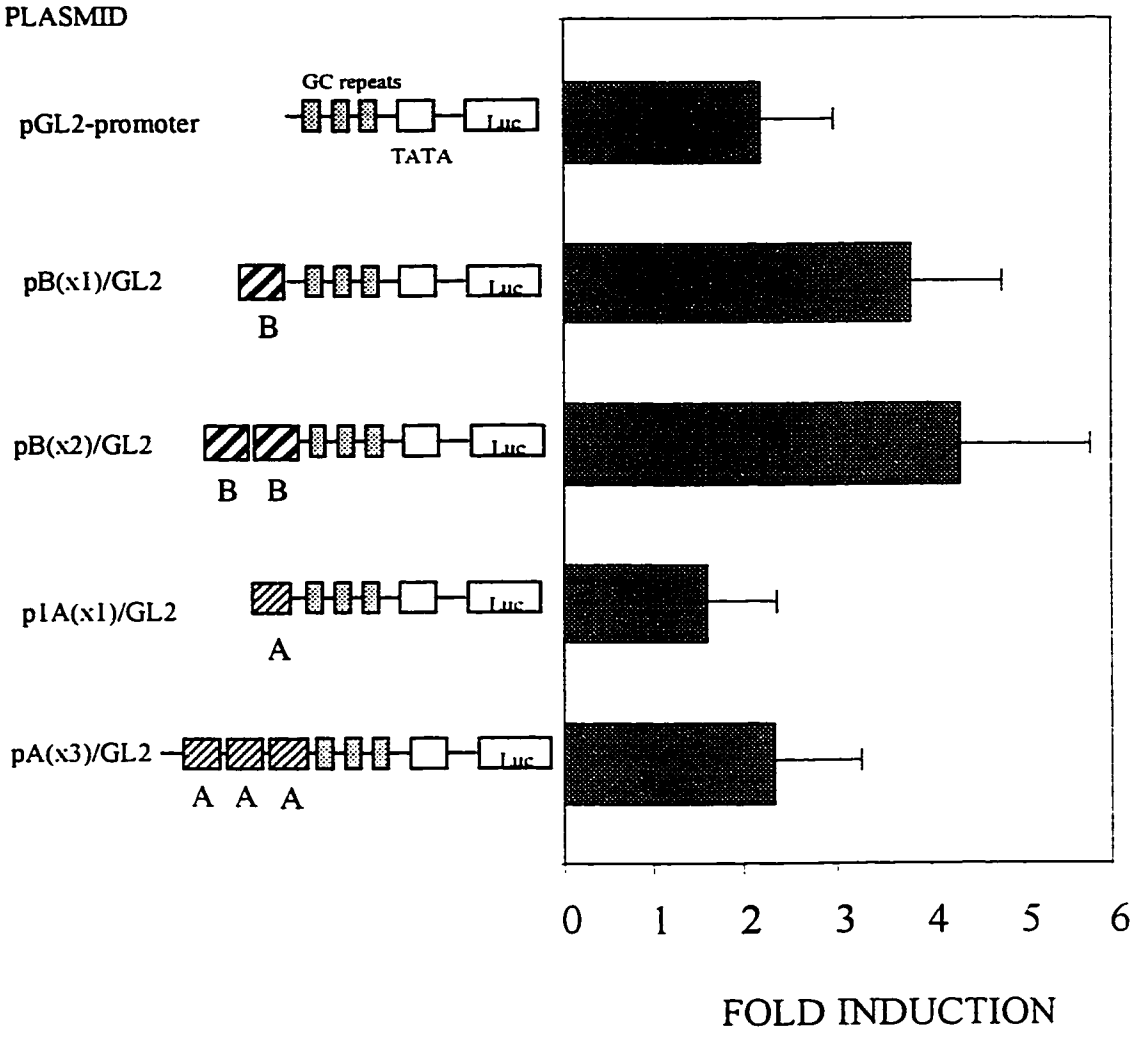

Figure 6-6. The B site of the SV40 enhancer is necessary for response to ORCA/p62. The indicated luciferase reporter plasmids were cotransfected into BSC40 cells in the presence or absence of an ORCA/p62 expression vector as described in Materials and Methods. Values shown are the fold induction of luciferase activity in the presence of ORCA/p62, representing the averages (± SEM) of at least three independent transfections done in duplicate.

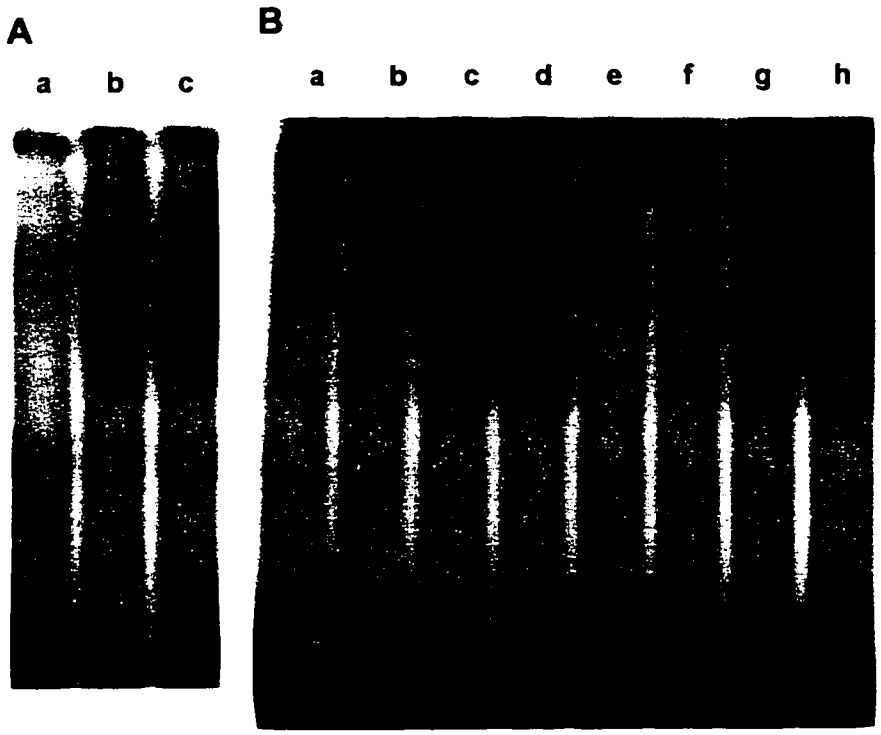

Figure 6-7. The mobility of protein/DNA complexes on the SV40 enhancer/promoter do not change in the presence of ORCA/p62. **A**. Gel retardation analysis of a labeled DNA fragment containing the SV40 enhancer and 21 base pair repeats incubated with nuclear extracts prepared from COS cells transfected with pSG5 (lane b) or pORCA/SG5 (lane c). Lane a, probe incubated in the absence of extract. **B**. Gel retardation analysis of the labeled DNA fragment described in A. Probe was incubated in the absence (lanes a-d) or presence (lanes e-h) of 500 μg of purified ORCA/p62. Binding reactions also contained 1 μg (lanes b and f), 2 μg (lanes c and g), or 4 μg (lanes d and h) of nuclear extract prepared from untransfected COS cells. Lanes a and e did not contain nuclear extract.

known, ORCA/p62 has been reported to bind the Ras-GTPase activating protein (Park *et al.*, 1995), as well as a novel cytokine receptor induced in Epstein-Barr virus-infected B lymphocytes (Devergne *et al.*, 1996). These observations suggest that ORCA/p62 is part of a large family of factors that play a general role in signal transduction in the cell. That ORCA/p62 also stimulates the transcription of the SV40 enhancer adds another functional dimension to this protein and suggests that ORCA/p62 provides a link between cell surface signaling and specific gene transcription.

The mechanism by which ORCA/p62 potentiates SV40 enhancer-mediated transcription is unknown, but a number of possibilities can be envisioned. ORCA/p62 shares a small region of homology with the transcriptional coactivator CBP (residues 136-154 of ORCA/p62 and residues 1715-1722 of CBP), suggesting a possible similarity in their mechanisms of action. This region is part of a domain in CBP that has been shown to bind TFIIB (residues 1680-1812) (Kwok *et al.*, 1994). However, this region of CBP homology is not essential for transactivation the SV40 enhancer (Fig. 6-3), rather part of the N-terminal SH2-binding domain (residues 29-50) appears to be required. Moreover, ORCA/p62 does not possess any intrinsic transactivation potential in mammalian cells when tethered to the promoter (J. Capone, unpublished observations). ORCA/p62 may activate transcription by binding directly or indirectly to the SV40 enhancer. However, we have been unable to detect binding of ORCA/p62 to SV40 enhancer DNA, or a supercomplex of ORCA/p62 and nuclear factors bound to DNA. These results suggest that our assay conditions were not conducive to the binding of ORCA/p62 to the probe DNA, that binding is weak or transient, or that ORCA/p62 functions indirectly by modifying other *trans*-acting factors. Thus, it is conceivable that

when overexpressed, ORCA/p62 could lead to the phosphorylation of one or more transcription factors that bind and transactivate the SV40 enhancer/promoter.

The SV40 enhancer contains binding sites for a number of ubiquitous and cell-specific transcription factors (see Fig. 6-1) including transcription enhancer factor 1 (TEF-1) (Xiao *et al.*, 1991), TEF-2 (factor GT-IC; Xiao *et al.*, 1987), AP1 (Lee *et al.*, 1987), octamer binding transcription factors (Rosales *et al.*, 1987), TCIIA/NF-κB and TC-IIB/KBF1 (Kanno *et al.*, 1989; Macchi *et al.*, 1989). There is evidence that AP1, TCIIA, and TCIIB may mediate response of the SV40 promoter to phorbol esters (Lee *et al.*, 1987; Kanno *et al.*, 1989; Macchi *et al.*, 1989; Sen and Baltimore, 1986b). What role these factors play in ORCA/p62-mediated transcriptional activation is unknown, and awaits future binding and transfection studies. ORCA/p62 has a tightly associated or intrinsic Ser/Thr protein kinase activity. Thus, ORCA/p62 may activate one or more SV40 enhancer-binding transcription factors by phosphorylation. Alternatively, since part of the SH2-binding domain of ORCA/p62 is required, perhaps ORCA/p62 initiates a signaling cascade that then results in transcription factor phosphorylation.

In summary, we have identified a cellular factor that is known to interact with components of the cell surface signal transduction pathways and also activates the SV40 early promoter/enhancer. At least part of the SH2-binding domain located in the N-terminal 50 amino acids of ORCA/p62 is required for this transactivating ability. Shortening the enhancer element to a single 72 base pair repeat reduces the activity of ORCA/p62, suggesting that ORCA/p62-mediated transactivation requires the cooperation of multiple *cis*-acting elements of the SV40 enhancer. Our data indicate that the B domain of the enhancer is necessary, but may not be sufficient, in mediating this

transcriptional activity. Many of the enhansons identified in the SV40 enhancer are also found associated with other viral and cellular enhancers and promoters (Jones *et al.*, 1988; Jiang *et al.*, 1997; MacLellan *et al.*, 1994). Therefore, p62/ORCA may be a more general regulator of gene transcription.

6.4 Bibliography

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 Discussion

Peroxisome proliferators induce profound changes in gene expression in association with proliferation of peroxisomes, and ultimately tumorigenesis, in rodents (Reddy and Lalwani, 1983). Because of the ubiquity of peroxisome proliferators and the potential for carcinogenicity, there is strong interest in understanding the mechanism of action of peroxisome proliferators and in assessing possible health risks to humans from exposure to these compounds.

It is becoming increasingly apparent that PPARs not only mediate the cellular responses to hypolipidemic drugs and nongenotoxic carcinogens, but also play fundamental roles in regulating 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; Lee *et al.*, 1995). PPARs can be activated by a variety of structurally diverse peroxisome proliferators, as well as by natural and synthetic fatty acids (Chapter 2, this thesis; Issemann *et al.*, 1990; Dreyer *et al.*, 1992; Göttlicher *et al.*, 1992; Keller *et al.*, 1993). Many of these compounds have been shown to be *bona fide* PPAR ligands (Krey *et al.*, 1997; Lehmann *et al.*, 1995; Kliewer *et al.*, 1995; Forman *et al.*, 1995; Devchand *et al.*, 1996). Since fatty acids have the ability to directly regulate gene expression via PPARs, it is now apparent that they possess hormone-like properties. PPAR signaling is linked and integrated with other hormone response pathways,including those for thyroid hormone and retinoic acid via their respective receptors. Therefore, there is considerable interest in elucidating the physiological roles of PPARs and their pathways of activation. This project was designed to obtain a better understanding of how PPARs activate transcription and of

how their activity is regulated. Chapters 2 and 3 of this thesis are studies of PPAR function applying two complementary systems. Chapter 2 investigates PPAR DNA-binding and transcriptional activity using transient transfection assays in mammalian cells. Chapter 3 presents a similar study using yeast as a model system. In Chapters 4 and 5, both yeast and mammalians cells are used to investigate the regulation of peroxisome proliferator-responsive genes.

The results in Chapter 2 show that diverse PPARs differentially mediate a transcriptional response to peroxisome proliferators via PPREs. In the monkey kidney cell line COS-1, mPPARα, rPPARα and xPPARα were able to activate a luciferase reporter gene linked to the HD- or AOx-PPRE in response to peroxisome proliferators, suggesting a common mechanism for the coordinated regulation of peroxisome proliferator-responsive genes. No drug-dependent induction of expression was seen by xPPARβ for either PPRE. Interestingly, xPPARγ was effective with the AOx-PPRE but not with the HD- PPRE. Despite the differential activity of the PPAR isoforms, all bound to both PPREs *in vitro*. The DNA-binding activity by PPARs requires the presence of auxiliary cofactors, one of which is RXRα. Cooperative DNA binding and heterodimerization between hRXRα and each of the xPPARs could be seen with both PPREs. Our results demonstrate that PPAR/PPRE binding and cooperativity with RXRα (and other cofactors) are obligatory, but not necessarily sufficient, for peroxisome proliferator-dependent transcription induction, and that distinct PPREs can selectively mediate induction by particular PPARs.

The results presented in Chapter 2 confirm and extend the results of other studies reporting that PPARs bind to DNA through cooperativity with auxiliary cofactors. These

studies also found that RXR potentiates the binding of PPAR to PPREs and is necessary for maximal PPAR-mediated transactivation (Kliewer *et al.*, 1992b; Bardot *et al.*, 1993; Gearing *et al.*, 1993; Keller *et al.*, 1993). PPAR and RXRα interact in solution in the absence of target DNA as shown by immunoprecipitation (Kliewer *et al.*, 1992b), and *in vivo* as shown by genetic assays detecting protein-protein interactions (Miyata *et al.*, 1994). Further evidence that PPAR and RXR cooperate *in vivo* to activate transcription via PPREs comes from studies carried out in yeast.

Studies of nuclear hormone receptor function in mammalian cells are complicated by the presence of endogenous nuclear hormone receptors and their ligands. The yeast *Saccharoymyces cerevisiae* is devoid of endogenous nuclear receptors and retinoids. Yeast has provided a model system that has aided in dissecting interactions between various nuclear hormone receptor heterodimeric partners, and thus work in yeast complements studies in mammalian cells (reviewed in Butt and Walfish, 1996). A number of nuclear hormone receptors have been shown to function in yeast in both ligand-dependent and ligand-independent manners (Hall *et al.*, 1993). Furthermore, yeast molecular genetics has allowed the rapid cloning of mammalian cofactors that cooperate with nuclear hormone receptors (reviewed in Horwitz *et al.*, 1996). At present, there are more than 150 known human nuclear hormone receptors. Once the human genome is sequenced, it is predicted that this number will increase to 500 (Butt and Walfish, 1996). Yeast also provides an efficient cell-based system to identify heterodimeric partners and to discover novel ligands for orphan receptors.

Chapter 3 of this thesis describes an investigation of PPAR function using yeast as a model system. Cosynthesis of both mPPARα and hRXRα was necessary to activate

the expression of a reporter gene linked to either the AOx- or the HD-PPRE. Either receptor expressed alone was essentially inactive. Moreover, the HD-PPRE seems to be a more efficient response element *vis-à-vis* the AOx-PPRE. The integrity of the AOx DR1 repeat is essential, because altering either response element abolished PPARα/RXRα-mediated transactivation. The integrity of both DR1 repeats of the HD-PPRE is essential for full activity, because altering the third or fourth response element dramatically reduced reporter gene activity. The small amount of remaining activity of the two reporter gene constructs containing mutant HD-PPREs is probably due to the ability of PPARα and RXRα to form heterodimers on the overlapping upstream DR1 (Chu *et al.*, 1995).

Transactivation of the PPRE-linked reporter gene occurs in the absence of exogenously added ligand. This is not surprising, because several other nuclear hormone receptors exhibit some ligand-independent activity (Heery *et al.*, 1993; Hall *et al.*, 1993). Alternatively, yeast may contain endogenous PPAR activators. This hypothesis is supported by the observation that PPARα interacts strongly with the coactivators p300 and SRC-1 in yeast, in the absence of added ligand (Dowell *et al.*, 1997). A number of hypolipidemic drugs and fatty acids, including Wy-14,643, nafenopin, petroselinic acid, docasahexaeoic acid, linoleic acid, and elaidic acid were tested for their ability to activate mPPARα in yeast. Of the fatty acids tested, only petroselinic acid (C18:ω12) was able to potentiate mPPARα/hRXRα transactivation. This fatty acid increased reporter gene activity by an additional two- to three-fold. The mPPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 had no effect on transactivation by mPPARγ2/hRXRα heterodimers in yeast either alone or in combination with 9-*cis* retinoic acid (Kassam *et al.*, 1998). The

reason that, besides petroselinic acid, none of the potent peroxisome proliferators and fatty acids previously shown to activate mPPARα and γ in mammalian cells could do so in yeast is unclear. It may be due to poor uptake of these compounds, rapid metabolism in yeast, and/or the inability of yeast to convert these compounds to proximate PPAR activators. The finding that Wy-14,643 is a *bona fide* PPARα ligand (Devchand *et al.*, 1996), and that this compound was able to potentiate association between SRC-1 and mPPARα in yeast (Dowell *et al.*, 1997), argues against these possibilities. For some of the fibrate hypolipidemic drugs, however, 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, other studies suggest 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). These findings are consistent with our observation that potentiation of PPARα/RXRα activity by petroselinic acid requires intact peroxisomes, but not necessarily the integrity of the peroxisomal β-oxidation system (Chapter 3, this thesis).

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 mPPARα/hRXRα function in yeast. PPARα/RXRα heterodimers may respond differently to 9-*cis* retinoic acid compared to RAR/RXR heterodimers and RXR homodimers in yeast, possibly due to the absence of specific coactivators. Indeed, other groups have noted differences in the response of TR/RXR heterodimers to 9-*cis* retinoic acid in yeast compared to mammalian cells (Hall *et al.*, 1993; Walfish *et al.*, 1996). This

has been attributed to the absence of corepressor proteins in yeast (Butt and Walfish, 1996). Henry and coworkers (1995) have also demonstrated that rPPARα and RXRα heterodimers function in yeast in a ligand-independent manner. No additional response to peroxisome proliferators or 9-*cis* retinoic acid was noted.

Taken together with the findings of other groups, the results presented in Chapters 2 and 3 of this thesis clearly demonstrate a convergence of the peroxisome proliferator- and retinoid-dependent signaling pathways on PPRE-like elements. Thus, it is likely due to the role of RXRα as a coregulator that places it at the center of lipid metabolism (Kliewer *et al.*, 1992a; 1992b; Zhang *et al.*, 1992; Wolf and Phil, 1993).

In Chapter 2, our comparison of the activities of the xPPAR isoforms on the AOx- and HD-PPREs demonstrated that with xPPARγ, activity can depend on the nature of the PPRE. Accordingly, mPPARγ2 synthesized in yeast bound cooperatively with hRXRα *in vitro* with equal affinities to both PPREs; however, it is a more efficient transactivator of the AOx-PPRE *in vivo* (Kassam *et al.*, 1998). These results are in contrast to those obtained with mPPARα, where the HD-PPRE is the stronger response element in yeast. The AOx- and the HD-PPREs are fairly divergent. There are differences both in the sequences of the TGACCT-like repeats, as well as in the flanking nucleotides (Tugwood *et al.*, 1992; Zhang *et al.*, 1992; Chu *et al.*, 1995). Moreover, while the AOx-PPRE contains two direct repeats in a DR1 configuration, the HD-PPRE contains four direct repeats (two DR1s with an overlapping DR2). It is likely that some or all of these differences underlie the target gene specificity observed with xPPARγ, mPPARγ, and perhaps other PPAR isoforms. Ligand activation and interactions with basal transcription

factors or coactivators may be influenced by differences in receptor-coregulator-DNA interactions imparted by different target PPREs.

The ability of xPPARγ to interfere with the *in vivo* induction of transcription mediated by rPPARα or xPPARα implies that PPAR isoforms may act as both repressors and activators of specific target genes. Thus, one PPAR isoform may transdominantly inhibit the others, depending on their relative abundance in a particular tissue. Similar observations were noted for the human and mouse homologues of PPARα and NUC1 (Kliewer *et al.*, 1994; Jow and Mukherjee, 1995). While hPPARα is a transcriptional activator in the presence of fibrates and ETYA, hNUC1 is not. Cotransfecting a constant amount of hPPARα and an increasing amount of hNUC1 resulted in a dose-dependent abrogation of hPPARα transcriptional activity (Jow and Mukherjee, 1995). This effect could be overcome by the addition of excess hPPARα expression plasmid. Since the two receptors bind as hRXRα heterodimers to a PPRE with similar affinities, it has been proposed that hNUC1 represses hPPARα by sequestering a limiting transcription factor, possibly a coactivator.

A distinctive feature of the PPAR subfamily is that the ligand-binding domains among the various PPAR subtypes exhibit greater sequence divergence than that observed among the subtypes of the TR or RAR subfamilies (Lemberger *et al.*, 1996). Similarly, for a given PPAR subtype, the divergence among species is greater than the interspecies differences seen in the TR or RAR subtypes. This implies that the ligand-binding domain for PPAR has evolved more rapidly than those of the TRs and RARs (Dreyer *et al.*, 1993). Therefore, one would expect that the three PPAR subtypes have

divergent ligand-binding specificities and distinct biological functions (Lemberger *et al.*, 1996). Coactivator-dependent receptor ligand assay (CARLA) has been used to systematically identify proximate PPAR ligands, revealing an amazing ability of the PPAR ligand-binding domains to accommodate a wide variety of molecular structures (Krey *et al.*, 1997). These findings show that PPARs differ from the other members of the RAR/TR subfamily with their stringent ligand specificities, and suggest that PPARs have evolved under different selection pressures. This study also identifies important overlap in ligand recognition between the three PPAR subtypes, particularly with polyunsaturated fatty acids. Several subtype-selective and subtype-specific ligands were also identified. Therefore, the regulation of PPAR target genes may be governed by multiple factors, including the tissue distribution and ligand affinity of the subtypes.

Ligand recognition by PPAR subtypes has, in general, been preserved through evolution (Krey *et al.*, 1997). However, species-specific differences do exist in the affinity of a receptor subtype for a given ligand (Krey *et al.*, 1997; Lehmann *et al.*, 1995; Forman *et al.*, 1995). It remains to be investigated whether these differences in the affinity for natural ligands reflect species-specific peculiarities, such as peroxisome proliferation. There are considerable species differences with respect to chemically-induced peroxisome proliferation and tumorigenesis. Human liver is refractory to the pathological effects of peroxisome proliferators observed in rodents. These species differences may be due to the relative abundance of the PPAR isoforms and/or species differences in ligand binding of a particular isoform. Interestingly, the relative levels of PPARα in human liver have been shown to be more than 10-fold lower than that observed in mice (Palmer *et al.*, 1998). Therefore, it is speculated that the low level of

PPARα expression in liver may be insufficient to compete effectively with other proteins that bind to PPREs, such as other PPAR subtypes, COUP-TFs and HNF-4.

PPAR function is subject to differential modulation by multiple nuclear hormone receptors that can recognize PPREs, including COUP-TFI (Miyata *et al.*, 1993), HNF-4 (Winrow *et al.*, 1994), TRα homodimers, TRα/RXRα heterodimers (Hunter *et al.*, 1996), and the orphan receptor ROR/RZR which binds to the HD-PPRE as a monomer[6] (Schräder et al., 1996). Moreover, PPARs can heterodimerize with partners other than RXR, such as TRα (Bogazzi *et al.*, 1994) and LXRα (Miyata *et al.*, 1996; Willy *et al.*, 1995). To identify other cellular factors that bind PPREs and/or interact with PPARα, we have developed a genetic screening system in yeast for the direct identification of PPRE-binding proteins. As described in Chapter 4, we identified COUP-TFII as an HD-PPRE-binding factor. Since the yeast strain was engineered to also express mPPARα, one would expect to obtain RXRα in such a screen. Surprisingly, COUP-TFII was the only factor that we identified. It is possible that the structure of COUP-TFII mRNA is particularly suited for efficient translation in yeast. In this regard, a long or GC-rich 5'-untranslated region seems to inhibit efficient synthesis of some mammalian proteins in yeast[7]. Any resulting secondary structure may cause dissociation from the ribosome.

Remarkably, COUP-TFII is a potent transcriptional activator of PPRE-linked reporter genes in yeast. However, COUP-TFII does not activate transcription via the HD-PPRE in mammalian cells but does interfere with activation mediated by PPAR/RXR heterodimers, as we had shown previously with COUP-TFI (Miyata *et al.*, 1993).

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[6] C. Winrow, J. Capone, and R. Rachubinski, unpublished observations
[7] S. Marcus, J. Capone, and R. Rachubinski, unpublished observations

COUP-TFs generally function as transcriptional repressors in mammalian cells. However, COUP-TFI and COUP-TFII can activate transcription in certain cell types and promoter contexts by a number of mechanisms (Gaudet and Ginsburg, 1995; Kimura *et al.*, 1993; Rodríguez *et al.*, 1997; Power and Cereghini, 1996; Ktstaki and Talianidis, 1997). Moreover, COUP-TFII has been shown to function as a constitutive transcriptional activator not only in yeast, but also *in vitro* (Malik and Karathanasis, 1995). These observations suggest that COUP-TFII possesses intrinsic activation potential. We hypothesized that the repression by COUP-TFII seen in mammalian cells is due to one or more transcriptional corepressors not present in yeast.

To determine if differential COUP-TF activity is mediated through the actions of auxiliary proteins, we used the yeast two-hybrid interaction cloning system to identify novel COUP-TFII-interacting proteins. Chapter 5 of this thesis describes the identification of a factor that bound COUP-TFII *in vitro* and appeared to convert COUP-TFII from a transcriptional repressor into an activator in mammalian cells. This protein, which we call ORCA (for Orphan Receptor CoActivator) is identical to p62, a ligand for the tyrosine kinase signaling molecule $p56^{lck}$ (Joung *et al.*, 1996). In the past, the term "coactivator" has been rather loosely applied. A number of criteria have been proposed to classify a factor as a coactivator (Horwitz *et al.*, 1996). Thus, a coactivator may be defined as: i) a limiting factor that enhances transcriptional activity of nuclear receptors without altering basal activity; ii) this factor should directly interact with nuclear receptors in a ligand-dependent manner; and iii) it should contact the basal transcriptional machinery, acting as a "bridging" molecule. The CBP and NCoA family of nuclear proteins meets several of these criteria. However, based on the above

definition, subsequent studies suggest that ORCA/p62 may not be a *bona fide* coactivator of COUP-TFII. Rather, it appears that ORCA/p62 stimulates transcription from the SV40 early promoter/enhancer. Since COUP-TFII was expressed from the SV40 promoter/enhancer in mammalian cells, an ORCA/p62-dependent increase in COUP-TFII mRNA and protein levels results in a stimulation of reporter gene activity. Moreover, simply increasing the amount of cotransfected COUP-TFII stimulates reporter gene activity[8]. The mechanism of this transcriptional activation is unclear, since a PPRE does not appear to be required. The stimulation of reporter gene transcription could be a result of COUP-TFII directly interacting with components of the basal transcription machinery and/or factors bound to the proximal promoter. As discussed below, results from other groups support this hypothesis.

COUP-TFs have been shown to repress transcription by a variety of direct and indirect mechanisms. Conversely, a number of recent studies indicate that COUP-TFs also activate transcription by diverse mechanisms. For example, COUP-TFI and COUP-TFII have been shown to activate transcription by an indirect mechanism involving interactions with octamer-binding proteins near the transcription start site (Power and Cereghini, 1996). Therefore, given the appropriate promoter structure, COUP-TFI and COUP-TFII action can be mediated by protein-protein interactions that do not necessarily require direct binding to DNA. Another study demonstrated that COUP-TFs act as auxiliary cofactors for HNF-4 homodimers to enhance hepatic gene expression (Ktistaki and Talianidis, 1997). It is suggested that, in this case, COUP-TFs function to bring the HNF-4 activation surface into a more optimal configuration to facilitate entry of other

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[8] S. Marcus, J. Capone, and R. Rachubinski, unpublished observations

components of the preinitiation complex. Taken together, these observations suggest that in addition to their DNA-binding properties, the ability of COUP-TFs to interact with other proteins is also highly promiscuous.

Studies of the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase promoter have also revealed a dual role for COUP-TFI in the regulation of this gene. COUP-TFI has been shown to repress the induction of the HMG-CoA synthase gene by PPAR via competition for PPRE binding (Rodríguez *et al.*, 1997). Moreover, COUP-TFI was able to activate transcription of a reporter gene construct containing the minimal HMG-CoA synthase promoter with coordinates -62 to +28 (relative to the transcription start site) in a liver cell-specific manner. This fragment contains only a GC box and a TATA box. No direct COUP-TFI-DNA interactions could be detected by gel shift experiments with a DNA probe containing this promoter region and COUP-TFI produced in hepatoma cell extracts. It has been speculated that the PPRE-independent transcriptional activating ability of COUP-TF may result from modification by (a) the binding of a liver-specific ligand, (b) interaction of COUP-TF with a hepatocyte-specific transcription factor, or (c) a post-translational modification such as phosphorylation (Rodríguez *et al.*, 1997).

Shibata and coworkers (1997) have demonstrated that COUP-TFI interacts with the corepressors NCoR and SMRT. Transfection in HeLa cells of a Gal4 DNA-binding domain fused to the putative ligand-binding domain of COUP-TFI repressed the basal transcription of a reporter gene containing Gal4-binding sites. Significantly, cotransfection of COUP-TFI relieved the gal4-COUP-TFI-mediated repression in a dose-dependent manner. COUP-TFIΔ35, which lacks the C-terminal 35 amino acids containing the repressor domain, failed to relieve this repression. Furthermore,

overexpression of NCoR or SMRT potentiated the silencing activity of COUP-TFI and relieved the COUP-TFI-mediated squelching of Gal4-COUP-TFI activity (Shibata *et al.*, 1997). Similarly, the COUP-TFII-mediated increase in transcription seen in BSC40 cells may actually be a derepression due to the squelching of putative corepressors by excess COUP-TFII. This explanation seems the most plausible because the increase in reporter gene activity is not dose-dependent. Rather, there appears to be a critical dose of COUP-TFII in the cell at which luciferase activity increases dramatically.

The diverse effects of COUP-TFs on different genes suggests that this family of nuclear receptors is multifunctional and performs a number of essential biological functions. It is unclear what physiological role ORCA/p62 plays in the function of COUP-TFII. In addition to COUP-TFII, ORCA/p62 also bound *in vitro* to COUP-TFI, mPPARα, and mPPARγ but not to RXRα or TR[9], suggesting that it may play a role in the function of certain nuclear receptors. COUP-TFII is phosphorylated *in vivo* as shown by cell culture labeling with [γ-^{32}P]ATP, followed by immunoprecipitation of cell extracts[10]. Phosphorylation of COUP-TF has been shown to be essential for DNA binding to DR1 response elements (Brodie *et al.*, 1996). Moreover, activated MAP kinase can phosphorylate bacterially-synthesized COUP-TFII *in vitro*[11] . Others have reported the phosphorylation of mPPARγ *in vivo* at a consensus MAP kinase site in the amino terminal domain (Zhang *et al.*, 1996; Adams *et al.*, 1997). It is interesting to note in this regard that ORCA/p62 possesses a tightly associated or intrinsic Ser/Thr protein kinase (Park *et al.*, 1995). However, we could not detect any phosphorylation of COUP-TFII by

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[9] S. Marcus, J. Capone, and R. Rachubinski, unpublished observations
[10] S. Marcus, J. Capone, and R. Rachubinski, unpublished observations
[11] S. Marcus, E. Shibuya, J. Capone, and R. Rachubinski, unpublished observations

ORCA/p62 when both proteins were purified from *Escherichia coli* and incubated together in the presence of [γ-^{32}P]ATP, suggesting that the protein kinase activity of ORCA/p62 is due to an associated cellular factor. Moreover, the presence of cotransfected ORCA/p62 did not alter the phosphorylation state of COUP-TFII *in vivo*[12]. It is possible that, in the cell line used in this experiment (the monkey kidney cell line COS-1), COUP-TFII is already maximally phosphorylated.

ORCA/p62 is probably a multifunctional protein. It was originally identified based on its interaction with p56^{lck}, a T-cell-specific *src* family tyrosine kinase required for T-cell signal transduction. However, the function of ORCA/p62 in cell surface signal transduction is not yet known. ORCA/p62 has recently been shown to belong to a novel class of ubiquitin-binding proteins, suggesting a physiological role for ORCA/p62 in the connection of mitogenic signals to the ubiquitination-mediated specific protein degradation pathway (Vadlamudi *et al.*, 1996). That ORCA/p62 also stimulates the transcription of the SV40 enhancer, described in Chapter 6 of this thesis, adds another functional dimension to this protein and suggests that ORCA/p62 provides a link between cell surface signaling and specific gene transcription. At least part of the SH2-binding domain located in the N-terminal 50 amino acids of ORCA/p62 is required for this transactivating ability. This finding suggests that ORCA/p62 initiates a signaling cascade that leads to the phosphorylation of one or more transcription factors that bind and transactivate the SV40 enhancer, for example TCIIA/NF-κB (Macchi *et al.*,1989). Moreover, many of the *cis*-acting elements identified in the SV40 enhancer are found

[12] S. Marcus, J. Capone, and R. Rachubinski, unpublished observations

associated with other viral and cellular enhancers and promoters (Jones *et al.*, 1988). Therefore, ORCA/p62 may be a more general regulator of gene transcription.

The magnitude of ORCA/p62-mediated transcriptional activation was greatest when the full SV40 enhancer was used, including both 72-base pair repeats. Indeed, the fold induction of transcription was marginal when a single copy of the 72-base pair repeat and 5'-flanking region was used. These results suggest that the cooperation of multiple *cis*-acting elements of the SV40 enhancer is required for ORCA/p62-mediated transcriptional induction. This is not surprising because a linear increase of transcription occurs with multiple copies of the SV40 enhancer (Zenke *et al.*, 1986). While dimers of either the A or B domain create some enhancer activity, the effect is reduced compared to the wild-type enhancer. Moreover, wild-type activity is still not achieved even with multimers of domains A or B (Zenke *et al.*, 1986). Accordingly, our data indicate that the B domain of the enhancer is necessary, but may not be sufficient, in mediating ORCA/p62-dependent transcriptional activity.

7.2 Summary and Conclusions

Chemically induced overexpression of the genes encoding peroxisomal proteins can lead to profound changes in fatty acid metabolism, peroxisome proliferation, hepatomegaly, and carcinogenesis. Studies of the promoter regions of two genes encoding peroxisomal β-oxidation enzymes led to the identification of the AOx- and HD-PPREs, which consist of direct repeats of the consensus sequence TGACCT. Transcriptional induction by peroxisome proliferators is mediated by PPARs, members of the nuclear hormone receptor superfamily, by binding directly to PPREs. In addition to the genes encoding peroxisomal proteins, PPARs regulate the expression of genes

involved in multiple metabolic pathways, as well as genes important for differentiation. Moreover, PPREs are subject to regulation by other members of the nuclear hormone receptor superfamily.

Research presented in this thesis aimed at determining the mechanisms of transcriptional activation by PPARs and how the transcription of PPRE-linked genes, particularly HD, is regulated. Multiple factors that interact with both the HD- and AOx-PPREs mediate response to peroxisome proliferators, while others modulate this response. A schematic summary is presented in Fig. 7-1. Binding of PPARs to PPREs requires the presence of auxiliary cellular factors, one of which is RXRα. PPARα and RXRα bind cooperatively to DR1 repeats of the consensus sequence TGACCT. These two nuclear receptors are present in rat liver and form complexes *in vitro* with both PPREs. They can mediate peroxisome proliferator responsiveness when the PPRE is linked to a heterologous reporter gene. Heterologous PPARs (α, β, and γ) can bind to both PPREs, but this binding is not necessarily sufficient for transactivation *in vivo*. This finding allows for the possibility of repression mediated by different PPAR subtypes through competition for DNA binding. Thus, as described in Chapter 2 of this thesis and presented in Fig. 7-2, xPPARγ is able to *trans*-dominantly inhibit the function of xPPARα or rPPARα.

Further evidence that PPAR and RXRα cooperatively activate transcription via PPREs came from studies in yeast. Yeast has proven to be a useful tool with which to study nuclear hormone receptor function and regulation, because this organism is devoid of endogenous nuclear receptors and their ligands. PPARα and RXRα synthesized in yeast cooperatively activate the transcription of a PPRE-linked reporter gene. Either

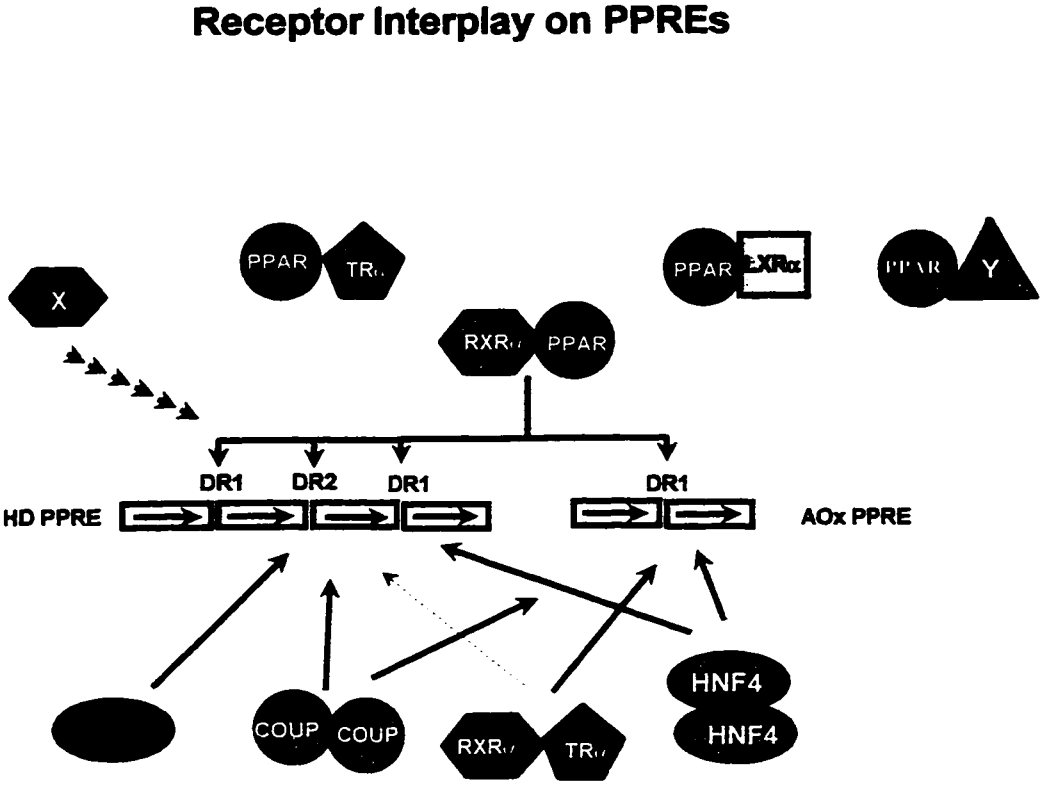

Figure 7-1. Summary of nuclear receptor interactions and interplay on the AOx- and HD-PPREs. X and Y represent putative cellular factors which may also be involved in the function of PPAR.

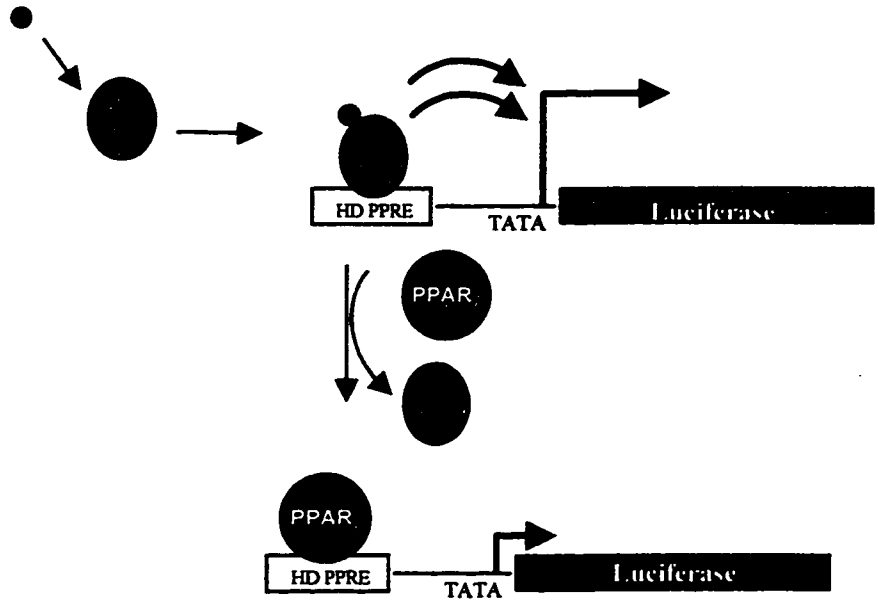

Figure 7-2. xPPARγ *trans*-dominantly inhibits the transactivation mediated by xPPARα or rPPARα.

receptor synthesized alone is essentially inactive. Cooperative transactivation occurs in the absence of exogenously added peroxisome proliferator or fatty acid, suggesting that either yeast contain endogenous PPAR activators, or that yeast lack putative corepressors found in mammalian cells. Peroxisome proliferators have no effect on transactivation by PPARα/RXRα heterodimers in yeast. Of the fatty acids tested, only petroselenic acid potentiates PPARα/RXRα-mediated transcriptional activation. Similar studies carried out in a yeast strain lacking peroxisomes and a strain deficient in β-oxidation indicate that peroxisomes, but not an intact β-oxidation system, is required for potentiation by petroselenic acid.

As depicted in Fig. 7-1, a number of other nuclear hormone receptors can bind to the HD-PPRE and/or AOx-PPRE, including COUP-TFI, COUP-TFII, HNF-4, ROR/RZR, TR homodimers, and TR/RXR heterodimers. Moreover, PPAR can heterodimerize with other nuclear receptors in addition to RXRα, including TR and LXRα. To identify other factors that contribute to a maximal transcriptional response to peroxisome proliferators via the HD-PPRE, either alone as with factor X or in cooperation with PPAR as with factor Y, a genetic screening system in yeast was developed for the identification of novel PPRE-interacting proteins. Using this system, COUP-TFII was identified as a factor that interacts with the HD-PPRE. Surprisingly, COUP-TFII is a strong transcriptional activator of PPRE-linked reporter genes in yeast. However, COUP-TFII does not activate transcription via PPREs in mammalian cells, rather it can antagonize PPARα/RXRα-mediated signaling, as shown schematically in Fig. 7-3. These results are similar to those obtained previously with COUP-TFI. A genetic screening system in yeast detecting protein-protein interactions was used to

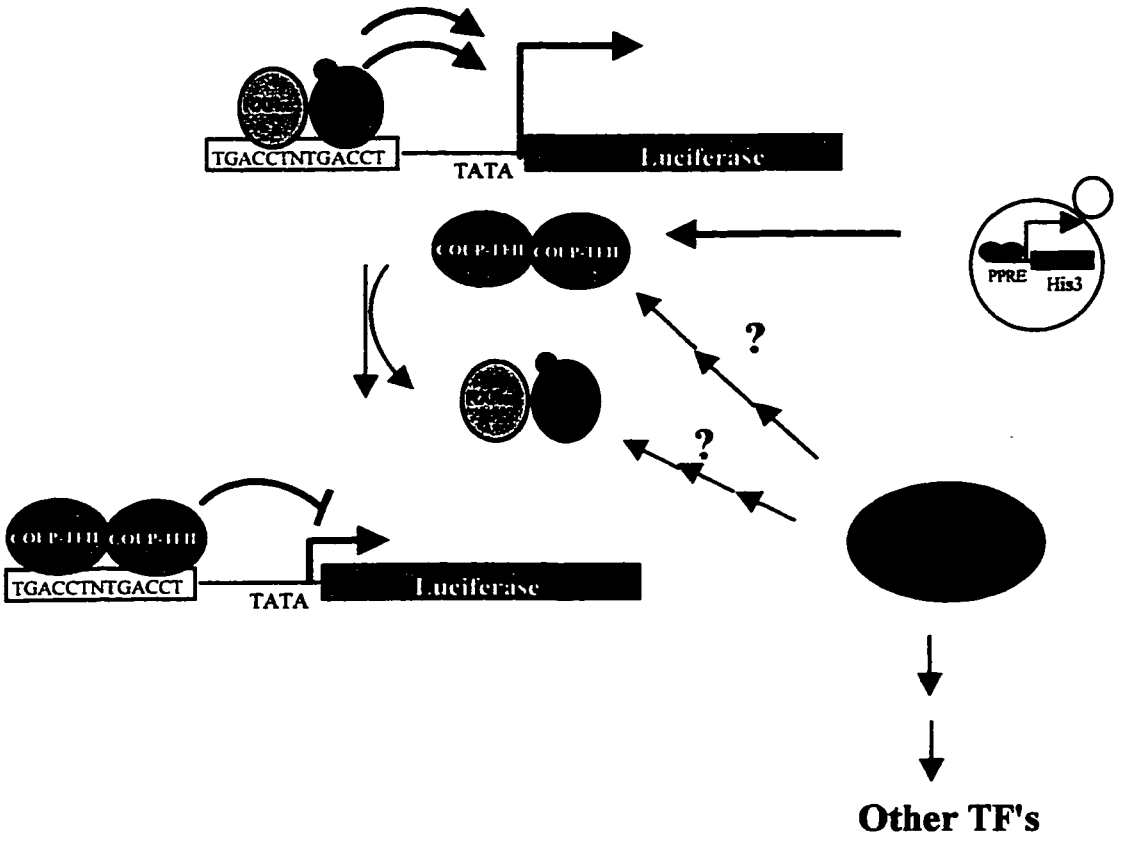

Figure 7-3. Summary of Chapters 3, 4, 5, and 6. PPARs require RXRα to bind PPREs and activate transcription. COUP-TFII was identified as a PPRE-binding factor by genetic selection in yeast. COUP-TFII activates transcription via PPREs in yeast, but potently antagonizes PPAR/RXRα-mediated transactivation in mammalian cells. A genetic screening system in yeast detecting protein-protein interactions was used to identify novel COUP-TFII-interacting proteins and putative corepressors. ORCA/p62, a ligand of the tyrosine kinase signaling molecule $p56^{lck}$, was identified as a factor that interacts with COUP-TFII and several other nuclear hormone receptors. Overexpression of ORCA also leads to transactivation of the SV40 early enhancer/promoter. Therefore, ORCA/p62 may link COUP-TFs and other transcription factors with cell surface signaling pathways.

identify novel COUP-TFII-interacting proteins and putative corepressors. ORCA/p62, a ligand of the tyrosine kinase signaling molecule $p56^{lck}$, was identified as a factor that interacts with COUP-TFII and several other nuclear hormone receptors. Overexpression of ORCA/p62 also leads to transactivation of the SV40 early enhancer/promoter. While it is not yet clear whether ORCA/p62 is a *bona fide* COUP-TFII coactivator, we speculate that ORCA/p62 may link COUP-TFs and perhaps other transcription factors with cell surface signaling pathways. The conclusions of Chapters 3, 4, 5 and 6 are summarized schematically in Fig. 7-3.

With the discovery of an increasing number of PPREs, it is becoming apparent that various PPAR subtypes cooperate in different tissues to play central roles in the maintenance of energy homeostasis: PPARγ is a master regulator of adipocyte differentiation and therefore controls fat storage, while PPARα controls lipid catabolism, particularly in the liver. The finding that certain fatty acids, and metabolites of fatty acids, are PPAR ligands that can induce the formation of peroxisomes suggests that peroxisome proliferation is ultimately a natural adaptation of the cell to changes in nutritional status. Thus, physiological situations that result in elevated levels of plasma triglycerides and fatty acids, such as fasting, stress, and a high-fat diet, are likely to cause activation of PPARα to stimulate the peroxisomal fatty acid oxidative pathway (Lemberger *et al.*, 1996). The identification of RXRα, TR, and LXRα as PPAR dimerizing partners signifies crosstalk between hormone signaling pathways and nutritional status (*e.g.* fatty acid and cholesterol levels). Finally, identification of nuclear receptor coactivators and cointegrators has shed some light on how nuclear receptor

signaling is integrated with cell surface signaling pathways, to create a net transcriptional response.

The picture in Fig. 7-1 is likely to become increasingly complex over time as new nuclear hormone receptors continue to be identified. The promiscuous binding of nuclear receptors to degenerate response elements reflects a common theme to the expression of many inducible genes (Lucas and Granner, 1992). Moreover, the combination of multiple enhancers and silencers with various promoters, all of which contain binding sites for a wide variety of transcription factors, affords multiple levels of regulation. These combinatorial mechanisms enable responses to diverse signals, such as hormones and mitogens, so that genes can be regulated coordinately, as well as individually. The AOx- and HD-PPREs are good examples for the study of these basic mechanisms of gene regulation.

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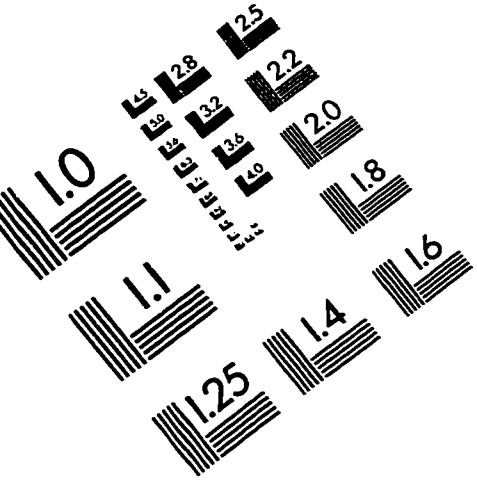
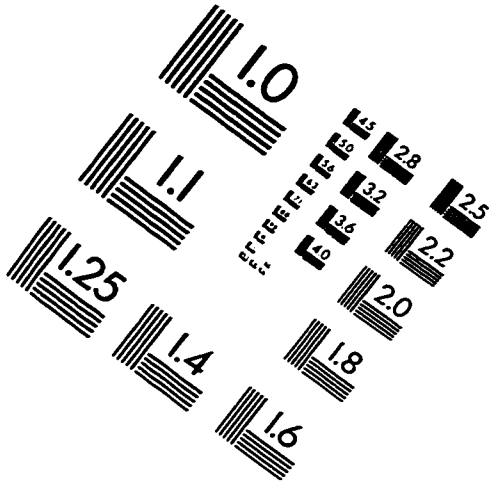
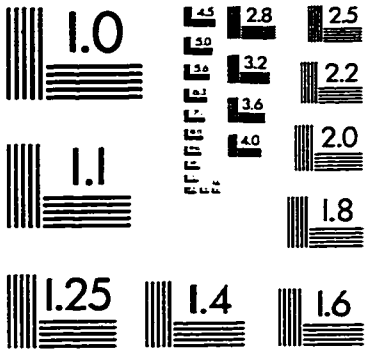
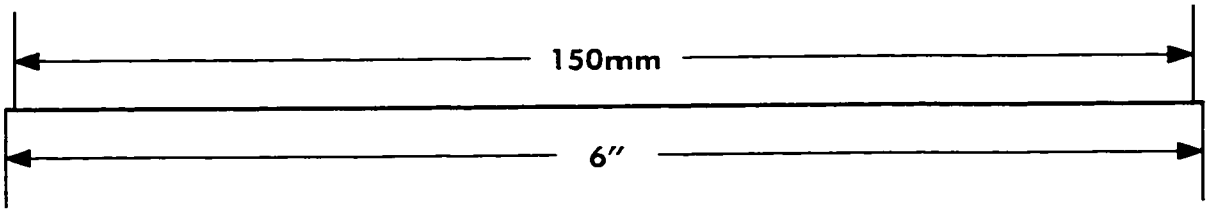
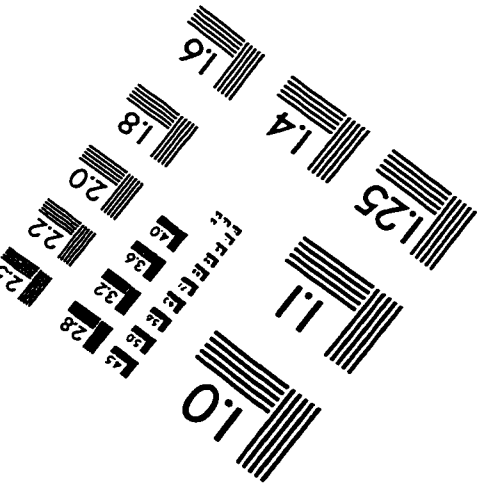
IMAGE EVALUATION
TEST TARGET (QA-3)

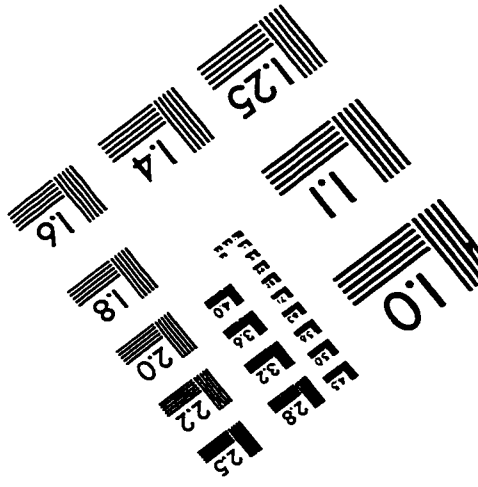












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