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THE MOLECULAR MECHANISM OF TRANSCRIPTIONAL ACTIVATION BY THE PEROXISOME PROLIFERATOR ACTIVATED-RECEPTOR α

By

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ABSTRACT

The peroxisome proliferator-activated receptor (PPARα) is a member of the nuclear receptor superfamily, a growing class of transcriptional activators. Many of these proteins, including PPARα, function as intracellular receptors for hormones and upon the binding of agonist, activate the transcription of target genes. PPARα is an integral regulator of fatty acid metabolism and as such is activated by fatty acids resulting in the stimulation of the expression of genes involved in the release, cellular uptake, and β-oxidation of free fatty acids. The research presented in this thesis details the discovery and characterization of mechanistic aspects by which PPARα mediates the transcriptional activation of target genes.

Transcriptional activators bind to specific DNA binding sites in the promoter region of target genes to effect changes in the rate of transcription. Included in the presented work are the discovery and the characterization of protein-DNA interactions that are involved the activation of transcription by PPAR. Firstly, this includes the identification of PPARs as the factors that mediated the activation of a target gene by free fatty acids. PPARs were shown to bind to their appurtenant DNA binding sites as a heterodimer with the retinoid X receptor (RXR). While the binding of this complex to DNA was required for PPAR mediated signalling, binding was not sufficient for transcriptional activation implying that the DNA binding site itself acted as an allosteric regulator. Secondly, evidence is presented that identifies two other nuclear receptors that

bound to the same DNA binding sites as PPAR. These factors, the chicken ovalbumin upstream promoter-transcription factor 1 and the hepatocyte nuclear factor 4, modulated the activity of PPARα resulting in the abrogation and potentiation, respectively, of PPARα activity.

Once bound to specific DNA sequences, transcriptional activators mediate their effects through a network of protein-protein interactions that ultimately controls the transcriptional process. Included in this thesis are studies that established that PPARα interacts with its DNA binding partner RXRα *in vivo* in the absence of exogenous agonist and DNA binding. This suggested that PPAR and RXR bound to DNA as a preformed heterodimer. To discover novel interacting partners for PPARα, the yeast two-hybrid screen was utilized. Two novel PPARα-protein interactions were identified. This included the discovery and cloning of a novel nuclear receptor, the liver X receptor that interacted with PPARα. Furthermore, an interaction was documented between PPARα and the receptor interacting protein 140, a putative mediator of the transcriptional activation activity of nuclear receptors. In both cases, the liver X receptor and receptor interacting protein 140, antagonized the transcriptional activation mediated by PPARα. These studies have succeeded in increasing the known repertoire of proteins that modulate and effect the activity of PPARα through protein-protein interactions.

The findings presented in this thesis have contributed to the understanding of the network of protein-DNA and protein-protein interactions that both potentiate and antagonize the activity of PPARα. This has helped further our understanding of the mechanism by which fatty acids directly regulate the activity of PPARα and control the expression of target genes to elicit adaptive changes in metabolism.

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To my family, I can't thank you enough for supporting me over the years and putting up with my career as a professional student. Finally, to Princess Genevieve, the epilogue to this story belongs to us...

My only regret is not cloning the moose PPARα.

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μ	micro
βgal	β-galactosidase
2YT+Amp	2YT media containing 100μg/ml ampicillin
8(S)-HETE	8(S)-hydroxyeicosatetraenoic acid
15d-PGJ_2	15d-$\Delta^{12,14}$-prostaglandin J_2
22-*(R)*-HC	22-*(R)*-hydroxycholesterol
a.a.	amino acid
Ab	antibody
AF-1	activation function 1
AF-2	activation function 2
AOx	acyl-CoA oxidase
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
CBP	CREB (cAMP response element binding protein) binding protein
cDNA	DNA complementary to mRNA
Ci	Curie(s)
CoA	coenzyme A
COUP-TF	chicken ovalbumin upstream promoter - transcription factor I
cpm	counts per minute
CPS	carbamyl-phosphate synthetase I
CTP	cytidine triphosphate

Da	dalton
dATP	deoxyadenosine triphosphate
ddH_2O	distilled deionized H_2O
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DR(x)	direct repeats of TGACCT motifs with a spacing of x nucleotides
dsDNA	double-stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift analysis
ER	estrogen receptor
GAD	GAL4 activation domain
GBD	GAL4 DNA binding domain
GST	glutathione S-transferase
GTP	guanosine triphosphate
h	hour(s)
HBSS	hepes-buffered saline solution
HD	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HNF-4	hepatocyte nuclear factor-4
HRE	hormone response element

IPTG	isopropylthio-β-D-galactoside
kbp	kilobase pair
LXR	liver X receptor
M	molar
MCS	multiple cloning site
min	minute(s)
mol	mole(s)
M_r	relative molecular mass
mRNA	messenger RNA
N-CoR	nuclear receptor corepressor
N-CoA	nuclear receptor coactivator
NP40	Nonidet P-40
nt	nucleotide(s)
NTP	nucleoside triphosphate(s)
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
p/CAF	p300/CBP associated factor
p/CIP	p300/CBP interacting protein
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
pol II	RNA polymerase II

PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-responsive element
RAR	retinoic acid receptor
RIP140	receptor interacting protein 140
RNA	ribonucleic acid
RNase A	ribonuclease A
rRNA	ribosomal RNA
RXR	retinoic acid receptor
RXR	retinoid X receptor
s	second(s)
SDS	sodium dodecyl sulphate
SMRT	silencing mediator for retinoid/thyroid hormone receptors
SRC-1	steroid receptor coactivator 1
STAT1	signal transducer and activator of transcription 1
SV40	simian virus 40
TAF	TBP-associated factor(s)
TBP	TATA element binding protein
TE	10mM Tris-HCl, 1mM EDTA
TF	transcription factor
TR	thyroid hormone receptor
Tris	tris(hydroxymethyl)aminoethane
VDR	vitamin D receptor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

'Corpora non agunt nisi fixata'

- Paul Ehrlich

1. INTRODUCTION

The transcriptional regulation, both spatial and temporal, of thousands of genes in higher eukaryotes is essential for maintaining homeostasis. This precise regulation is accomplished with a limited complement of factors that are not only shared amongst related signalling pathways but also mediate the signalling of diverse effectors. To achieve the differences in regulation between all genes, the promoter region of each gene binds a unique subset of these factors to effect regulatory control. This subset of factors, both ubiquitous and specialized, that binds DNA and regulates the transcription of a gene, has been referred to as the "enhanceosome". Intrinsic to this model are the implications that these activators of transcription act in a synergistic manner and that the content of a gene's enhanceosome is dictated by the presence of specific DNA binding sites for each factor (Nakashima et al., 1999; reviewed in Carey, 1998).

The effect of the enhanceosome is to regulate the initiation of transcription by RNA polymerase II (pol II). Transcriptional initiation involves the recruitment and formation of a complex of the transcription factor IID (TFIID), transcription factor IIA (TFIIA) and the pol II holoenzyme bound to DNA at the transcriptional start site. Currently, two models describe the formation of this complex (reviewed in Greenblatt, 1997; and Pugh, 1996; Pugh, 1996). In the first model, based on results from higher eukaryotes, the transcription factor IID (TFIID) binds to the promoter start site, recognizing the TATA box, an A-T rich DNA sequence. Subsequently, transcription factor IIA (TFIIA) binds to the bound TFIID. Sequential recruitment of

transcription factor IIB (TFIIB), pol II, transcription factor IIE (TFIIE) and finally transcription factor IIH (TFIIH) completes the formation of a complex competent to initiate transcription.

Recent studies in yeast allude to an alternative sequence of events (reviewed in Greenblatt, 1997; and Pugh, 1996). These studies suggest that a preformed pol II holoenzyme (pol II plus the transcription factors TFIIA, TFIIB, TFIIE, TFIIF, TFIIH) may be directly recruited to the transcriptional start site. Since the recruitment of TFIID and TFIIA is important in higher eukaryotes, the complex of TFIID/TFIIA bound to DNA may recruit the pol II holoenzyme instead of the sequential recruitment of additional factors in higher eukaryotes.

Thus, the existence of a holoenzyme in yeast may be indicative of significant differences in the initiation of transcription in yeast and higher eukaryotes. However, the detection of an RNA polymerase II holoenzyme in higher eukaryotes that contains the requisite TFIID associated factors for initiation of transcription alludes to conceptual similarities between the initiation of transcription in yeast and mammalian cells (Ossipow et al., 1995).

The activation of transcription requires that a number of barriers to the initiation process must be overcome including those presented by chromatin structure and the recruitment of the general transcription factors/pol II to the promoter region (reviewed in Ptashne and Gann, 1997; Grunstein, 1997). Transcriptional activators are proposed to act by recruiting the transcriptional machinery to the site of transcriptional initiation. Indeed, the tethering of transcription factors to DNA is sufficient to recruit pol II and activate transcription. This localization is proposed to enable the transcription factors to compete

with histones for binding to DNA. Not surprisingly, the destabilization of chromatin structure represents an important regulatory mechanism in the regulation of transcription. In fact, the difference between recruitment of the transcriptional machinery and chromatin remodeling is difficult to distinguish with the findings that an associated factor of TFIID has the ability to acetylate histones, a modification that destabilizes chromatin (Mizzen et al., 1996).

1.1 THE LIGAND-DEPENDANT COMPONENTS OF THE ENHANCESOME

The viability of complex eukaryotes requires that gene regulation be coordinated amongst multiple cells, tissues and organs. To this end, higher eukaryotes have evolved the ability to regulate the expression of target genes with extracellular signals such as hormones. One strategy involves the binding of these signals directly to ligand-activated transcription factors known as nuclear receptors. Nuclear receptors comprise one of the largest families of transcriptional activators and are generally regulated by the binding of soluble extracellular ligands that enter cells by diffusion across the cell membrane. The complex of holo-nuclear receptors bound to their appurtenant DNA binding sites results in the activation of the transcription of target genes. Thus, the inclusion of the binding site of a nuclear receptor in a target gene's promoter confers transcriptional regulation by specific extracellular signals (reviewed in Tsai and O'Malley, 1994).

The research described in this thesis addresses the molecular mechanism of action of the nuclear receptor known as the peroxisome proliferator-activated receptor α (PPARα). PPARα is activated by agonists that include fatty acids, permitting the regulation of the transcription of genes involved in fatty acid metabolism in response to dietary status. Thus, the inclusion of PPARα binding sites in the promoter of target genes permits the coordination of gene regulation in response to the metabolic needs of an organism (Nakashima et al., 1999; reviewed in Limberger et al., 1996).

1.2 NUCLEAR RECEPTORS

1.2.1 Historical Perspective

The nuclear receptor superfamily is one of the largest known classes of transcription factors. As implied by the name, this family of proteins performs dual roles: that of a receptor for extracellular ligands and that of a transcription factor. The synthesis of these two roles affords organisms a means to maintain homeostasis by directly altering gene expression in response to extracellular signals.

While the existence of potent regulators of cellular processes such as steroids, retinoids, thyroid hormones and vitamin D_3 were known in the early 1900s, it was not until 1966 that the existence of soluble nuclear binding proteins for these ligands was demonstrated (Jensen et al., 1966). With the identification of hormonally responsive genes during the 1970s coupled with findings that these hormones were targeted to their tissues by high affinity receptors, the cloning and the characterization of the glucocorticoid receptor established for the first time that these two phenomena could be attributed to the same protein (Hollenberg et al., 1985; Yamamoto, 1985).

Over the past thirteen years, the number of identified nuclear receptors continues to grow. In fact, a survey completed in 1996 identified 63 different genes that encoded nuclear receptors in diverse organisms such as nematodes, arthropods and vertebrates (primates, rodents, amphibians, fish) (Laudet, 1997). Notable examples include the estrogen receptor (ER), the thyroid hormone receptor (TR), the retinoic acid receptor (RAR), the retinoid X receptor (RXR) whose agonists include estrogen, thyroid hormone, all-*trans* retinoic acid, 9-*cis* retinoid acid respectively(Greene et al., 1986; Green et al., 1986; Weinberger et al., 1986; Sap et al., 1986; Petkovich et al., 1987; Mangelsdorf et al.,

1990). Some examples of the cellular processes in which nuclear receptors play a role include growth, development, fatty-acid metabolism, bile acid synthesis, inflammation and atherosclerosis (Janowski et al., 1996; Devchand et al., 1996; Ricote et al., 1998; Tontonoz et al., 1998; Nagy et al., 1998). The potential for continued exponential growth in our understanding of nuclear receptors and their functions promises to continue in light of the number of predicted nuclear receptors (>200) in the nematode *Caenorhabditis elegans* for which neither a function has been attributed nor a vertebrate homologue identified (Sluder et al., 1999).

1.2.2 Architecture of a Nuclear Receptor

The nuclear receptor superfamily shares a conserved amino acid sequence homology that is composed of four individual domains: A/B; C; D and E/F (Fig. 1.1, *Panel A*) (reviewed in Limberger et al., 1996). These conserved domains mediate the various functions of nuclear receptors including DNA binding, ligand binding and transcriptional regulation. While the gross structure and function of a domain is similar between two given receptors, the determinants of functional specificity confer distinct biological activities to each receptor. For example, while the ligand binding domains of the estrogen receptor (ER), the thyroid hormone receptor (TR), the retinoid X receptor (RXRα), the peroxisome proliferator-activated receptor (PPAR) and the retinoic acid receptor (RAR) share a conserved structure and ability to bind ligand with the consequent activation of transcription, the specific ligand binding determinants of each receptor result in transcriptional activators that respond to distinct extracellular signals to effect different biological responses (Wurtz et al., 1996).

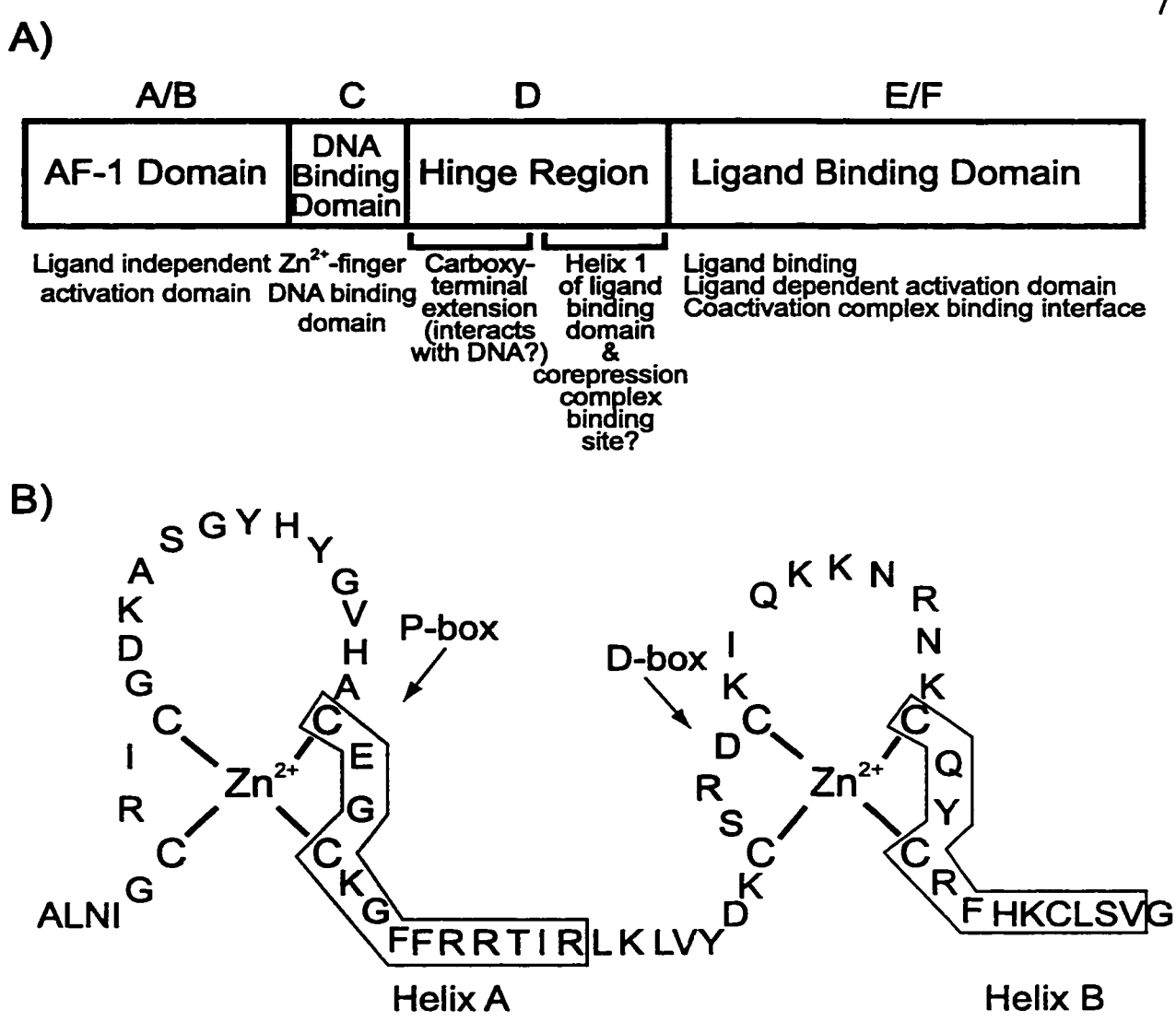


Figure 1.1: Schematic representation of the functional domains of mouse PPARa. Panel A. Schematic representation of the domains of the nuclear receptor PPARa and corresponding functions. Panel B. Schematic representation of the structural features of the DNA binding domain of mouse PPARa. The amino acids involved in forming the a-helices are boxed while the residues contained in the P- and D-boxes are shaded.

1.2.2.1 AF-1 Domain

Nuclear receptors contain two modular domains that are able to activate transcription, the N-terminal activation function-1 domain (AF-1) and the C-terminal AF-2 domain that is described in Section 1.2.1.4. The AF-1 or A/B domain (Fig. 1.1, *Panel A*) is the least conserved region amongst nuclear receptors and contains an intrinsic activation domain that appears to function independently of ligand binding (reviewed in Tsai and O'Malley, 1994). The biological relevance of this AF-1 activity is unknown, as is the regulation of this activity. However, recent studies have begun to characterize a cooperative relationship between the AF-1 domain and the AF-2 domain, a role in which the AF-1 domain potentiates the activity of the ligand-dependent activity of the AF-2 domain (refer to Section 1.2.4.4) (Oñate et al., 1998; Shao et al., 1998).

1.2.2.2 DNA Binding Domain

The C domain or DNA binding domain is the most highly conserved region amongst all nuclear receptors (Fig. 1.1, *Panel A*). The DNA binding domains of all nuclear receptors contain 8 absolutely conserved cysteine residues (Laudet, 1997). These cysteines coordinate two Zn^{2+} atoms forming two Zn^{2+} finger structures that contain two perpendicular α-helices (Fig. 1.1, *Panel B*: Helix A and Helix B) (Rastinejad et al., 1995). Helix A encompasses a region spanning the 3rd and 4th cysteines of the first Zn^{2+} finger while Helix B spans the 7th and 8th cysteines of the second Zn^{2+} finger.

The sequence of the consensus DNA binding site of a nuclear receptor is TGACCT. However, since most nuclear receptors bind DNA as dimers, functional DNA binding sites are composed of two repeats of the TGACCT half-site separated by 0 to 5 intervening bases (reviewed in Mangelsdorf and Evans, 1995). The Helix A of the DNA

binding domain of each of the dimerized nuclear receptors lies in the major groove of the DNA and makes specific base contacts with the TGACCT half-site (Bourguet et al., 1995). In fact the amino acids lying in this region comprise the P-box, a region biochemically implicated in determining the binding site specificity (reviewed in Zilliacus et al., 1995).

Since the DNA binding domains of nuclear receptors are sufficient to bind appurtenant binding sites as dimers, this domain must also contain dimerization determinants. In fact, biochemical and structural studies have identified the D-box, the amino acids which lie between the 5^{th} and 6^{th} cysteines of the second Zn^{2+} finger (Fig. 1.1, *Panel B*), as the region that contains residues that make important contacts between DNA binding partners (Rastinejad et al., 1995; Zilliacus et al., 1995). Of note is the fact that the ligand binding domain also contains additional dimerization determinants (refer to Section 1.2.2.4).

1.2.2.3 Hinge Region

The D domain or hinge domain, forms a link between the DNA binding domain and the E domain or ligand binding domain (Fig. 1.1, *Panel A*). This domain has been implicated in the binding of transcriptional repressors (refer to Section 1.3.3.1) and the formation of additional interactions with the DNA binding site.

The hinge region of nuclear receptors was originally identified as a linker region between the minimal DNA binding domain and the minimal ligand binding domain. In fact, with more detailed biochemical analysis and the determination of the structure of the RXR/TR dimer bound to DNA, the N-terminal portion of the hinge region appears to contain an α-helical region referred to as the carboxy-terminal extension (CTE) of the

DNA binding domain (reviewed in Rastinejad et al., 1995). This region contains the T-box and the A-box, motifs important in the DNA binding properties of receptors that bind to DNA as monomers. In fact, the CTE of TR makes contacts with the DNA just outside of the core DNA binding site identifying an additional DNA binding region in nuclear receptors (Rastinejad et al., 1995).

The structural determination of the ligand binding domains of TR, RAR and RXRα have led to the classification of the carboxyl portion of the hinge region as the first of 12 α-helical regions that comprise the ligand binding domain (Wurtz et al., 1996; Renaud et al., 1995; Bourguet et al., 1995; Wagner et al., 1995). Helix 1 is an integral structural component of the ligand binding domain. In fact, in the structures of the ligand binding domains of RXR and RAR, Helix 1 makes specific contacts with Helix 3, Helix 5 and Helix 8 (Wurtz et al., 1996).

It has become clear that the hinge region of nuclear receptors is not just a link between the ligand binding and DNA binding domains. This region contributes to both DNA binding and structural integrity/regulation of the ligand binding domain. It is tempting to speculate about the functional importance of the proximity of the CTE and Helix 1 and the potential for allosteric interactions between the DNA binding domain and the ligand binding domain. However, clearly the hinge region should be partitioned between the DNA binding domain and the ligand binding domain to better reflect the molecular resolution of our understanding of the functional domains of nuclear receptors.

1.2.2.4 Ligand Binding Domain

The C-terminal ligand binding domain of nuclear receptors serves two primary functions: the binding of ligands and the consequent activation of transcription (Fig. 1.1,

Panel A). The recent determination of the structures of the ligand binding domains of PPAR, RXR, RAR, ER and TR demonstrate that all three share a common fold of an antiparallel α-helical 'sandwich' composed of three layers of α-helices (Nolte et al., 1998; Uppenberg et al., 1998; Wagner et al., 1995; Renaud et al., 1995; Bourguet et al., 1995; Brzozowski et al., 1998). A comparison of the ligand binding domain structures reveals that this region is composed of 12 conserved α-helical regions (reviewed in Wurtz et al., 1996). In fact, sequence alignment of the ligand binding domains of representative nuclear receptors has led to the proposal that the α-helical 'sandwich' fold is conserved amongst nuclear receptors (Wurtz et al., 1996).

The extreme C-terminus of the ligand binding domain has been identified as essential for ligand-dependent activation and as such termed the activation function-2 domain (AF-2) (Fig. 1.2). The AF-2 domain is located in Helix 12 and is comprised of a highly conserved motif of ΦΦXEΦΦ (where Φ represents a hydrophobic residue; LLQEIY in mouse PPARα) (Danielian et al., 1992; Wurtz et al., 1996). It is proposed that ligand-dependent activation involves the formation of a novel binding interface upon the binding of ligand in which the presence and integrity of the AF-2 domain is absolutely required. Furthermore, a novel class of proteins termed coactivators has been identified that interacts with the holo-ligand binding domains of nuclear receptors to mediate/effect the activation of transcription. These coactivators require the presence and integrity of the AF-2 domain, suggesting that they recognize and bind to a surface formed upon the rearrangement of the AF-2 domain (reviewed in Shibata et al., 1997).

Thus, the AF-2 domain plays an integral role in two events: an agonist-dependent conformational change in the ligand binding domain and the subsequent creation of a

coactivator binding surface (Fig. 1.2). The determination of the structures of the holo-ligand binding domains of TR, RAR, PPAR and ER demonstrate the integral role that the AF-2 domain plays in both of these events (Nolte et al., 1998; Uppenberg et al., 1998; Shiau et al., 1998; Wagner et al., 1995; Renaud et al., 1995; Brzozowski et al., 1998). In these structures, agonist binds in a pocket of the ligand binding domain and the α-helical AF-2 domain caps this pocket. The interactions that stabilize the position of the AF-2 domain over the ligand binding pocket involve conserved hydrophobic residues of the AF-2 domain (Brzozowski et al., 1998; Renaud et al., 1995; Nolte et al., 1998; Shiau et al., 1998). Since the structures of the apo-ligand binding domains of RXR, RAR and ER reveal that the AF-2 domain is not positioned over the ligand binding pocket, it is proposed that the positioning of the AF-2 domain over the ligand binding pocket is the critical conformational change required for the recruitment of coactivators and the subsequent activation of transcription (Fig. 1.2) (Nolte et al., 1998; Renaud et al., 1995; Bourguet et al., 1995).

The structure of holo-PPARγ and holo-ER bound to the minimal nuclear receptor binding domain of a coactivator illustrates the role the AF-2 domain plays in forming the coactivator binding interface upon the binding of agonist (Nolte et al., 1998; Shiau et al., 1998). Upon agonist binding, not only does the AF-2 domain cap the ligand binding pocket, but it also contributes to the formation of the surface to which the coactivator binds along with Helices 3, 4 and 5. In fact, the 1st hydrophobic residue and the conserved Glu of the AF-2 domain (consensus sequence: $\underline{\Phi}\Phi X\underline{E}\Phi\Phi$) make stabilizing contacts with the coactivator. The role these residues play in coactivator binding alludes

to their absolute requirement for agonist-activated transcriptional activation by nuclear receptors (Danielian et al., 1992).

Of considerable debate, however, is the question of the position of the AF-2 domain in the apo-ligand binding domain. While the structures of the apo-ligand binding domains of RAR, RXR, TR and ER demonstrate variable positioning of the AF-2 domain including projection into solution and contact with crystallographic partners, none of these structures depict the AF-2 domain occupying the active conformation (Wagner et al., 1995; Bourguet et al., 1995; Tanenbaum et al., 1998; Brzozowski et al., 1998; Nolte et al., 1998; Renaud et al., 1995). Interestingly, the structure of the antagonist bound form of ER, presumably stabilized in the inactive conformation, depicts the AF-2 domain occupying the hydrophobic coactivator binding pocket formed by helices 3, 4 and 5 (Shiau et al., 1998; Brzozowski et al., 1998). Therefore, in the inactive state, the AF-2 domain may block the coactivator binding pocket.

In summary, the ligand binding domain may effect ligand dependent transcriptional activation according to the following model (Fig. 1.2). The binding of agonist results in the repositioning of the AF-2 domain over the ligand binding pocket. This rearrangement of the AF-2 domain results in the uncovering of a hydrophobic coactivator binding pocket and the positioning of the AF-2 domain such that it contributes to the formation of the coactivator binding surface.

The ligand binding domain of nuclear receptors is also involved in forming a dimerization interface between pairs of receptors. The findings in initial studies that identified amino acids that disrupted the binding interface have been tempered by realization that these residues are involved in forming critical contacts that stabilize the

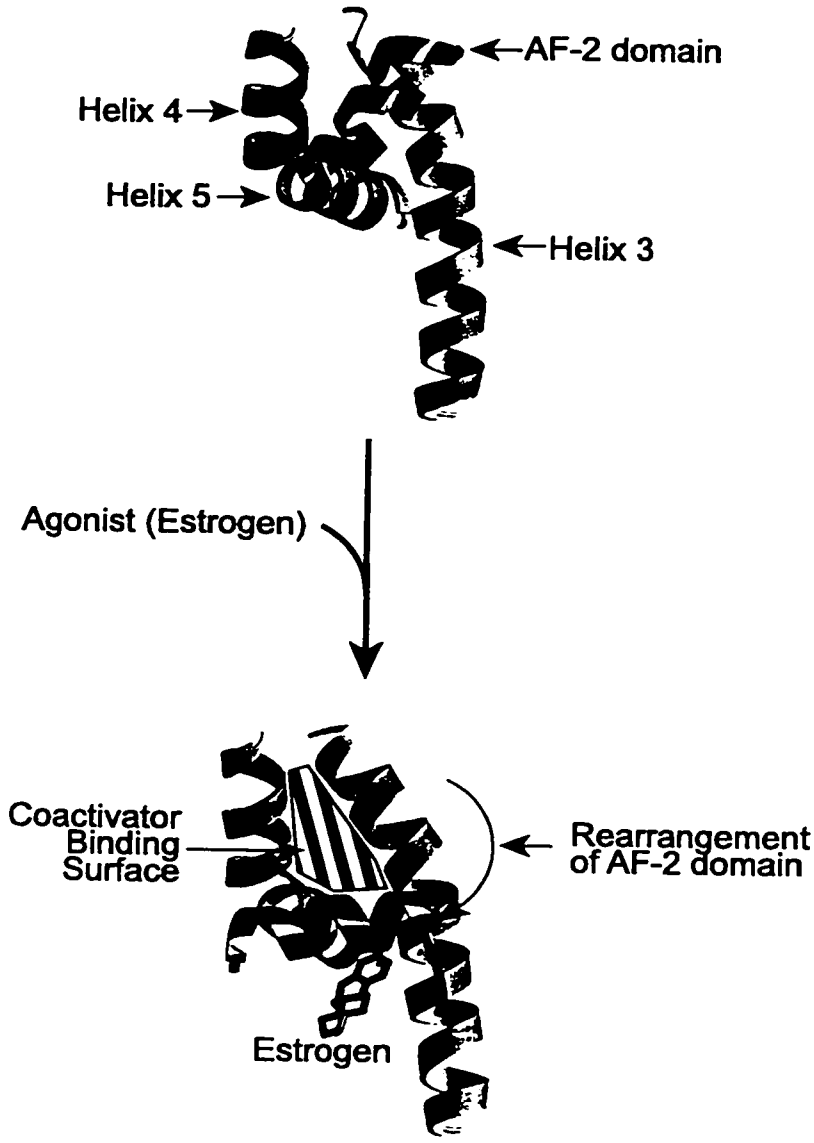


Figure 1.2: Molecular mechanism of ligand-dependent activation of nuclear receptors. Schematic model of the conformational changes induced upon agonist binding that result in the repositioning of the AF-2 domain to unmask/create the coactivator binding surface. The conformation of the inactive and active states are based upon the coordinates of the structures of the antagonist-bound and agonist-bound structures of the estrogen receptor respectively (Brzozowski et al., 1998).

core of the ligand binding domain. Thus, mutation of these residues probably resulted in perturbation of the structural integrity of the domain and only affected receptor dimerization indirectly (reviewed in Wurtz et al., 1996). The crystal structure of RXRα revealed that the ligand binding domains were present as dimers, forming contacts between Helices 10 (Bourguet et al., 1995). Interestingly, Helix 10 contains residues conserved amongst nuclear receptors and is exposed on the surface of the ligand binding domain (Wurtz et al., 1996). In fact, a point mutant of PPARα in the putative Helix 10 region results in a loss of the ability to interact with RXRα (Juge-Aubry et al., 1995). Furthermore, a construct of RXRα in which the C-terminal half of Helix 10 is deleted, is unable to dimerize with PPARα *in vitro* (Dowell et al., 1997b). Taken together, these results suggest that Helix 10 may be involved in the formation of a dimerization interface between nuclear receptors.

1.2.3 Mechanisms of Transcriptional Activation by Nuclear Receptors

In vitro studies have demonstrated that nuclear receptors stabilize the formation of the transcriptional initiation complex (reviewed in Shibata et al., 1997; and Bagchi et al., 1992). Since a number of nuclear receptors interact *in vitro* with transcription factors such as the TATA binding protein (TBP, a subunit of TFIID) and TFIIB, nuclear receptors may stabilize the transcriptional initiation complex and activate transcription by recruiting these factors (Ing et al., 1992; Baniahmad et al., 1993; Blanco et al., 1995; Ingles et al., 1991).

Our understanding of nuclear receptor mediated transcriptional activation has been greatly furthered by the identification of proteins that interact with nuclear receptors to mediate their function (Hörlein et al., 1995; Chen and Evans, 1995; Oñate et al., 1995).

In fact, the association with these proteins is dictated by the binding of agonist (Fig. 1.3). In the absence of agonist, a complex of proteins associates with the DNA bound receptor and actively represses transcription (Hörlein et al., 1995). Upon the binding of ligand to the nuclear receptor, this corepressor complex, dissociates from the holo-nuclear receptor (Hörlein et al., 1995). The ligand bound nuclear receptor then recruits 'coactivator' proteins to mediate transcriptional activation (Oñate et al., 1995). Interestingly, the corepression complex includes proteins that have histone deacetylase activity while many coactivators have histone acetylase activity (Heinzel et al., 1997; Alland et al., 1997; Nagy et al., 1997; Spencer et al., 1997; Bannister and Kouzarides, 1996). In fact, the binding of the corepression complex to TR bound to DNA is required for full repression of the target gene and the histone acetylase activity of the coactivation complex is essential for transcriptional activation (Hörlein et al., 1995; Korzus et al., 1998).

The correlation between the recruitment of histone acetylation/deacetylation activities and transcriptional activation/repression by nuclear receptors, strongly suggests that chromatin remodeling is an integral part of transcriptional regulation by nuclear receptors. However, the *in vivo* modification of histones by these complexes has not directly been determined (Pazin and Kadonaga, 1997). While the histone acetyltransferase activity is required for transcriptional activation by nuclear receptors, the disruption of chromatin, presumably by acetylation of histones is not sufficient for transcriptional activation (Wong et al., 1997). The ability of TR to disrupt chromatin requires the presence of ligand and an intact AF-2 domain and is not linked to processive transcription (Wong et al., 1997). This would further suggest that the histone acetyltransferase activity in the ligand recruited coactivation complex is responsible for

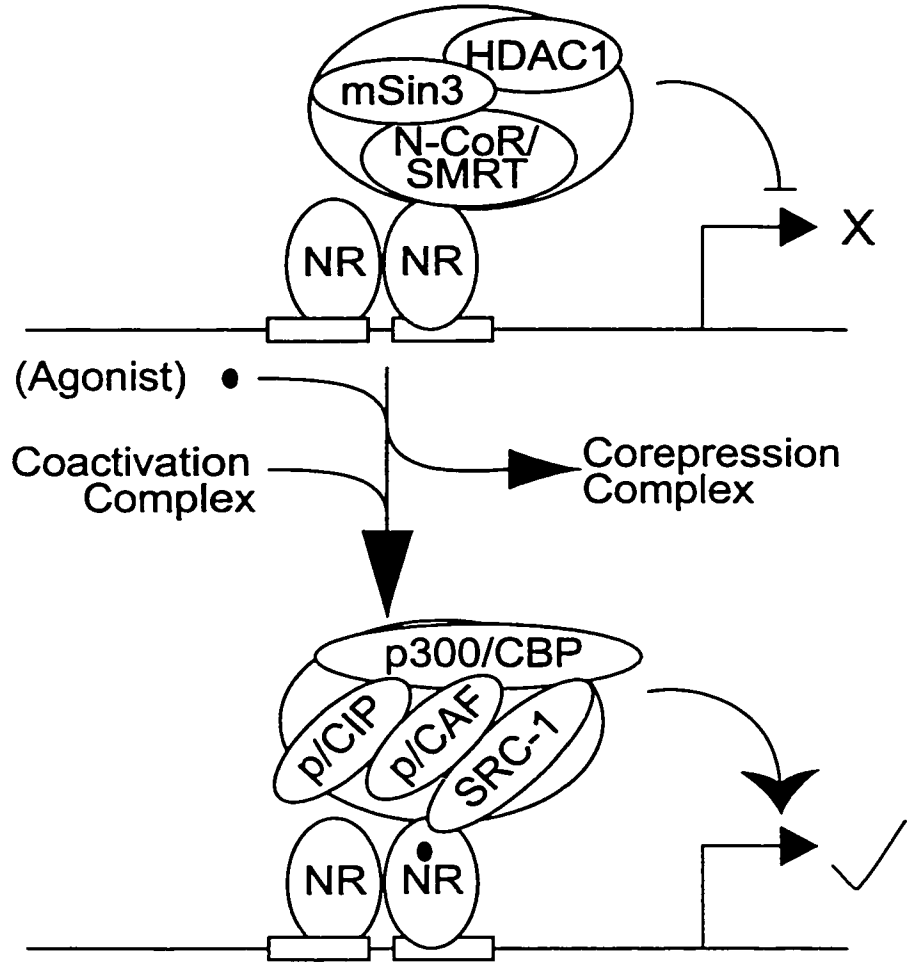


Figure 1.3: Current model of transcriptional activation by nuclear receptors.
This schematic depicts the current model of transcriptional regulation by nuclear receptors. In the absence of agonist, the receptors are bound to DNA in the inactive conformation and are complexed to the corepression complex. The corepression complex is composed of N-CoR, SMRT, mammalian Sin3 homologue (mSin3) and histone deacetylase 1 (HDAC1). The histone deacetylase activity is attributed to the maintenance of chromatin structure and repression of transcription. The ligand binding domain undergoes a conformational change that concomitantly displaces the corepression complex and creates a coactivator binding surface. The binding of agonist (•) stabilizes the active conformation of the ligand binding domain and the subsequently recruited coactivation complex which is composed of factors with histone deacetylase activity such as steroid receptor coactivator-1 (SRC-1), p300/CPB associated factor (p/CAF), p300/CBP interacting protein (p/CIP), and the cointegrator p300/CBP.

this disruption of chromatin. However, the possibility exists that the targets of the acetylase/deacetylase activities may be proteins other than histones. In fact, the basal transcription factors TFIIF and the β subunit of $TAF_{II}E$ have been shown to be substrates *in vitro* (Imhof et al., 1997).

A recent study attempted to differentiate between the roles played by the coactivators and the nuclear receptor ER in the initiation of transcription and subsequent rounds of reinitiation (Kraus and Kadonga, 1998). It was demonstrated that ER and the recruited coactivator acted cooperatively to promote productive initiation of transcription that includes formation of the preinitiation complex, initiation of RNA synthesis and promoter clearance. While the ER promoted subsequent rounds of transcription, the coactivator had no effect. Thus, nuclear receptors and coactivators play distinct roles in the processes of the initiation of transcription and the reinitiation of subsequent rounds of transcription.

1.2.3.1 Cooperativity between AF-1/AF-2 Activation Domains

Recent studies have demonstrated that the N-terminal AF-1 domain and the C-terminal AF-2 domain act cooperatively to activate transcription in response to ligand. The phosphorylation status of the AF-1 domain of PPARγ was demonstrated to decrease the affinity of the ligand binding domain for agonist and to impair agonist-dependent transcriptional activation (Shao et al., 1998). This effect was not mediated by the binding of the AF-1 domain to the coactivation complex, nor by the interaction of the AF-1 domain with the ligand binding domain leading to the proposal that the regulatory mechanism was intramolecular. However, a recent study demonstrated that distinct regions of CBP interacted with the AF-1 and AF-2 regions of PPARγ lead to the

hypothesis that the cooperativity between the two activation domains is a result of multiple interactions with the CBP/coactivation complex (Gelman et al., 1999). In support of an interaction between the AF-1 domain and coactivators, the progesterone receptor was documented to interact with the coactivation complex through this region (Oñate et al., 1998). This interaction potentiated both AF-1 and AF-2 mediated signalling. Furthermore, the presence of both domains cooperatively increased the transcriptional activation by the progesterone receptor (Oñate et al., 1998). While these studies suggest that transcriptional activation mediated by the coactivation complex involves the cooperative action of both nuclear receptor activation domains, the mechanism by which this occurs remains unclear.

1.2.4 Coactivators and Corepressors of Nuclear Receptors

The existence of proteins whose interaction with nuclear receptors is dependent upon the presence or absence of agonist has been documented for a number of years (Eggert et al., 1995; Fondell et al., 1996; Hlachmi et al., 1994; Cavaillès et al., 1994; Puigserver et al., 1998; Casanova et al., 1994). Recently, a growing number of these proteins have been identified and characterized (reviewed in Shibata et al., 1997). Essentially, they comprise two multiprotein complexes that appear to mediate the repressive and activating effects of nuclear receptors. The corepression complex binds to DNA-bound nuclear receptors in the absence of agonist and actively represses transcription by possibly stabilizing chromatin. The action of agonist binding results in the displacement of the corepressor complex and recruitment of the coactivation complex (Fig. 1.3). Currently, the presence of at least four coactivators, all able to acetylate histones, is required in a functional coactivation complex (Korzus et al., 1998).

1.2.4.1 Corepression Complex

In the absence of agonist, nuclear receptors such as RAR and TR, actively repress transcription of target genes (Shibata et al., 1997). Three proteins have been identified that interact with these DNA bound receptors and mediate their repressive effects (Fig. 1.3). These proteins, termed corepressors, include N-CoR and SMART and the recently discovered SUN-CoR (small unique nuclear receptor corepressor) (Glass et al., 1997; Heery et al., 1997; Zamir et al., 1997a). The mechanism of repression of both N-CoR and SMRT appears to be through the recruitment of a complex of proteins that includes mammalian Sin3 orthologues (mSin3) and most importantly histone deacetylase 1 (HDAC1) (Nagy et al., 1997; Alland et al., 1997; Heinzel et al., 1997). The presence of

HDAC1 in this corepression complex alludes to chromatin stabilization as a possible mechanism of the active repression by these DNA-bound receptors. While SUN-CoR contains an intrinsic transcriptional repression activity, it is unknown if this corepressor acts as an alternative to N-CoR/SMRT or as a component of the corepression complex (Zamir et al., 1997a).

The recent discovery that N-CoR interacts with TFIIB, and the TATA binding protein associated factors $TAF_{II}32$ and $TAF_{II}70$, suggests that N-CoR interacts with basal transcription factors to inhibit transcription. In fact, N-CoR disrupted the functional interaction between TFIIB and $TAF_{II}32$ that is required for initiation of transcription. Thus, it appears that N-CoR inhibits transcription by not only stabilizing chromatin but also through interactions with components of the transcriptional initiation complex (Muscat et al., 1998).

Helix 1 of the ligand binding domain of TR has been proposed to be the binding site of N-CoR and SMRT (Chen and Evans, 1995; Hörlein et al., 1995; Wagner et al., 1995). This region is conserved in both TR and RAR and was named the CoR box (Wagner et al., 1995). However, the residues in the CoR box that were identified as essential for N-CoR binding are actually involved in forming contacts between Helix 1 and the core of the ligand binding domain (Hörlein et al., 1995; Kurokawa et al., 1995; Wurtz et al., 1996). While these residues do not interact directly with N-CoR, this does not exclude Helix 1 from playing an essential role in the formation of a corepressor binding. (Wurtz et al., 1996) In fact, the protein L7/SPA was discovered to interact with this region and shown to disrupt the repressive activity of both N-CoR and SMRT, possibly by competing with corepressors for binding to nuclear receptors (Jackson et al.,

1997). Furthermore, peptides that overlap this region of TR are effective in inhibiting the repression of transcription mediated by corepressors, presumably by competing for binding to corepressors (Baniahmad et al., 1995; Tong et al., 1996; Zhang et al., 1998).

Interestingly, the DNA binding site can affect whether the corepressor is released upon agonist binding. The complex of RAR/RXR can bind to direct repeats of the TGACCT half site separated by a spacing of 1 and of 4 nucleotides, DR1 and DR4 response elements respectively. Although this complex bound to a DR4 response element activates transcription in response to the RAR agonist all-*trans* retinoic acid, this complex bound to a DR1 response element is unresponsive to all-*trans* retinoic acid. In fact, the binding of ligand displaces the corepression complex bound to RAR/RXR on a DR4 response element but the corepression complex cannot be dislodged by agonist when RAR/RXR is bound to a DR1 element (Kurokawa et al., 1995). Therefore, the DNA binding site allosterically regulates the ability of the bound nuclear receptor to activate/repress transcription of target genes in response to ligand.

1.2.4.2 Coactivation Complex

If the coactivation complex that RAR recruits is typical of nuclear receptors, this complex is composed of at least four coactivators (Fig. 1.3) (Korzus et al., 1998). These factors include the steroid receptor coactivator-1 (SRC-1), CREB binding protein (CBP), p300/CBP associated factor (p/CAF) and p300/CBP interacting protein (p/CIP).

CBP and its homologue p300 are referred to as cointegrators since they are essential coactivators of a number of disparate transcriptional activators including signal transduction and inducer of transcription-1 (STAT-1), AP-1 (Fos/Jun complex) and the cAMP response element binding protein (CREB) in addition to nuclear receptors

(Chakravarti et al., 1996; Kamei et al., 1996; Chrivia et al., 1993; Hanstein et al., 1996). p300/CBP has an intrinsic histone acetylase activity, that while required for transcriptional activation by CREB and STAT-1, is not essential for nuclear receptor mediated signalling (Bannister and Kouzarides, 1996; Korzus et al., 1998). Since p300/CBP interacts with SRC-1, p/CIP and p/CAF, p300/CBP is hypothesized to act as a 'platform' for the binding of the requisite complement of coactivators to mediate signalling by a transcriptional activator (Torchia et al., 1997; Yao et al., 1996; Hanstein et al., 1996; Smith et al., 1996). While p300 and CBP display a great deal of functional overlap, recent evidence suggests that some signalling pathways are uniquely mediated by either p300 or CBP (Kawasaki et al., 1998).

SRC-1, also referred to as F-SRC-1, GRIP1 and TIF2, was isolated as a ligand-dependent nuclear receptor interacting protein (Oñate et al., 1995; Kamei et al., 1996; Takeshita et al., 1996; Hong et al., 1997; Voegel et al., 1996). As with p300/CBP, SRC-1 contains an intrinsic histone acetylase activity, although this activity is not essential for RAR mediated signalling (Spencer et al., 1997; Korzus et al., 1998). This coactivator appears to be unique to the coactivation complex recruited by nuclear receptors and not required or utilized by CREB, AP-1 or STAT-1 (Korzus et al., 1998).

p/CIP and p/CAF were both isolated as CBP interacting proteins (Yang et al., 1996; Torchia et al., 1997; Anzick et al., 1997; Chen et al., 1997; Takeshita et al., 1997; Li et al., 1997). As with CBP and SRC-1, p/CAF and p/CIP have an intrinsic histone acetylase activity (Yang et al., 1996; Torchia et al., 1997; Chen et al., 1997). While the histone acetylase activity of p/CIP is not required for RAR mediated activation, the activity of p/CAF is essential (Korzus et al., 1998). The fact that the loss of histone

acetylase activity abrogates transcriptional activation signifies the important role that this activity plays in the activation of transcription by nuclear receptors.

The potential for understanding the role p/CAF plays in the coactivation complex has been greatly furthered by two studies. The first describes the binding of p/CAF to the DNA binding domain of RAR in the absence of an intact AF-2 domain (Blanco et al., 1998; Korzus et al., 1998). The second study discovered that p/CAF exists in a complex of at least 20 polypeptides that included a number of TATA binding protein associated factors such as $TAF_{II}31$, $TAF_{II}30$, and $TAF_{II}20$ (Ogryzko et al., 1998). These findings increase the potential number of polypeptides contained in the coactivation complex and indicates that transcriptional activation by nuclear receptors not only involves chromatin remodeling but also interactions with components of the basal transcription machinery. Taken together, these studies have begun to differentiate the role of p/CAF in the coactivation complex that is distinct from that of both SRC-1 and p300/CBP.

1.2.4.3 Recruitment of the Coactivation Complex

SRC-1, p300/CBP, p/CIP and p/CAF demonstrate an agonist-dependent interaction with nuclear receptors (Chakravarti et al., 1996; Dowell et al., 1997a; Oñate et al., 1995; Korzus et al., 1998; Torchia et al., 1997). This interaction requires the presence and integrity of the AF-2 domain and is postulated that coactivators recognize the novel binding surface bounded by Helix 3, Helix 5 and the repositioning of the AF-2 domain upon agonist binding (Fig. 1.2) (Voegel et al., 1996; Kamei et al., 1996; Korzus et al., 1998; Torchia et al., 1997; Brzozowski et al., 1998; Feng et al., 1998). The minimal coactivator binding determinant that interacts with agonist-bound nuclear receptors is a

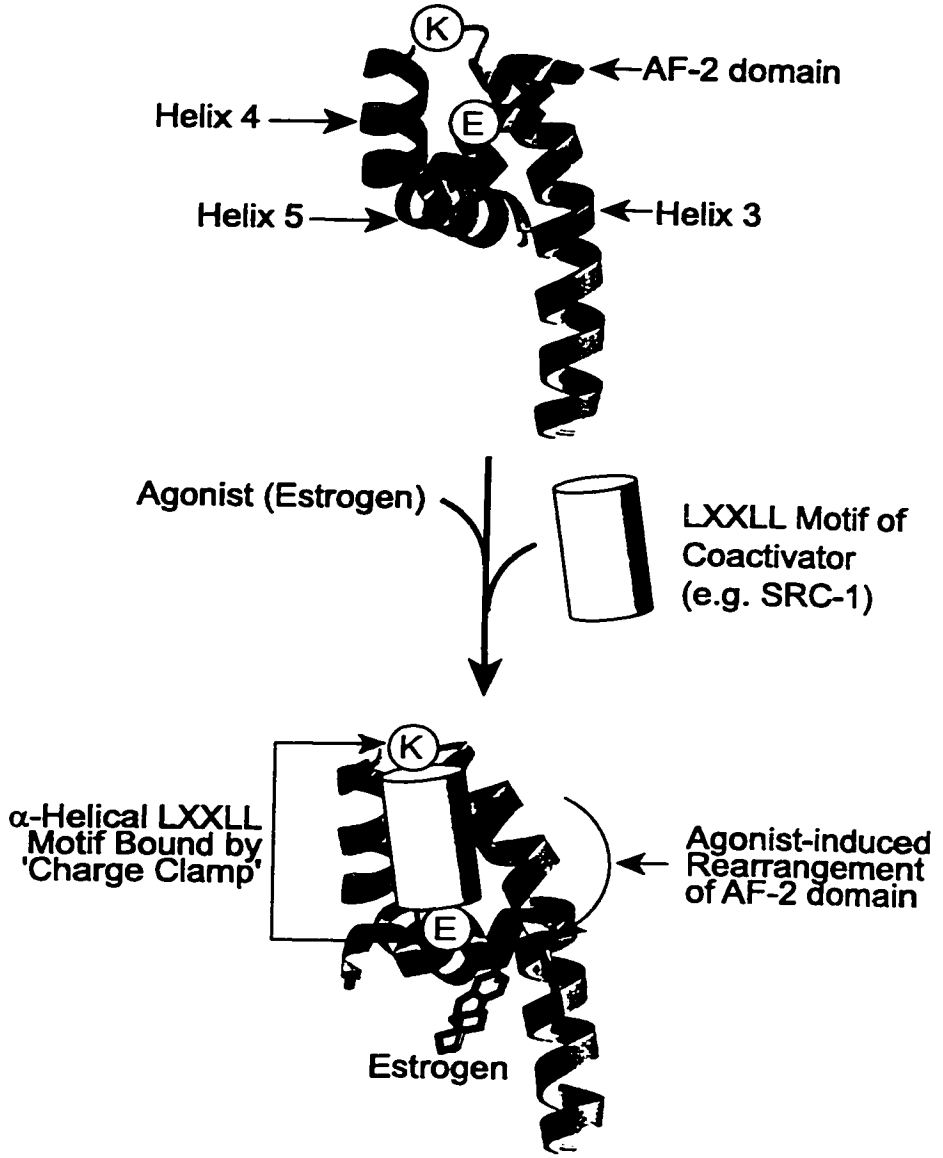


Figure 1.4: Molecular mechanism of ligand-dependent recruitment of coactivators. Schematic model of the binding of the LXXLL motif of a coactivator to the holo-ligand binding domain of a nuclear receptor. The conformational changes induced upon agonist binding result in the repositioning of the AF-2 domain creating a binding surface that binds the α-helical LXXLL motif of the coactivator SRC-1. The binding interface is composed primarily of hydrophobic contacts and the 'charge clamp' formed by the Glu and Lys, residues that are conserved amongst nuclear receptors. The conformation of the inactive and active states are based upon the coordinates of the structures of the antagonist-bound and agonist-bound structures of the estrogen receptor respectively while the position of the bound α-helical LXXLL motif is inferred from the structure of the holo-ligand binding domain of PPARγ bound to the LXXLL motif of SRC-1 (Brzozowski et al., 1998; Nolte et al., 1998).

leucine rich motifs of the sequence LXXLL (Heery et al., 1997). In fact, this motif, termed the NR box, was sufficient to mediate the interaction with holo-ligand binding domains. Mutation of the three motifs present in SRC-1 resulted in a complete loss of activation by nuclear receptors (Heery et al., 1997). While the NR box motif (LXXLL) is required for interaction with the holo-ligand binding domains of nuclear receptors, the amino acid sequences that flank these motifs alter the binding specificities of each nuclear receptor interacting domain (Ding et al., 1998; Leers et al., 1998; McInerney et al., 1998; Hong et al., 1999; Eng et al., 1999). Even though, all four of the known coactivators are capable of interacting with nuclear receptors in a ligand dependent manner, albeit with varying affinities, the significance of the existence of multiple sites of interaction within the coactivation complex remains unresolved.

The recently determined structure of the holo-ligand binding domain of PPARγ and ER bound to the LXXLL motif from SRC-1 supports the proposed mechanism of agonist-dependent recruitment of coactivators (Fig. 1.2) (Nolte et al., 1998; Shiau et al., 1998). Firstly, the LXXLL motif forms an α-helix that binds in the proposed binding pocket formed by Helix 3, Helix 4, Helix 5 and the repositioned AF-2 domain. Secondly, the bound α-helical LXXLL motif interacts with a 'charge clamp' composed of the conserved Glu of the AF-2 domain and a Lys located in Helix 3 that is highly conserved amongst nuclear receptors (Nolte et al., 1998; Shiau et al., 1998). The fact that agonist-activated nuclear receptors interact with LXXLL motifs, share structural homology and conserved key residues involved in LXXLL motif binding suggest that the structure of the LXXLL motif of SRC-1 bound to PPARγ and ER is indicative of a general

mechanism by which nuclear receptors recruit coactivators in response to agonist-binding (Heery et al., 1997; Nolte et al., 1998; Shiau et al., 1998).

1.2.4.4 Miscellaneous Nuclear Receptor Interacting Factors

A number of proteins have been identified that interact with nuclear receptors and lack a clearly defined function (reviewed in Shibata et al., 1997). Even though some of these factors, such as the receptor interacting protein 140 (RIP140), interact with nuclear receptors in a ligand dependent manner that requires the AF-2 core activating domain and LXXLL motifs, their function is difficult to define (Cavaillès et al., 1995). Furthermore, an RNA component of the coactivation complex has been identified (Lanz et al., 1999). While this molecule potentiates nuclear receptor-mediated transcription, the mechanism of this unexpected coactivator molecule remains to be characterized. The number of these proteins/molecules without a defined function alludes to the potential for discovery of additional components/functions of the coactivator/corepressor complexes and even novel regulatory mechanisms of nuclear receptor signalling.

1.3 THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR α

A historical treatment of the peroxisome proliferator-activated receptor (PPAR) will serve to illustrate the origins of the nomenclature and acronyms used in this field. When PPARα was first cloned in 1990, it was named for its ability to be activated by a class of compounds known as peroxisome proliferators (Issemann and Green, 1990). This class of structurally diverse compounds were grouped according to their ability to induce the proliferation of peroxisomes in the liver and the cortex of kidneys in rodents (reviewed in Reddy and Warren, 1982). Examples of such compounds include hypolipidemic agents, industrial plasticizers and even dietary status (high fat diet, starvation).

The induction of peroxisomes, cellular organelles defined by the presence of an oxidase and a catalase that generate and decompose hydrogen peroxide, was proposed to enhance the metabolism of fatty acids. In fact, many of the characterized peroxisomal enzymes are involved in either the catabolism or anabolism of fatty acids (reviewed in Lazarow and Fujiki, 1985), including a fatty acid β-oxidation pathway that is distinct from that of the mitochondria, and induced by peroxisome proliferators (Lazarow and deDuve, 1976). The enzymes of this pathway include the acyl-CoA oxidase, the hydratase/dehydrogenase bifunctional enzyme and a ketoacyl-CoA thiolase (Osumi et al., 1987; Ishii et al., 1987; Hijikata et al., 1990; Bodnar and Rachubinski, 1991). The increase of β-oxidation activity by peroxisome proliferators was attributed to the induction of the transcription of these genes (Lazarow et al., 1982; Furuta et al., 1982; Reddy et al., 1986). Eventually, DNA elements that mediate the activation by peroxisome proliferators were identified in the promoter regions of the acyl-CoA oxidase

gene (AOx) and the hydratase/dehydrogenase bifunctional enzyme gene (HD) (Zhang et al., 1992; Dreyer et al., 1992; Osumi et al., 1991). As such, these DNA elements were termed peroxisome proliferator-responsive elements (PPRE) and interestingly, contained consensus binding sites for nuclear receptors. The coincident cloning of PPARα enabled the demonstration that this nuclear receptor mediated the effects of peroxisome proliferators through these *cis*-acting DNA elements (Issemann and Green, 1990). Since the AOx PPRE and the HD PPRE were the first PPREs to be cloned, they have become the prototypical DNA binding sites of PPARα and are utilized in many model systems.

Over the past seven years, the PPAR family has not only grown to three members (α, β/δ, γ subtypes) but has also garnered increasing research attention; efforts that are well justified since PPARs have been identified as integral factors involved in fatty acid metabolism, adipogenesis, diabetes, inflammation and atherosclerosis (Limberger et al., 1996; Devchand et al., 1996; Ricote et al., 1998; Jiang et al., 1998; Tontonoz et al., 1994b; Tontonoz et al., 1998; Nagy et al., 1998; Lehmann et al., 1995).

1.3.1 The PPAR Family

The first PPAR to be cloned was the α subtype from mouse (Issemann and Green, 1990). Shortly thereafter, three PPARs were cloned from *Xenopus laevis*, the α, β and γ subtypes (Dreyer et al., 1992). To date, PPARs have been cloned from numerous vertebrates including mouse, rat, hamster, human, cattle and salmon (Zhu et al., 1993; Amri et al., 1995; Tontonoz et al., 1994a; Issemann and Green, 1991; Chen et al., 1993; Schmidt et al., 1992; Sher et al., 1993; Jow and Mukherjee, 1995; Mukherjee et al., 1994; Mukherjee et al., 1997b; Xing et al., 1995; Aperlo et al., 1995; Yanase et al., 1997; Vidal-Puig et al., 1997; Kliewer et al., 1994; Ruyter et al., 1997; Sundvold et al., 1997). All

PPARs have been classified as belonging to one of the α, β/δ or γ subtypes. Unlike PPARα and PPARβ/δ, three different isoforms of PPARγ exist (Tontonoz et al., 1994a; Zhu et al., 1993; Fajas et al., 1998). PPARγ2 contains an additional 30 N-terminal amino acids compared to PPARγ1, the difference resulting from alternative promoter usage (Zhu et al., 1995). The PPARγ3 isoform mRNA encodes a protein indistinguishable from PPARγ1 and similar to PPARγ2, this mRNA arises from an independent promoter (Fajas et al., 1998). The δ subtype, has been tentatively grouped with the β subtype from *X. laevis* although they do not share a high degree of homology. However, neither a δ isoform has been identified in *X. laevis*, nor a β subtype in higher vertebrates indicating that they may be related. For the sake of simplicity, the δ and β subtypes will be referred to as PPARβ. Sequence comparison of the cloned PPARs demonstrates that the homology of the three subtypes of PPAR within the same organism is higher than the similarity between the same subtype in different organisms (reviewed in Limberger et al., 1996), making the confusion regarding the β/δ subtype understandable.

1.3.2 Cellular Localization of PPARs

Most nuclear receptors are constitutively localized to the nucleus with the exception of some whose translocation is triggered by the binding of ligand (Tsai and O'Malley, 1994). Immunocytochemical studies have revealed that PPARs are constitutively localized in the nucleus (Dreyer et al., 1993) but sequence analysis has not identified a definitive nuclear localization signal (Dingwall and Laskey, 1991; Schoonjans et al., 1996c). While PPARs appear to be constitutively localized to the nucleus, the presence of endogenous ligands that cause translocation cannot be discounted.

1.3.3 Tissue Distribution of PPARs

Each PPAR subtype has a specific tissue distribution pattern, including the two isoforms of PPARγ. Interestingly, the expression patterns overlap implying a coincidence of functions in the same tissue and permitting an interplay between the signals mediated by the different PPARs. PPARα is predominately expressed in liver, kidney, heart and brown adipose tissue (Issemann and Green, 1990; Dreyer et al., 1992; Mukherjee et al., 1994). The β subtype is ubiquitously expressed with mRNA transcripts detected in heart, brain, central nervous system, intestine, muscle, spleen, kidney, lung, adrenal gland with lower levels found in liver, white and brown adipose tissue, and testes (Schmidt et al., 1992; Dreyer et al., 1992; Amri et al., 1995; Kliewer et al., 1994; Cullingford et al., 1998). The transcript for PPARγ1 is found predominately in liver, kidney and heart while the PPARγ2 and PPARγ3 transcripts are found predominately in heart, colon and adipose tissue (Mukherjee et al., 1997b; Kliewer et al., 1994; Tontonoz et al., 1994a; Fajas et al., 1998). Recently, the PPARγ transcript has been detected in monocytes/macrophages (Tontonoz et al., 1998; Ricote et al., 1998).

1.3.4 Architecture of the Peroxisome Proliferator-activated Receptor

1.3.4.1 AF-1 Domain of PPAR

The highly divergent N-terminal region of nuclear receptors contains a ligand independent activation domain (reviewed in Limberger et al., 1996). Very little is known about the AF-1 domain of PPARα or PPARβ. As described later in this thesis, an activation activity attributed to this region of PPARα was detected in yeast and an N-terminal deletion of this region abolished this activity (Miyata et al., 1996). This deletion of PPARα was utilized in the yeast two-hybrid screen, an assay that requires a

transcriptionally inactive bait protein. In support of these findings, a survey of PPARα and PPARγ constructs used in yeast two hybrid screens, reveals that in all cases similar N-terminal deletions of PPARs were used (Puigserver et al., 1998; Zhu et al., 1996; Zhu et al., 1997; Dowell et al., 1997a; Mizukami and Taniguchi, 1997).

The N-terminal region of PPARγ has been shown to contain a ligand independent activation ability with the potency of this AF-1 activity differing between the γ1 and γ2 isoforms. Furthermore, insulin appears to stimulate this AF-1 activity (Werman et al., 1997). The AF-1 activity of this region is also affected by its phosphorylation status. MEK/mitogen-activated kinase was identified to phosphorylate the γ1 and γ2 isoforms at the same serine residue. Phosphorylation of this serine not only inhibited the AF-1 activity of this region, but also the ligand dependent activation activity of PPARγ (Camp and Tarfuri, 1997; Adams et al., 1997). The inhibitory effect of the phosphorylation status of this region on ligand-dependent activity appears to be effected by a consequent reduction in the affinity of the ligand-binding domain for agonist (Shao et al., 1998). Interestingly, this phosphorylation site is not conserved in the α and β PPAR subtypes, providing a subtype specific regulatory mechanism.

1.3.4.2 DNA Binding Domain of PPAR

Most nuclear receptors, bind DNA as dimers and the D-box, which lies between the 5th and 6th cysteines in the second zinc finger contains residues that make important contacts between DNA binding partners (Rastinejad et al., 1995; Zilliacus et al., 1995). Notably, the D-box of PPARs is unique in that it only contains 3 amino acids compared to the 5 found in most nuclear receptors (reviewed in Limberger et al., 1996). PPARα binds to DNA with the retinoid X receptor (RXRα) as a requisite binding partner. RXRα

also acts as the binding partner for the thyroid hormone receptor (TR) and the vitamin D_3 receptor (VDR). However, it appears that the PPAR/RXR dimer is bound to DNA in the opposite orientation compared to the TR/RXR and VDR/RXR dimers (Juge-Aubry et al., 1997; IJpenberg et al., 1997; DiRenzo et al., 1997). It is proposed that the unique nature of the PPAR D-box is responsible for the orientation of the PPAR/RXR dimer bound to DNA (Limberger et al., 1996).

1.3.4.3 Hinge Region of PPAR

The CTE of TR makes contacts with the DNA just outside of the core DNA binding site (Rastinejad et al., 1995). This includes not only contacts with the DNA backbone, but also a direct base contact between a lysine and the 3rd residue flanking the core DNA binding site. This lysine (**K***) lies in the motif **K***XKL which is conserved in all PPARs. A recent study has demonstrated that the minimal DNA binding domain of PPARγ requires the inclusion of this putative CTE (Hsu et al., 1998). While this study did not identify the homology between TR and PPARs in this region and the possible role that the conserved Lys may play, deletion analysis demonstrated that the region containing this Lys was required for DNA binding (Hsu et al., 1998). With the evidence that the nucleotides that flank the core DNA binding site of PPARα and PPARγ influence DNA binding, it is possible that the putative CTE of PPARα interacts with the nucleotides in this region and may even make specific base contacts similar to TR (Bourguet et al., 1995; Palmer et al., 1995; Juge-Aubry et al., 1997; IJpenberg et al., 1997). Alternatively, this region may simply be required for the structural integrity of the minimal DNA binding domain. Not withstanding, the determination of whether this

region in PPAR interacts with DNA could identify a potential mechanism by which the DNA binding site allosterically modulates the activity of a bound PPAR.

1.3.4.4 Ligand Binding Domain of PPAR

The presence of the region encompassing the putative Helix 12 and the conserved AF-2 domain, is required for the ligand dependent interaction between PPAR and coactivators (Dowell et al., 1997a; Zhu et al., 1997; Treuter et al., 1998; Schulman et al., 1998). However, only in the case of PPARγ has the presence of Helix 12 and the AF-2 core domain been shown to be required for ligand dependent transcriptional activation (Zhu et al., 1997; Zamir et al., 1997b). Interestingly, ligand binding does not require this region of PPARα, suggesting that while this region is required for the formation of a coactivator binding surface, it is not required for ligand binding (Dowell et al., 1997b).

1.3.5 PPAR Ligands

Upon cloning PPARα, it was realized that its tissue expression pattern correlated with the localization of the effects of peroxisome proliferators. This prompted the hypothesis that PPARα mediated the effects of these compounds in a similar manner to previously characterized nuclear receptors such as the estrogen receptor (Issemann and Green, 1990). In fact, peroxisome proliferators such as Wy 14,643, nafenopin, clofibrate and polyunsaturated fatty acids were potent activators of PPARα (Issemann and Green, 1990; Göttlicher et al., 1992; Keller et al., 1993). With the cloning of all three PPAR subtypes, it became apparent that each subtype had a unique but overlapping spectrum of activators (Krey et al., 1993). Further study revealed that eicosanoids and arachidonic acid derivatives also activated PPARs (Yu et al., 1995). Only recently have activators of PPARs been shown to be ligands. While the highest affinity ligands are synthetic

compounds, natural compounds have been shown to bind and activate PPARs. While most ligands have been identified through the determination of binding constants, functional assays have also been used to identify PPAR 'ligands'. It is important to distinguish the fact that while the former identifies PPAR ligands, the latter only identifies receptor agonists and not antagonists nor negative antagonists.

1.3.5.1 Ligand Binding Determinants of Holo-PPARγ

The recent determination of the structure of the ligand binding domain of PPARγ bound to the agonist BRL49653 reveals that agonist binds in a large hydrophobic pocket within the ligand binding domain (Nolte et al., 1998). Amongst the many contacts that the ligand binding domain makes with BRL49653, two key His residues coordinate binding of the carboxyl group of BRL49653. These interactions are proposed to be conserved with the broad spectrum of acidic compounds that act as PPARγ agonists including fatty acids and their derivatives.

1.3.5.2 Identification of Ligands by Classical Binding Studies

The first natural ligand of PPARα to be identified was leukotriene B_4, a chemoattractant of leukocytes involved in mediating the inflammatory response (Devchand et al., 1996). The binding of leukotriene B_4 was shown to be specific and the dissociation constant (K_d) was ~90 nM. Additionally, the ability of Wy14,643 to compete with leukotriene B_4 for binding to the ligand binding domain identified the potent peroxisome proliferator Wy14,643 as a PPARα ligand. Further studies have identified fatty acids such as linolenic, linoleic and arachidonic acid as PPARα ligands with K_d's in the micromolar range. Furthermore, the eicosanoids 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE), a lipoxygenase metabolite of arachidonic

acid, phytanic acid and 15d-$\Delta^{12,14}$-prostaglandin J_2 (15d-$\Delta^{12,14}$-PGJ_2) are PPARα ligands (Kliewer et al., 1997; Ellinghaus et al., 1999).

The only natural high affinity ligands of PPARγ to be identified to date are 15d-$\Delta^{12,}$14-PGJ_2, 9-hydroxyoctadecadienoic acid and 13-hydroxyoctadecadienoic acid. The affinity of 15d-$\Delta^{12,}$14-PGJ2 for PPARγ was determined to be a K_d of 325 nM (Forman et al., 1995; Nagy et al., 1998). Although this compound acts as an agonist for both α and γ PPAR subtypes, it is a more potent PPARγ agonist (Yu et al., 1995). The hydroxyoctadendienoic acids are oxidized metabolites of linoleic acid and components of the low density lipoprotein complex. While these compounds bound to and activated PPARγ, their K_d's and half-maximal effective concentrations were in the 10-20 μM range, leaving the biological relevance of these ligands in question (Nagy et al., 1998).

A number of synthetic compounds have been identified as high affinity PPAR ligands in addition to Wy 14,643 (Devchand et al., 1996). The thiazolidinedione BRL49653, a compound that increases the sensitivity of tissues to insulin, is a PPARγ ligand with a K_d of 43 nM (Lehmann et al., 1995). A novel fibrate GW 2433 has been identified as the first high affinity ligand of PPARβ with a K_d of 13 nM (Brown et al., 1997). In this same screen, another novel fibrate, GW2331 was identified as a high affinity ligand for both PPARα and PPARγ with K_d's of 140 nM and 300 nM respectively (Kliewer et al., 1997). The continued discovery of synthetic subtype specific ligands will enable the investigation of the biological consequences of the activation of each PPAR subtype.

1.3.5.3 Ligand Induced Conformational Changes

The model of nuclear receptor activation involves the binding of agonist in a pocket of the ligand binding domain. Ligand binding stabilizes the active conformation of the receptor in which Helix 12 is repositioned over the ligand binding pocket forming the coactivator interface (Wurtz et al., 1996). This conformational change can be detected as a change in the proteolytic degradation pattern of this region. A number of PPARα activators were assessed for the ability to induce a conformational change in the ligand binding domain. Wy 14,643, eicosatetraynoic acid, LY-171883 and clofibrate all induced conformational changes as detected by changes in the proteolytic degradation pattern of the ligand binding domains of PPARα (Dowell et al., 1997b). Currently, this is the only report that ligands of PPARα induce a conformational change in the ligand binding domain.

1.3.5.4 Coactivator-dependent Receptor Ligand Assay

This assay is based upon the fact that a coactivator binding interface is formed upon ligand binding (Fig 1.2). In this case, an agonist is defined as a compound able to cause recruitment of the coactivator SRC-1 to the ligand binding domains of all three *X. laevis* subtypes (Krey et al., 1997). These studies revealed that PPARα ligands included Wy 14,643, LTB_4, polyunsaturated fatty acids and eicosanoids. PPARβ ligands comprised polyunsaturated fatty acids, bezafibrate and of the eicosanoids, only 8(S)-HETE. Ligands identified for PPARγ include polyunsaturated fatty acids, 15d-$\Delta^{12,14}$-PGJ_2, and ciprofibrate.

1.3.5.5 Ligand Induced Complex Assay

Another recently documented effect of the PPARα ligand Wy 14,643, is the ability to induce the binding of RXR and PPAR to a PPRE (Forman et al., 1997). While this effect of ligand has never been characterized for PPAR/RXR before, it has been documented that the binding of RXR/RXR homodimers to DR1 response elements requires the presence of the RXRα ligand 9-*cis* retinoic acid (Zhang et al., 1992b). This ligand induced complex assay was used to characterize a number of compounds as potential ligands. PPARα ligands identified include the fibrates Wy14,643, ciprofibrate and clofibrate, the unsaturated fatty acids linoleic, linolenic, arachidonic, and various eicosanoids. Interestingly, a number of prostaglandins that activate PPARα, do not appear to bind. Although prostaglandin A_1 (PGA_1), PGA_2, PGB_2, PGD_2, PGE_2, $PGF_{2\alpha}$ and 15d-$\Delta^{12,14}$-PGJ_2 activate PPARα, they do not act as ligands in this assay. Polyunsaturated fatty acids such as linoleic, linolenic and arachidonic acids were the best PPARβ ligands by this assay. While PPARβ was activated by PGA_1, PGA_2, and 15d-$\Delta^{12,14}$-PGJ_2, none of these compounds induced binding of the PPARβ/RXRα complex to DNA. Thus, the α and β isoforms demonstrate overlapping ligand spectrum according to this assay. Interestingly, according to this assay, compounds which activate PPARs may not necessarily be ligands.

1.3.5.6 PPAR Ligands: Synopsis

A number of assays have been utilized to identify potential ligands of PPAR without validating their results with the determination of a K_d. While the results of the different assays agree that PPAR ligands include fibrate compounds, polyunsaturated fatty acids and eicosanoids, there are some significant differences. For example,

leukotriene B_4 was identified as a PPARα ligand with a K_d~90 nM (Devchand et al., 1996). However, the ligand induced complex assay did not recognize leukotriene B_4 as a ligand (Forman et al., 1997). Some of these discrepancies arise from the confusion between PPAR ligands and agonists. A ligand of a PPAR simply refers to the specific interaction between these two molecules, the affinity of which is defined by the K_d. However, the definition of an agonist implies a binding event and a resulting biological effect such as coactivator recruitment. Thus, the coactivator-dependent receptor ligand assay approximates the means by which ligands effect a biological response, but since ligands of receptors also act as antagonists and even negative antagonists, this assay only identifies PPAR agonists. The ligand induced complex formation assay is an uncharacterized function of PPAR ligands of which very little is known, much less whether it is a relevant biological phenomenon. Since this assay was unable to identify, leukotriene B_4 as a PPARα ligand, conclusions must be cautiously derived from this assay. Since nuclear receptors are by their nature receptors, ligands exist that act as agonists, antagonists and negative antagonists. Therefore, care must be taken not to interchange the term ligand and agonist.

1.3.6 DNA Binding Site of PPAR

As alluded to in earlier sections, PPARs bind to specific DNA binding sites in order to activate transcription. These DNA binding sites or peroxisome proliferator-responsive elements (PPREs) are located in the promoter regions of target genes. While this section provides an overview of DNA binding, Section 3.0 provides a more detailed commentary. The two PPREs that will be referred to frequently through out this work are the acyl-CoA oxidase (AOx PPRE) and the bifunctional hydratase/dehydrogenase (HD

PPRE). As previously mentioned, these PPREs were the first to be identified and regulate genes involved in peroxisomal β-oxidation.

1.3.6.1 Structure of DNA Binding Sites

The basic binding site of PPAR is composed of two consensus nuclear receptor binding half-sites of the sequence TGACCT. The DNA binding sites of nuclear receptors that bind as dimers are composed of two of these repeats that can exist in an various orientations such as inverted (TGACCT-AGGTCA), everted (AGGTCA-TGACCT) and direct (TGACCT-TGACCT). The heterodimer of PPAR/RXR binds to a DR1 element, a direct repeat separated by 1 nucleotide. In all characterized PPREs, the core DNA binding site conforms to the DR1 structure, however there are variations in the composition of the bases of the TGACCT repeats (Fig. 1.3).

A binding site selection study of the binding site of PPARα/RXRα revealed that the consensus binding sequence of the core DNA binding site is TNACCC N TGACCN. The terminology referring to this region as the core DNA binding site is utilized to reflect the growing appreciation of the importance of the 3' flanking region in modulating PPAR activity (Palmer et al., 1995; Juge-Aubry et al., 1997; IJpenberg et al., 1997; DiRenzo et al., 1997). The nucleotide between the two half-sites is assumed to simply act as a spacer, however the PPARα/RXRα complex had a greater affinity for the presence of an T over a G at this site (Castelein et al., 1997). It is unknown if PPARγ exhibits a different preference for nucleotides in the core DNA binding site.

1.3.6.2 Peroxisome Proliferator-responsive Elements

The first PPREs to be identified were those of the AOx and HD genes (Zhang et al., 1992; Dreyer et al., 1992; Osumi et al., 1991). These PPREs were isolated according

GENE	PPRE SEQUENCE	FUNCTION OF GENE PRODUCT
Acyl-CoA oxidase	TGACCTtTGTCCT	- first step of peroxisomal β-oxidation
Hydratase/Dehydrogenase bifunctional Enzyme*	TCTCCTtTGACCTat-TGAACTaTTACCT	- second and third step of peroxisomal β-oxidation
Acyl-CoA Synthetase	TGACTGaTGCCCT	- converts fatty acids to acyl-CoA derivatives
Cytochrome P450 A6	TCAACTtTGCCCT	- formation of dicarboxylic acids by ω-oxidation
Mitochondrial HMG-CoA Synthase	AGACCTtTGGCCC	- ketogenesis
Medium-chain Acyl-CoA Dehydrogenase	TCACCTtTACCCG	- first step of mitochondrial medium-chain β-oxidation
Liver-Fatty Acid Binding Protein	TGACCTaTGGCCT	- liver fatty acid binding protein
Adipocyte Protein 2	TGAACTgTGATCC	- adipose tissue fatty acid binding protein
Malic Enzyme	TCAACTtTGACCC	- malate decarboxylation
Phosphoenoyl Pyruvate Kinase	AGACCTtTGGCCG	- gluconeogenesis and glyceroneogenesis
Lipoprotein Lipase	TGCCCTtTCCCCC	- hydrolysis of triglyceride rich particles
Apolipoprotein A-I	TGACCCcTGCCCT	- protein component of high density lipoprotein (HDL)
Apolipoprotein A-II	CAACCTtTACCCT	- protein component of HDL
CONSENSUS	TGACCTt/aTGACCT	

Figure 1.5: Peroxisome proliferator-responsive elements (PPREs).
Depiction of the characterized PPREs from the promoters of various genes and the functions of those gene products.
* the HD PPRE is complex element composed of two DR1 elements separated by two nucleotides

to the criteria that these elements mediated the action of peroxisome proliferators and were present in the promoter regions of genes that were activated by peroxisome proliferators. These elements were selected in an unbiased manner since at that time it was unknown that nuclear receptors mediated the effects of peroxisome proliferators. With the cloning of PPARα and the discovery that PPAR bound to the PPRE as a heterodimer with RXR, additional criteria were added to the definition of a PPRE. This included the ability of the PPAR/RXR complex to bind to this sequence and the ability of this candidate response element to mediate the cooperative action of PPAR and RXRα in mediating transcriptional activation by peroxisome proliferators.

In addition to the identification of PPREs regulating the AOx and HD enzymes of peroxisomal β-oxidation, PPREs of genes involved in other fatty acid metabolic processes have been identified. This implicates PPARs in the regulation of mitochondrial β-oxidation, ketogenesis, gluconeogenesis/glyceroneogenesis, intracellular fatty acid transport, conversion of malate to pyruvate, esterification of fatty acids, release of fatty acids, extracellular fatty acid transport, microsomal ω-hydroxylation of fatty acids, and transport of fatty acids into the mitochondria (medium chain acyl-CoA dehydrogenase (Gulick et al., 1994), mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (Rodríguez et al., 1994), phosphoenolpyruvate carboxykinase (Tontonoz et al., 1995), fatty acid binding protein (Poirier et al., 1997), adipocyte P2 (Tontonoz et al., 1994a), malic enzyme (Castelein et al., 1994), acyl-CoA synthetase (Schoonjans et al., 1995), lipoprotein lipase (Schoonjans et al., 1996a), apolipoprotein A-II (Vu-Dac et al., 1996), cytochrome P450 4A6 (Muerhoff et al., 1992, Aldridge et al., 1995), cytochrome p450

4A1 (Palmer et al., 1995) and muscle carnitine palmitoyltransferase I (Mascaro et al., 1998)).

Interestingly, the 3' flanking region of the core DNA binding site appears to greatly influence the binding of the PPAR/RXR complex and to even determine what PPAR subtype may signal through this element (Palmer et al., 1995; IJpenberg et al., 1997; Juge-Aubry et al., 1997). The implication that PPREs are subtype specific has enormous implications in helping to resolve the apparent paradox between the overlapping expression patterns and agonist specificity with the distinct biological functions of PPAR subtypes.

1.3.7 Mechanism of Transcriptional Regulation by PPAR

It is presumed that the agonist-dependent activation of target genes by PPAR is similar to that of other nuclear receptors (Fig. 1.2). While PPAR recruits coactivators in a typical manner, the role of corepressors in the regulation of PPAR activity remains unresolved and in fact may be atypical.

1.3.7.1 PPAR Coactivation Complex

A number of components of the coactivation complex appear to interact with PPARα and γ. These include the cointegrators p300/CBP and the nuclear receptor specific coactivator SRC-1 (Nolte et al., 1998; Gelman et al., 1999; Mizukami and Taniguchi, 1997; Dowell et al., 1997a; Krey et al., 1997; Zhu et al., 1996; Schulman et al., 1998). Recently, PGC-1, a novel coactivator of PPARγ signalling, has been identified in brown fat tissue and is linked to adaptive thermogenesis (Puigserver et al., 1998). It remains to be determined whether PPARs require the presence of p/CAF, p/CIP and the histone acetylase activity of p/CAF, as is the case with RAR.

1.3.7.2 PPAR Corepression Complex

Sequence alignment with PPARα and RXRα demonstrates that neither contain an COR box in the hinge domain, addition, N-CoR was shown to not be required in the regulation of PPARγ (Lavinsky et al., 1998).

To date, N-CoR and SMRT have not been conclusively shown to bind to DNA bound complexes of PPARα, PPARγ and RXRα, an observation supported by the fact that findings that neither RXRα nor PPAR contain the COR box in the hinge region (DiRenzo et al., 1997; Hörlein et al., 1995). While one study found that N-CoR did not interact with PPARα or PPARγ *in vitro* (DiRenzo et al., 1997), another reports that PPARγ interacts with both N-CoR and SMRT (Zamir et al., 1997b). Interestingly, SMRT and not N-CoR appears to be involved in the repression of PPARγ by phosphorylation by the MEK/mitogen activated kinase (Lavinsky et al., 1998). Further study is required to assess the role of the corepression complex in PPAR signalling. The inability of PPAR to recruit the corepressor complex in the typical manner may be the result of the involvement of an as yet unidentified factor/corepressor or alternative regulation of PPAR activity.

1.3.7.3 Recruitment of Basal Transcription Factors

To date, an interaction between PPAR and basal transcription factors has not been demonstrated. However, the PPAR heterodimerization partner RXRβ, interacts with TFIIB *in vivo* in a ligand dependent manner suggesting that the recruitment of transcription factors may be used by the functional complex of PPAR/RXR to activate the transcription of target genes (Leong et al., 1998).

1.4 BIOLOGICAL ROLE OF THE ALPHA SUBTYPE OF PPAR

The cloning of a novel nuclear receptor that was activated by peroxisome proliferators, identified this protein as a candidate transcriptional regulator of peroxisomal β-oxidation and perhaps fatty acid metabolism in general (Issemann and Green, 1990). The cataloguing of genes for which a functional PPRE has been identified continues to grow and suggests a role for PPARα as a transcriptional regulator of many processes involved in fatty acid catabolism such as serum triglyceride transport, liberation of fatty acids, intracellular fatty acid transport, import of fatty acids into the mitochondria, peroxisomal β-oxidation, microsomal ω-oxidation, mitochondrial β-oxidation, ketogenesis and gluconeogenesis (reviewed in Schoonjans et al., 1996b). The number of processes regulated by PPARα and the identification of PPARα in disparate and evolutionarily distant vertebrates such as fish, rodents, amphibians and primates with a similar activation profile by agonists supports such an integral and conserved role for PPARα.

The production of a PPARα null mouse has permitted the study of the physiological role of PPARα *in vivo*. PPARα null mice are viable and healthy demonstrating the fact that this nuclear receptor is not essential for growth, differentiation and development (Lee et al., 1995). Interestingly, PPARα null mice do not experience any of pleiotropic effects associated with exposure to peroxisome proliferators. In fact, the genes of the peroxisomal β-oxidation pathway are not induced (Lee et al., 1995; Ren et al., 1997; Aoyama et al., 1998; Peters et al., 1997b). In addition, while the constitutive expression of the peroxisomal β-oxidation genes remained unaffected in PPARα null mice, the constitutive expression of genes of the mitochondrial β-oxidation pathway was

reduced (Aoyama et al., 1998). Therefore, PPARα not only appears to play a role in regulating adaptive changes in fatty acid metabolism but also its constitutive maintenance. These studies not only demonstrate that PPARα is responsible for mediating the induction of genes of fatty acid metabolism by peroxisome proliferators but also that PPARα is an integral regulatory component of fatty acid catabolism.

1.4.1 Mediator of the Pleiotropic Effects of Peroxisome Proliferators

Exposure of rodents to peroxisome proliferators results in the induction of specific genes, hepatomegaly and an induction in number and size of peroxisomes. However, mice which are PPARα-null do not experience any of these pleiotropic effects when exposed to the peroxisome proliferator Wy 14,643 (Lee et al., 1995). Furthermore, while all three PPAR subtypes are expressed in liver and can be activated in varying degrees by Wy 14,643, the β and γ subtypes were unable to compensate for the loss of PPARα. Therefore, while there is a great deal of functional overlap *in vitro,* there appears to be a higher degree of subtype specificity *in vivo*. Thus, PPARα is essential to the induction of the genes of peroxisomal β-oxidation and organelle biogenesis in liver.

1.4.2 Attenuator of the Inflammatory Response

The discovery that leukotriene B_4, a chemoattractant of leukocytes to sites of inflammation, was a PPARα agonist implicated PPARα as a modulator of the inflammatory response (Devchand et al., 1996). In fact leukotriene B_4 is an α-subtype specific agonist that activates the expression of the genes of the ω- and β-oxidation of fatty acids. Since, the length of the inflammatory response is governed by the levels of inflammatory mediators, the activation of PPARα inducible genes would result in the catabolism of leukotriene B_4 and reduce the duration of the inflammatory response. In

fact, PPARα-null mice experienced prolonged inflammatory responses compared to wild type mice. Therefore, these findings identify PPARα as a novel pharmaceutical target to regulate the inflammatory response.

1.4.3 Mediator of Peroxisome Proliferator Induced Hepatocarcinogenesis

Peroxisome proliferators are strong hepatocarcinogens in rodents that appear to act as tumor initiators although they do not directly modify DNA (reviewed in Rao and Reddy, 1991). The mechanism of carcinogenesis is proposed to result from the lipid peroxidation and DNA modification caused by H_2O_2 and hydroxyl radicals produced in hepatocytes exposed to peroxisome proliferators. This increase may result from the increase in β-oxidation of fatty acids without a compensatory increase in catalase levels (Rao and Reddy, 1991; Cattley and Glover, 1993; Clayson et al., 1994). However, long-term exposure of humans to hypolipidemic pharmaceutical peroxisome proliferators does not result in proliferation of peroxisomes nor hepatocellular carcinomas (De La Iglesia et al., 1982; Roberts et al., 1998).

The cloning of mouse PPARα suggested that the carcinogenic effects could be mediated by this receptor (Issemann and Green, 1990). In fact, Peters *et al.*, 1997, using a PPARα-null mouse demonstrated that PPARα is required for peroxisome proliferator induced carcinogenesis. All of wild type mice exposed to Wy 14,643 developed hepatocellular carcinomas while the PPARα-null mice were not affected (Peters et al., 1997a). While humans express PPARα, the reason for the lack of peroxisome proliferation and hepatocarcinogenesis induced by peroxisome proliferators is unknown (Sher et al., 1993).

The discovery that p300 is a component of the coactivation complex recruited by PPARα has led to an additional proposed mechanism of PPARα mediated carcinogenesis (Dowell et al., 1997a). The cointegrator p300 is a factor that is involved in mediating signalling by not only nuclear receptors but also the tumor suppressor protein p53 (Gu et al., 1997; Lill et al., 1997; Avantaggiati et al., 1997). Competition for this limiting factor between p53 and PPARα was suggested to potentially contribute to hepatocarcinogenesis (Dowell et al., 1997a). In fact, antagonism resulting from a competition for p300 between two signalling pathways that both require p300 has been demonstrated for the RAR and AP-1 (Fos/Jun) signalling pathways (Kamei et al., 1996). The sequestration of limiting coactivation complex components may in fact upset the balance between the numerous signalling pathways that share common components leading to inhibition of signalling, aberrant regulation of genes and even transformation events.

1.5 BIOLOGICAL ROLES OF PPARβ AND PPARγ

PPARγ is an integral factor in the transcriptional regulation of the differentiation of preadipocytes in mature adipocytes (Tontonoz et al., 1994b; reviewed in Mandrup and Lane, 1997). Not only are PPARγ agonists capable activating adipogenesis, but also, many adipocyte specific genes contain functional PPREs, some of which are responsive only to the PPARγ subtype (Forman et al., 1995; Kliewer et al., 1995; Lehmann et al., 1995; Tontonoz et al., 1995; Juge-Aubry et al., 1997). PPARγ is also implicated in the promotion of differentiation of macrophages into foam cells, an adipocyte-like cell (Tontonoz et al., 1998; Nagy et al., 1998). This is of special significance since the progression of atherosclerotic plaques is believed to involve the conversion of

macrophages that have infiltrated arterial lesions and subsequent conversion into foam cells, a process integral to the pathogenesis of atherosclerosis (reviewed in Ross, 1993).

To date, PPARβ remains without a characterized biological function. While its tissue expression pattern is unique in that it is expressed in neural tissues, it is also coexpressed, albeit at low levels with the α and γ subtypes (Schmidt et al., 1992; Dreyer et al., 1992; Amri et al., 1995; Kliewer et al., 1994; Cullingford et al., 1998).

1.6 THE FUTURE PROSPECTS OF PPARα

As the number of genes that are regulated by PPARα increases, our appreciation of the integral role PPARα plays in fatty acid metabolism continues to grow. A reflection of this importance is the attention PPARα now garners as a pharmaceutical target for the treatment of disorders associated with fatty acid metabolism such as atherosclerosis. The continued identification of PPARα regulated genes will not only continue to clarify the physiological role of PPARα but also elucidate the full consequences of pharmaceuticals targeted to PPARs. Intrinsic to our understanding of the role of PPARα is resolving the paradox posed by the existence of PPAR subtype specific biological effects and the existence of an apparent overlap in PPAR subtype agonist specificity, response element utilization, and tissue expression patterns. Recent studies have begun to allude to differences in response element specificity between the different PPAR subtypes (Juge-Aubry et al., 1997). However, whether these differences are biologically relevant remains to be determined. While the focus of subtype specific *cis*-acting elements is focused on PPREs, very little is known about the influence these elements have in the context of the full promoter regions of target genes. Finally, a full understanding of the function of PPARα will require an appreciation of the means by

which PPARα activity and carbohydrate metabolism are integrated. The coordination of fatty acid metabolism with that of carbohydrates will require regulation of PPARα activity. Not only will the defining of the mechanism and action of PPARα lead to the ability to design powerful pharmaceuticals but also, ultimately lead to an understanding of energy metabolism as a whole.

1.7 THIS THESIS

At the outset of the research presented in this thesis, B. Zhang, a member of our research group, had just identified the DNA element in the promoter of the hydratase-dehydrogenase gene (HD PPRE) that conferred responsiveness to peroxisome proliferators (Zhang et al., 1992). Interestingly, it was noted that this element contained sequences recognized by ligand-activated nuclear receptors. The previous cloning of nuclear receptors that were activated by peroxisome proliferators, the PPARs, presented a putative transcriptional activator that could mediate the effects of peroxisome proliferators (Issemann and Green, 1990; Dreyer et al., 1992). It is at this point that my involvement in this research began.

I first set out to establish that PPARs were involved in mediating the effects of peroxisome proliferators in the regulation of the HD gene. The results presented in Section 3.0 demonstrated for the first time that PPARα, β and γ bound to the HD PPRE as well as the AOx PPRE to mediate the effects of peroxisome proliferators (Marcus et al., 1993). The binding of PPARs to DNA was shown to bind as a heterodimer with RXRα (Marcus et al., 1993). These results added PPAR to the growing list of nuclear receptors, such as RAR, VDR and TR, that dimerized with RXRα to bind their appurtenant DNA binding sites and contributed to the growing appreciation that RXRα acted as a promiscuous DNA binding partner for many nuclear receptors (Zhang et al., 1992a; Kliewer et al., 1992b). Although the binding of PPAR to DNA was necessary, complementary *in vivo* work by S. Marcus demonstrated that it was not sufficient for transcriptional activation. Thus, this entailed one of the first demonstrations that the

DNA binding site could act as an allosteric modulator of the transcriptional activity of bound nuclear receptors.

In order to better understand the cooperative binding of PPAR and RXR to the AOx and HD PPREs, the *in vivo* studies outlined in Section 4.0 were aimed at determining if PPAR interacted with RXRα. To this end, the yeast two-hybrid system was used to demonstrate that RXRα and PPARα interact *in vivo* (Miyata et al., 1994). This interaction did not require binding to DNA nor the presence of exogenous agonist such as is the case of RXRα homodimers (Zhang et al., 1992b). This data supported a model of PPARα forming a heterodimer with RXRα that was then capable of recognizing the AOx and HD PPREs. Not only did this entail the first demonstration of the *in vivo* interaction of PPARα and RXRα, but also, this was the first demonstration of an interaction between two nuclear receptors in the yeast two-hybrid system.

Nuclear receptors other than the heterodimer of PPAR/RXR recognize DNA elements of the same structure as a PPRE. Section 4.0 describes the investigation of two such candidate nuclear receptors, the chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) and the hepatocyte nuclear factor 4 (HNF4) (Winrow et al., 1994). The described studies entail the first demonstration of COUP-TF1 and HNF4 binding to the HD and AOx PPREs (Miyata et al., 1993; Winrow et al., 1994). While COUP-TF1 antagonizes PPAR/RXR mediated signalling, presumably by competing for binding to the PPRE, the binding of HNF4 to the HD PPRE results in activation of transcription of the target gene. Therefore, the transcription of genes regulated by PPARα is not only dependent upon a balance between the activity of PPARα/RXRα bound to a PPRE, but

also the antagonistic and potentiating roles of multiple nuclear receptors that also recognize and bind to the PPRE.

Since the yeast two-hybrid system was previously validated as a means to detect the *in vivo* interaction between PPARα and a binding partner, this system was used to genetically screen for novel PPARα interacting proteins. Through this screen, it was hoped that a greater understanding of the mechanism of PPARα mediated signalling could be achieved through the identification of novel protein-protein interactions. Section 5.0 describes the cloning of a novel nuclear receptor, the liver X receptor α (LXRα), which interacted with PPARα (Miyata et al., 1996). Although LXRα bound as a heterodimer with RXRα to DR4 response elements, LXRα did not bind any of the various consensus nuclear receptor binding sites, including PPREs, as a heterodimer with PPARα. In fact, the presence of LXRα antagonized binding of the PPAR/RXR heterodimer to PPREs *in vitro* and inhibited the activity of PPAR *in vivo*. This inhibition presumably resulted from the formation complexes of PPAR/LXR and RXR/LXR that do not recognize PPREs, thus reducing the amounts of PPAR and RXR available to bind to PPREs. LXRα has since been characterized as a key regulator of cholesterol metabolism (Janowski et al., 1996; Peet et al., 1998). Understanding the coordination and regulation of metabolic pathways at the level of transcription requires the determination of whether the interplay between PPAR and LXR mediated signalling is manifested biologically.

Section 6.0 describes the first description and characterization of the interaction between PPARα and the receptor interacting protein 140 (RIP140) (Miyata et al., 1998). This interaction was detected using the yeast two-hybrid screen in a similar manner to that of the discovery of LXRα. RIP140 was originally characterized as a coactivator that

interacted with ER in an agonist-dependent manner (Cavaillès et al., 1995). Interestingly, RIP140 interacted with PPARα in an agonist-independent manner *in vivo* and *in vitro*. Furthermore, RIP140 strongly antagonized PPARα/RXRα mediated transcriptional activation *in vivo*. Although RIP140 is referred to as a coactivator, the results of these studies demonstrate that this characterization may not take into account unknown complexities in the recruitment of coactivators by PPARα or that RIP140 may not be a typical coactivator such as SRC-1. Thus, RIP140 has been added to the list of proteins that interact with PPAR and effect the function of this nuclear receptor.

Finally, Section 7.0 details the novel proposal of applying the allosteric ternary complex model to the mechanism of nuclear receptor action (De Lean et al., 1980). This model arose from the inquiry into alternative explanations to the 'atypical' manner in which PPARα interacted with coactivators in the absence of agonist (Miyata et al., 1996; Dowell et al., 1997a). The allosteric ternary complex was originally developed to explain the mechanism of action of the β-adrenergic receptor, a G-protein coupled receptor (De Lean et al., 1980). Since, similar to the β-adrenergic receptor, nuclear receptors bind ligands (agonists/antagonists) and recruit effector molecules (coactivators), the allosteric ternary complex model is an appropriate model with which to describe the mechanism of nuclear receptor function. Not only does the application of this model to nuclear receptors provide a rigorous conceptual framework in which to describe their actions but it also provides a novel view of the recruitment of coactivators/corepressors and an alternative explanation for the agonist-independent interaction between PPARα and coactivators.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Specialized Chemicals and Reagents

This list identifies the sources of specialized reagents that were utilized during the conduct of the described research.

9-*cis* retinoic acid	Sigma Chemical Company
antibiotic cocktail – penicillin (5,000 U/ml) streptomycin (5,000 μg/ml)	Life Technologies
bovine serum albumin	Pharmacia
5-bromo-4-choloro-3-indoyl-β-D-galactoside (X-gal)	Biosynth AG
charcoal, Dextran coated	Sigma Chemical Company
coenzyme A	Sigma Chemical Company
Hoechst 33258	Fisher Scientific
isopropyl-β-D-thiogalactoside (IPTG)	Biosynth AG
L-glutamine	Life Technologies
luciferin	Biosynth AG
molecular weight standards:	
DNA: 1 kbp DNA ladder	Life Technologies
protein: low range SDS-PAGE standards	Bio-Rad
nucleoside triphosphates	Pharmacia
o-nitrophenyl-β-D-galactopyranoside (ONPG)	Sigma Chemical Company
phenylmethylsulphonylfluoride (PMSF)	Sigma Chemical Company
poly(dIdC•dIdC)	Pharmacia
RNase "A"	Pharmacia
salmon testes DNA, sonicated	Sigma Chemical Company
serum:	
calf	Life Technologies
fetal bovine	Sigma Chemical Company
trypsin-EDTA	Life Technologies
Wy 14,643	ChemSyn Laboratories

2.1.2 Radiochemicals

L-[^{35}S]-methionine (1151 Ci/mmol; 10 μCi/μl) NEN Life Science Products

[α-^{32}P]dATP (3,000 Ci/mmol; 10 μCi/μl) Amersham Canada Ltd.

2.1.3 Oligonucleotides

The oligonucleotides described in Table 2.1 and Table 2.2 were used for electrophoretic mobility shift analysis, DNA sequencing and vector construction.. All oligonucleotides were synthesized by the Mobix Central Facility, McMaster University with the exception of MalE which was purchased from New England Biolabs.

2.1.4 Polyclonal anti-RXRα Serum

The rabbit polyclonal serum directed against human RXRα was a gift of Dr. R. Evans (Mangelsdorf et al., 1990).

2.1.5 Cloning Vectors

pGEM-5Zf, pGEM-7Zf: These vectors were utilized as intermediates in subcloning procedures as well as *in vitro* expression vectors (Promega).

2.1.6 *In Vitro* Expression Vectors

2.1.6.1 Nuclear Receptor Expression Vectors

pGEM-hCOUP-TF1: This expression vector was a gift from Dr. B. O'Malley and contains the coding region of human COUP-TF1 inserted into the *Sma*I/*Eco*RI sites of pGEM 7Zf(+) (Wang et al., 1989).

pGEM-tCOUP: A truncated form of human COUP-TF1 (amino acids 51 to 413), expressed from the SP6 promoter, was generated by inserting the *Sma*I/*Aat*II fragment from pGEM-hCOUPTF1 into the *Eco*RV/*Aat*II sites of pGEM-5Zf (Promega). The fusion of the *Eco*RV/*Sma*I ends creates a translation start site.

Table 2.1: Oligonucleotides: dsDNA Probes for EMSA

The following pairs of complementary oligonucleotides were designed such that once annealed, they created double-stranded DNA binding sites for nuclear receptors. The non-complementary regions of the annealed oligonucleotides create *Bam*HI/*Bgl*II sticky ends which were filled in with nucleotides using the Klenow fragment of DNA Polymerase I. The incorporation of [α-^{32}P]dATP created a radiolabelled DNA probe for use in EMSA studies of nuclear receptor-DNA interactions. The depicted DNA probes include peroxisome proliferator-responsive elements (PPRE) and synthetic hormone response elements (HRE) composed of direct repeats of the TGACCT half site separated by a varying number of nucleotides (DR0-DR5).

NUMBER	OLIGONUCLEOTIDE SEQUENCE	DESCRIPTION
AB4952 AB4953	5' GATCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA 3' 3' GAGAGGAAACTGGATAACTTGATAATGGATGTAAACTCTAG 5'	HD PPRE[1]
AB4950 AB4951	5' GATCCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT 3' 3' GAAAGGGCTTGCACTGGAAACAGGACCAGGGGAAAACGATCTAG 5'	AOx PPRE[2]
AB8959 AB8960	5' GATCCTGACTTGTTCTGAGACCTTTGGCCCAGTTTTTCTGAGGCAGGCAGAGGA 3' 3' GACTGAACAAGACTCTGGAAACCGGGTCAAAAAGACTCCGTCCGTCTCCTCTAG 5'	HMG PPRE[3]
AB4582 AB4581	5' GATCTTCTGACCTTGACCTGG 3' 3' AAGACTGGAACTGGACCCTAG 5'	DR0 HRE
AB4584 AB4583	5' GATCTTCTGACCTCTGACCTGG 3' 3' AAGACTGGAGACTGGACCCTAG 5'	DR1 HRE
AB4586 AB4585	5' GATCTTCTGACCTCCTGACCTGG 3' 3' AAGACTGGAGGACTGGACCCTAG 5'	DR2 HRE
AB4588 AB4587	5' GATCTTCTGACCTCCTTGACCTGG 3' 3' AAGACTGGAGGAACTGGACCCTAG 5'	DR3 HRE
AB4590 AB4589	5' GATCTTCTGACCTCCTGTGACCTGG 3' 3' AAGACTGGAGGACACTGGACCCTAG 5'	DR4 HRE
AB4592 AB4591	5' GATCTTCTGACCTCCTGGTGACCTGG 3' 3' AAGACTGGAGGACCACTGGACCCTAG 5'	DR5 HRE

[1] HD PPRE: Peroxisome proliferator-responsive element identified in the promoter of the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene (Zhang et al., 1992).
[2] AOx PPRE: Peroxisome proliferator-responsive element identified in the promoter of the rat acyl-CoA oxidase gene (Dreyer et al., 1992).
[3] HMG PPRE: Peroxisome proliferator-responsive element identified in the promoter of the rat 3-hydroxy-3-methylglutaryl CoA synthase gene (Rodriguez et al., 1994). These oligos were also used to create pHMG(1X)luc.

Table 2.2: Oligonucleotides: Sequencing Primers

NUMBER	OLIGONUCLEOTIDE SEQUENCE	PURPOSE
AB9972	$^{5'}$GAGCAAAGGAAG$^{3'}$	Sequencing of the multiple cloning site (MCS) of pCPS (annealing position: nt –56 to nt –67 of rat carbamyl-phosphate synthetase basal promoter[1])
MalE	$^{5'}$GGTCGTCAGACTGTCGATGAAGCC$^{3'}$	Sequencing of the MCS of pMAL-2c (annealing position: nt 2781 to nt 2704)
AB2198	$^{5'}$GAGAGTAGTAACAAAGGTC$^{3'}$	Sequencing of the MCS of pGBT9 (annealing position: nt 836 to nt 854)
AB3313	$^{5'}$TCGATGATGAAGATACCCCA$^{3'}$	Sequencing of the MCS of pGAD424 (annealing position: nt 792 to nt 812)

[1] rat carbamyl-phosphate synthetase promoter (Lagacé et al., 1987)

pSG5-hHNF4: This vector, provided by Dr. F. Sladek, expresses the full length human HNF-4 coding sequence under the control of the T7 promoter (Sladek et al., 1990).

pSG5-xPPARα, pSG5-xPPARβ, pSG5-xPPARγ: These pSG5 based vectors contain the coding sequences of the *Xenopus laevis* PPAR subtypes α, β and γ under the control of the T7 promoter and were provided by Dr. W. Wahli (Dreyer et al., 1992).

pSG5-mPPARα, pSG5-hRXRα: These vectors performed dual roles as both eukaryotic and *in vitro* T7 controlled expression vectors of mouse PPARα and human RXRα (Tugwood et al., 1992; Marcus et al., 1993).

pB-rPPAR: The sequence of rat PPARα cloned into pBluescript SK(+) (Stratagene) and expressed from the T7 promoter was a gift from Dr. D. Noonan (Ligand Phrmaceuticals, San Diego) (Marcus et al., 1993).

2.1.6.2 RIP140 Expression Vector

pBRIP: This vector expresses full length RIP140 from the T3 promoter and was a gift from Dr. M. Parker (Cavaillès et al., 1995).

2.1.7 MBP Fusion Protein Expression Vectors

pMAL-2c: This vector expresses the cytosolic form of the maltose binding protein (MBP) under the control of the isopropyl-β-D-thiogalactoside inducible P_{tac} promoter (New England Biolabs).

pMAL-mPPARα: Expresses mPPARα fused to MBP and was created by inserting the *Bam*HI fragment from pSG5-mPPARα into the *Bam*HI site of pMAL-2c

pMAL-hRXRα: Expresses a fusion of human RXRα and MBP and was constructed by inserting the *Eco*RI fragment from pSG5-RXRα into the *Eco*RI site of pMAL-2c.

2.1.8 Eukaryotic Expression Vectors

2.1.8.1 Nuclear Receptor Expression Vectors

The following nuclear receptor expression vectors were utilized in transient transfection assays and in the overexpression of these proteins in COS-1 cells. pSG5 was utilized to normalize the addition of these expression plasmids in transient transfection assays (Green et al., 1988).

pSG5-mPPARα: This vector, a gift from Dr. S. Green, expresses mouse PPARα (Tugwood et al., 1992).

pSG5-RXRα: This expression vector, constructed by S. Marcus, contains the human RXRα coding sequence inserted into the *Eco*RI site of pSG5 (Marcus et al., 1993).

pRc/CMV-rPPARα: This expression vector of the rat PPARα was constructed by inserting the coding region of rPPARα from pB-rPPARα, into pRc/CMV (Invitrogen) (Marcus et al., 1993).

pSG5-xPPARα, pSG5-xPPARβ and pSG5-xPPARγ: These pSG5 based vectors express the α, β and γ subtypes of the *Xenopus laevis* PPARs (Dreyer et al., 1992).

2.1.8.2 RIP140 Expression Vector

pEFRIP: This RIP140 expression plasmid was supplied by Dr. M.G. Parker (Cavaillès et al., 1995) for use in *in vivo* studies of the effects of RIP140 on PPARα/RXRα mediated signalling. The parental vector, pEF-BOS (Mizushima and Nagata,

1990), was appropriately added to compensate for variations in the amount of pEFRIP utilized in transient transfection assays.

2.1.9 Luciferase Reporter Vectors

Mechanistic studies of PPARα required an easily assayed model of PPARα mediated transcriptional activation by peroxisome proliferators. To such an end, a number of vectors were constructed in which the luciferase gene from *Photinus pyralis* (deWet et al., 1987) was placed under the control of PPARα enhancer sequences and the basal promoter from the rat carbamyl-phosphate synthetase I (CPS) gene (Lagacé et al., 1987).

pCPSluc: This reporter construct was generated by B. Zhang (Zhang et al., 1992) and comprises the CPS basal promoter expressing the luciferase gene from *Photinus pyralis* (Lagacé et al., 1987; deWet et al., 1987). PPAR enhancer elements were inserted into the *Bam*HI site immediately upstream of the CPS basal promoter sequences. This reporter vector was chosen over comparable pGL2 (Promega) based constructs for the low levels of luciferase expression obtained in the absence of added exogenous factors while achieving high levels of induction in the presence of PPARα, RXRα and Wy 14,643.

pAOx(X2)luc: In this pCPSluc based vector constructed by B. Zhang, two copies of the AOx PPRE in the forward direction control the expression of the luciferase gene (Marcus et al., 1993).

pHMG(X1)luc: This vector was constructed by inserting the annealed oligonucleotides AB8959 and AB8960 (refer to Table 2.1) into the *Bam*HI site of pCPSluc (Zhang

et al., 1992) creating a reporter vector in which the luciferase gene is placed under the control of one copy of the mHMG-CoAS PPRE (Rodríguez et al., 1994).

2.1.10 Mammalian Cell Lines

The mammalian cell lines used were BSC-40 African green monkey kidney cells (Brockman and Nathans, 1974) and COS-1 African green monkey kidney cells which had been transformed with the simian virus 40 (Gluzman, 1981).

2.1.11 Yeast Strains

Two different *Saccharomyces cerevisiae* strains were utilized for the yeast two-hybrid studies: HF7c (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, gal4-52, gal80-538, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, URA3::GAL4*$_{17mers(x3)}$*-CyC1*$_{TATA}$*-lacZ*) and Y190 (*MATa, leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4ΔΔ80ΔΔ URA3 GAL-lacZ, LYS GAL-HIS3, cyh*^{r}) (Harper et al., 1993; Feilotter et al., 1994). Both strains contain two separate reporter genes (β-galactosidase and HIS3) under the control of the binding site or upstream activation sequence (UAS_G) of the yeast transcription factor GAL4. During the screening of the GAL4 activation domain-cDNA fusion library (refer to Methods: Genetic Screen for PPARα Interacting Proteins) the yeast strain HF7c was chosen for its lack of growth on His deficient media as compared to strain Y190, an indication of tighter regulation of the HIS3 reporter gene. However, Y190 produced easily detectable levels of β-gal activity making this strain ideally suited for quantitation of protein-protein interactions (refer to Methods: Yeast Two-hybrid system).

2.1.12 Yeast Expression Plasmids

The characterization of protein-protein interactions in the yeast two-hybrid system requires that the proteins of interest be fused to either the DNA binding domain (DBD) or the activation domain (AD) of the yeast transcription factor GAL4. pGBT9 and pGAD424, which express the GAL4 DBD and GAL4 AD respectively, contain unique restriction enzyme sites in which to insert protein coding sequences in order to create the appropriate fusion constructs (Clontech). pGBT9 and pGAD424 are shuttle vectors that can be selected for and propagated in both yeast and *E.coli*.

pGBT9: This vector constitutively expresses amino acids 1 to 147 of GAL4, the region that encompasses the GAL4 DBD.

pGAD424: The GAL4 AD (amino acids 768 to 881) tagged with the nuclear localization signal of the simian virus 40 large T antigen is constitutively expressed from this vector.

pCL1: This vector expresses full length GAL4 and was used as a positive control in β-galactosidase assays.

2.1.12.1 GAL4 DBD Fusion Expression Plasmids

pGBD-mPPARα: This fusion of mouse PPARα (amino acids 26 to 468) with the GAL4 DBD was constructed by inserting the *Eco*RI/*Bam*HI fragment from pSG5-mPPARα into the corresponding sites of pGBT9.

pGBD-ΔNmPPARα(Δ435): This construct encodes the GAL4 DBD fused to amino acids 83 to 435 of mouse PPARα and was generated by inserting the *Pvu*II fragment from pSG5-mPPARα into the *Sma*I site of pGBT9. Due to the cloning

strategy, the amino acids GDPSTCSQANSGRISYDL are appended to the C-terminus of mPPARα.

pGBD-ΔNmPPARα: A fusion the GAL4 DBD fused to amino acids 83 to 468 of mouse PPARα was generated by inserting the *Kpn*I/*Bam*HI fragment from pSG5-mPPARα into the *Kpn*I/*Bam*HI sites of pGBD-ΔNmPPARα(Δ435),

pGBD-RXRα: The fusion of human RXRα (amino acids 44 to 462) with the GAL4 DBD, was constructed by inserting the *Sma*I/*Pst*I fragment from pSKXR$_3$-1 (Mangelsdorf et al., 1990)into the same sites of pGAD424.

2.1.12.2 GAL4 AD Fusion Expression Plasmids

pGAD-mPPARα: This fusion of the GAL4 AD with amino acids 26 to 468 of mouse PPARα was generated by inserting the *Eco*RI/*Bam*HI fragment from pSG5-mPPARα into the corresponding sites of pGAD424.

pGAD-ΔNmPPARα: This vector expresses a fusion of the GAL4 DBD fused to amino acids 83 to 468 of mouse PPARα and was constructed by excising the *Eco*RI/*Bam*HI fragment from pGBD-ΔNmPPARα(Δ435) and inserting it into the corresponding sites of pGAD424.

pGAD-RXRα: The fusion of human RXRα (amino acids 44 to 462) with the GAL4 AD, was generated by inserting the *Sma*I/*Pst*I fragment from pSKXR$_3$-1 (Mangelsdorf et al., 1990) into the same sites in pGAD424.

2.1.12.3 GAL4 AD/cDNA Fusion Libraries

Once established, the yeast two-hybrid system was utilized to genetically screen for novel PPARα interacting proteins encoded in a cDNA library fused to the GAL4 AD.

Two such libraries were screened during the course of these studies including a human HeLa S3 cell Matchmaker library and a human liver Matchmaker library (#HL400A1 and #HL4002AB respectively; Clontech). Both libraries were obtained as *E. coli* DH10β cultures and were amplified and purified as described in the Methods section.

2.2 METHODS

2.2.1 Construction of Plasmids

The generation of all plasmid constructs described in the Materials section was carried out utilizing standard recombinant DNA techniques as described by Ausubel *et al*, 1989 and according to manufacturers' instructions. All restriction enzymes, DNA modifying enzymes and DNA polymerases were obtained from New England Biolabs.

2.2.2 Sequencing of Plasmid DNA

The sequence of plasmid DNA was determined by the dideoxynucleoside triphosphate chain termination method (Sanger et al., 1977) using the ^{T7}Sequencing Kit (Pharmacia) according to manufacturer's instructions.

2.2.3 Maintenance of *Escherichia coli*

Escherichia coli (E. coli) were grown in sterile 2YT liquid media (Ausubel et al., 1987) or on solid 2YT media (plates) that contained 100 μg/ml ampicillin (Ausubel et al., 1987). Generally speaking, *E. coli* liquid cultures were grown at 37°C in a rotary shaker (250 rpm) while plate cultures were maintained in a 37°C incubator.

2.2.4 Introduction of plasmid DNA into *E. coli* cells

2.2.4.1 Transformation using Calcium Chloride

The following technique of preparing competent *E. coli* and the subsequent transformation with plasmid DNA (Hanahan, 1983) was chosen since it results in a consistently high transformation efficiency. Briefly, 200 ml of LB media warmed to 37°C (Ausubel et al., 1987), was inoculated with a saturated 5 ml culture of DH10β *E. coli*. This culture was incubated at 37°C until the OD_{600} reached 0.5 at which point the culture was immediately cooled in an ice-water bath. During subsequent steps, the cells,

reagents and equipment were kept ice-cold. The cells were pelleted by centrifugation, resuspended in 20 ml of solution RF1 (100 mM RbCl, 30 mM $MnCl_2 \cdot 4H_2O$, 30 mM KOAc pH 7.5, 10 mM $CaCl_2 \cdot 2H_2O$, 15% glycerol (v/v), pH adjusted to 5.8 with glacial acetic acid) and incubated on ice for 1 h. Cells were then pelleted, resuspended in 8 ml of solution RF2 (10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM $CaCl_2 \cdot 2H_2O$, 15% glycerol (v/v), pH adjusted to 6.8 with NaOH) and 100 µl of this suspension was aliquoted into microfuge tubes that had been chilled to -70°C.

In order to transform these competent DH10β *E. coli*, DNA was added to aliquots of cells which had been thawed on ice. This mixture was sequentially incubated on ice for 40 min, 'heat shocked' at 42°C for 2 min and cooled on ice for 2 min. Finally, the cells were incubated in 1 ml of LB media for 1 h at 37°C and then plated onto 2YT+Amp plates. A successful preparation of cells demonstrated a transformation efficiency of $\geq 1 \times 10^7$ transformants/µg DNA.

2.2.4.2 Transformation by Electroporation

Rescuing plasmids from a preparation of yeast DNA by transformation of *E. coli* required the use of electroporation in order to obtain a transformation efficiency of approximately 1×10^{10} transformants/µg of DNA (Ausubel et al., 1987). Briefly, 50 ml of 2YT warmed to 37°C were inoculated with a 5 ml saturated culture of *E. coli* DH10β. This culture was grown until the OD_{600} reached 0.5 and immediately chilled in an ice-water bath. During subsequent steps, the cells, reagents and equipment were kept ice-cold. The cells were pelleted four times by centrifugation and sequentially resuspended in 500 ml of ddH2O, 10 ml of ddH2O, 30 ml of 10% glycerol and finally 0.5 ml of 10% glycerol.

In a microfuge tube, 100 µl of the cell suspension was mixed with 100 ng of DNA and transferred to an electroporation cuvette (BioRad: 0.2 cm electrode gap). The electroporation apparatus (BioRad) was set to 2.5 kV, 200 Ω and 25 µF. Immediately after application of the electrical pulse, 1 ml of SOC media (Appendix B, (Ausubel et al., 1987)) was added to the cuvette. The cells were transferred to a microfuge tube, incubated for 1 h at 37°C in a rotary shaker and finally plated onto 2YT+Amp plates.

2.2.5 Plasmid DNA Purification

2.2.5.1 Small Scale Preparation

The small scale or "miniprep" method of plasmid purification from *E. coli* was a modified "lysis by boiling" method (Sambrook et al., 1989). 3 ml of a saturated bacterial culture grown in 2YT+Amp (100µg/ml), was pelleted in a microfuge tube and resuspended in 350 µl of STET (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 5% Triton X-100 (v/v)) and 25 µl of lysozyme solution (10 mg/ml lysozyme, 10 mM Tris-HCl pH 8.0). After incubation in a boiling water bath for 40 s, the cellular debris was pelleted by centrifugation for 10 min at 12000 x g. This pellet was removed with a sterile toothpick and 200 µl of 7.5 M ammonium acetate and 600 µl of isopropanol was added to the supernatant. The plasmid DNA was precipitated by freezing in liquid N_2 for 5 min and pelleted by centrifugation for 10 min at 12000 x *g* at 4°C. The pellet was washed once with 70% ethanol, pelleted again and dried under vacuum. The plasmid DNA was resuspended in 20 µl of TE+RNAse (10 mM Tris-HCl pH 8, 1 mM EDTA, 20 µg/ml RNase "A").

2.2.5.2 Chloramphenicol Amplification of Plasmid DNA

Chloramphenicol was added to *E. coli* cultures in order to amplify plasmid DNA and consequently increase the yields obtained from large scale plasmid DNA preparations (Sambrook et al., 1989). A single *E. coli* colony was used to inoculate a 500 ml volume of 2YT+Amp. This culture was incubated at 37°C overnight without shaking. In the morning, the culture was shaken until the OD_{600} of the culture reached ~0.6. At this point, chloramphenicol dissolved in ethanol was added to a final concentration of 170 μg/ml and the culture was incubated overnight at 37°C in a rotary shaker. The following day the cells were harvested and the plasmid DNA purified by the method which follows.

2.2.5.3 Large Scale Plasmid DNA Purification

Large amounts of pure plasmid DNA, such as that required for transient transfection assays, was purified by anion exchange chromatography (Qiagen). Bacterial cultures grown in the presence of chloramphenicol were harvested and lysed by a modified alkaline lysis method (Birnboim, 1983). The plasmid DNA was purified from this cell lysate by purification on a Qiagen 500-tip column according to the manufacturer's instructions. The final pellet of purified plasmid DNA was resuspended in sterile TE pH 8.0, quantitated by fluorometry and the concentration adjusted to 1 mg/ml.

2.2.5.4 Amplification of Matchmaker Plasmid Library

In order to utilize the yeast two-hybrid system as a genetic screen, two different commercial cDNA fusion libraries were utilized. Since both libraries were obtained as *E. coli* cultures, amplification and purification of the plasmid library was required. The

libraries were amplified by plating 5 x 10^5 colony forming units onto sixty 10 cm 2YT+Amp plates. The bacteria were grown to subconfluence and then harvested by adding 10 ml of 2YT+Amp and scraping the cells off the plate with a glass "hockey stick". The collected cells were pelleted by centrifugation at 5000 x *g* and the plasmid DNA was extracted by the large scale plasmid purification technique previously described. The purified DNA was resuspended in TE pH 8.0 and quantitated by fluorometry.

2.2.6 DNA Quantitation by Fluorometry

Purified plasmid DNA, resuspended in TE pH 8.0, was added to 2 ml of TNE Buffer (10 mM Tris pH 7.4, 1 mM EDTA, 100 mM NaCl) containing 1 μg/ml Hoechst 33258 dye. The resulting fluorescence was measured in a Hoefer TKO 100 fluorometer standardized with calf thymus DNA and the concentration of plasmid DNA calculated.

2.2.7 Synthesis of Proteins *In Vitro*

In vitro synthesized proteins were utilized in *in vitro* bonding studies of protein-protein and DNA-protean interactions. The synthesis of RNA transcripts was carried out with the Riboprobe System (Promega) and the subsequent translation of proteins in reticulocyte lysate (Promega) according to the manufacturer's instructions. Radiolabelled proteins were produced by the addition of L-[^{35}S]-methionine to the translation reactions. The translated proteins were resolved by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography. Quantitation of the incorporated radioactivity using a Phosphorimager (Molecular Dynamics) permitted the normalization of the amounts of protein added in experiments.

2.2.8 Analysis of Protein-DNA Interactions

2.2.8.1 Radiolabeling of dsDNA Probes

Radiolabelled dsDNA probes were synthesized by annealing 20 pmol of two complimentary oligonucleotides (refer to Table 2.1). Once annealed, the oligonucleotides were designed to leave 5' noncomplimentary ends that correspond to the sticky ends of *Bam*HI and *Bgl*II restriction sites. In order to fill in the overhanging single stranded DNA, the Klenow fragment of DNA polymerase was used to synthesize dsDNA in the presence of 33 μM dGTP, dCTP, dTTP and 100 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; 10 μCi/μl). This reaction was allowed to proceed for 15 min at 25°C. The volume was made up to 100 μl with TE pH 8.0 and the unincorporated nucleotides were separated from the labelled dsDNA by elution through two Sephadex G-50 spin columns (Sambrook et al., 1989). The final eluates were collected and the incorporated radioactivity measured by liquid scintillation spectrometry.

Briefly, the G-50 spin columns were prepared by adding 1 ml of a Sephadex G-50 resin (Pharmacia) equilibrated in TE pH8.0 to a 1 cc syringe plugged with sianylized glass wool. The resin was washed twice with 100 μl of TE pH 8.0 by spinning for 4 min at 2000 x *g*.

2.2.8.2 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift analysis (EMSA) (Garner and Revzin, 1981) was performed to investigate protein-DNA interactions between nuclear receptors and DNA binding sites. Each 15 μl binding reaction contained Buffer C ((Andrews and Faller, 1991): from a 3 X stock: 20 m Hepes pH 7.9, 25% glycerol (v/v), 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1.5 mM $MgCl_2$ and 0.5 mM DTT), 4 μg of BSA, 4 μg of

sonicated salmon testes DNA, 8 μg of poly dI•dC-poly dI•dC (Pharmacia), 0.2 pmol of radiolabelled DNA probe and the protein(s) of interest. Two sources of protein were used in the described work. The first EMSA analysis described, utilizes PPARs obtained from the extracts of COS-1 cells transfected with PPAR expression vectors. In this case, the amount of protein added to each binding reaction was normalized with the addition of BSA. The remaining EMSA utilized proteins synthesized *in vitro* in reticulocyte lysate. In this case, 'naïve' translation reaction was added to normalize for the addition of protein and reaction buffer. The binding reactions were incubated at 28°C for 25 min and resolved on a non-denaturing 4% polyacrylamide gel (30:0.8 acrylamide: bisacrylamide, prerun for 4 h at 230 V prior to loading sample) in 0.25 X TBE buffer at 230 V/4°C. The gels were dried and autoradiographed to visualize complex formation.

2.2.9 Overexpression/Purification of Maltose Binding Protein Fusions

2.2.9.1 Overexpression of MBP Fusion Proteins

In vitro detection of protein-protein interactions were determined by performing solid-phase capture assays with maltose binding protein (MBP) fusions of PPARα and RXRα. The initial overexpression of the fusion proteins was performed by inoculating 1 L of 2YT+Amp media warmed to 37°C with 10 ml of a saturated culture of DH5α *E.coli* transformed with the fusion protein expression vector. The culture was grown to an OD_{600} of 0.5 and isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM to induce expression of the fusion proteins. After an induction period of 2 h, the cells were harvested by centrifugation and resuspended in 20 ml of Column Buffer (20 mM Tis-HCl, 200 mM NaCl, 1 mM EDTA). This cell suspension was equally divided between two 15 ml polystyrene tubes and the cells were lysed using a probe

sonicator (four 30 s bursts interspersed with 30 s incubations on ice). The cell debris was pelleted by centrifugation at 35,000 x *g* and the supernatant saved for column purification.

2.2.9.2 Purification of MBP Fusion Proteins

The MBP fusion proteins were purified from the cell lysate by affinity chromatography on a column composed of a solid matrix complexed to a maltose polymer (Amylose Resin, New England Biolabs). The lysate was loaded onto the column (flow rate 1 ml/min, 2.3 cm diameter column, 10 ml column volume previously equilibrated with 10 column volumes of Column Buffer) and washed with 10 column volumes of Column Buffer. The protein was eluted with Column Buffer containing 10 mM maltose and 1 ml fractions of the eluate collected. Protein elution was monitored by a 'microtitre Bradford Assay' in which 4 µl of Bradford Reagent (Bio Rad) was placed in a well of a 96 well microtitre plate along with 16 µl from each 1 ml fraction collected. Fractions that contained protein were further analyzed by SDS-PAGE and the appropriate fractions were pooled and dialyzed against Column Buffer+20% glycerol overnight. Finally, the protein concentration was quantitated by the Bradford Assay and checked once more by SDS-PAGE to ensure purity. The column was regenerated by washing with 1 column volume of ddH_2O, 3 column volumes of 0.1% SDS, 3 column volumes of ddH_2O and 3 column volumes of Column Buffer.

2.2.9.3 MBP Fusion Solid-Phase Capture Assay

To characterize the *in vitro* interaction between PPARα and RXRα and a radiolabelled, *in vitro* synthesized candidate protein; solid phase capture assays were performed. Essentially, this comprises a determination of the ability of the candidate

protein to bind to an MBP fusion of RXRα or PPARα complexed to amylose resin. Briefly, the binding substrate was prepared by incubating amylose resin with the MBP fusion (4.5 mg protein/ml resin) in a microfuge tube, washing twice with 10 bed volumes of Column Buffer and finally resuspending the MBP fusion complexed beads with Column Buffer as a 50% slurry. The binding reaction was composed of 20 µl of slurry, 5 µl of programmed reticulocyte lysate and 500 µl of Column Buffer supplemented with 0.2% (v/v) Nonidet-P40 and 2% bovine serum albumin (BSA). The binding reaction was incubated at room temperature for 30 min with continuous mixing. The beads were sequentially washed with 10 column volumes of: Column Buffer containing 0.2% (v/v) Nonidet-P40 and 2% BSA; Column Buffer supplemented with 0.1% (v/v) Nonidet-P40 and 2% BSA; and Column Buffer alone. Bound material was eluted by boiling the beads in SDS-PAGE loading buffer, analyzed by SDS-PAGE and visualized by autoradiography.

2.2.10 Determination of Protein Concentration by the Bradford Assay

Protein concentration was determined utilizing Bradford reagent (BioRad) and bovine serum albumin (Pharmacia) as a calibration standard (Brandford, 1976).

2.2.11 Maintenance of *Saccharomyces cerevisiae*

Various strains to *Saccharomyces cerevisiae* were cultured according to standard methods (Ausubel et al., 1987). Yeast lacking plasmids were cultured in YDP media or on YPD plates. However, yeast containing plasmids were cultured synthetic complete media (SC). This media lacked specific amino acids for which the presence of the plasmid marker could rescue the auxotrophic phenotype of the yeast. Typically, SC media lacked leucine, tryptophan or histidine

2.2.12 Introduction of DNA into Yeast

2.2.12.1 Transformation using Lithium Acetate

In order to introduce plasmids into yeast, the following low efficiency yeast transformation protocol was followed (Elble, 1992). Briefly, 1 ml of a fresh, saturated liquid culture of yeast was pelleted in a microfuge tube. 10 μg of sonicated salmon testes DNA (Sigma) and 1 μg of each plasmid was added to the pellet and vortexed. 0.5ml of PLATE solution (45% polyethylene glycol 3350 (PEG-3350: Sigma), 100 mM LiAc, 10 mM Tris-HCl pH 7.5, 1mM EDTA) was added and the mixture vortexed again. The cells were incubated overnight at room temperature and 100 μl of cell suspension was plated out on appropriate solid media the following day.

2.2.12.2 Transformation by Electroporation

Electroporation was utilized for the transformation of yeast with the human HeLa S3 cell GAL4 AD-cDNA fusion library (Ausubel et al., 1987). A 500 ml culture of yeast was grown overnight to a cell density of 1 x 10^8 cells/ml. This culture was centrifuged at 4,000 x *g* for 10 min at 4°C. The pelleted cells were resuspended in 80 ml of sterile ddH_2O. 10 ml of 10 X TE buffer (100 mM Tris pH 7.5, 10 mM EDTA) and 10 ml of 10 X LiAc stock solution (1 M LiAc pH 7.5) was added to the cell suspension and incubated at 30°C with gentle shaking for 45 min. 2.5 ml of 1 M DTT was added and the suspension incubated a further 15 min at 30°C with gentle shaking. The volume of the suspension was brought up to 500 ml with ddH2O. The cells were pelleted three times and sequentially resuspended in 500 ml of ddH2O, 50 ml of 1 M sorbitol and finally 0.5 ml of 1 M sorbitol. In a microfuge tube, 100 μl of the yeast cell suspension was mixed with 100 ng of plasmid DNA. This mixture was then transferred to an

electroporation cuvette (BioRad: 0.2 cm electrode gap). The electroporator (BioRad) was set at 200 Ω, 25 μF and 1.5 kV. 1 ml of ice cold 1 M sorbitol was added to the cuvette to rescue the yeast and this solution was plated out on appropriate SC plates containing 1 M sorbitol. In addition, 1 μl of this mixture was plated onto five SC(-Leu-Trp) plates to determine the total number of transformants and the transformation efficiency obtained.

2.2.12.3 Large Scale Yeast Transformation

The following method of transformation of yeast with the human liver cDNA-GAD fusion library was chosen for the large number of transformants obtained albeit at a lower transformation efficiency than by electroporation (Schiestl et al., 1993). A 50 ml starter culture of SC(-Leu) media was inoculated with HF7c yeast transformed with pGBD-NΔmPPARα and grown overnight to saturation. The cell density of this culture was determined and 2.5 x 10^9 cells were added to 500 ml of prewarmed YPD media. This culture was incubated at 30°C with shaking until the cell density reached 2 x 10^7 cells/ml. From this point on, unless noted, all equipment and solutions were ice cold and cells maintained on ice. Cells were pelleted three times by centrifugation for 10 min at 4000 x *g* and sequentially resuspended in 200 ml of ddH_2O, 10 ml of ddH_2O and finally 4.5 ml of 100 mM LiAc. Added to this final suspension was 5 ml of sonicated salmon testes DNA (Sigma: previously boiled for 5 min and flash chilled on ice), 24 ml of 50% w/v PEG-3350 (Sigma), 3.6 ml of 1 M LiAc, 2 ml of ddH_2O and 270 μg of Matchmaker liver cDNA plasmid library. The cells were incubated for 30 min at 30°C followed by a heat shock of 20 min at 42 °C. Finally, the yeast cells were pelleted and resuspended in 30 ml of ddH_2O and 0.5 ml of this mixture was plated onto each of sixty

SC(-Leu-Trp-His) plates. In addition, 1 μl of cells was plated onto five SC(-Leu-Trp) plates to determine the total number of transformants and the transformation efficiency obtained.

2.2.13 Yeast Two-hybrid System I: Protein-Protein Interactions

The yeast two-hybrid system was utilized to characterize the interaction between PPARα and RXRα. The premise of this system is that two interacting proteins, one fused to the GAL4 DBD and the other to the GAL4 AD and coexpressed in yeast will result in functional reconstitution of GAL4 (Fields and Song, 1989). A heterologous reporter gene such as β-galactosidase under the control of the GAL4 DNA binding site, will be activated by this artificially reconstituted GAL4. To this end, expression plasmids of GAL4 DBD-(RXRα or PPARα) and GAL4 AD-(RXRα or PPARα) fusion proteins were transformed into the *S. cerevisiae* strain Y190 by the low efficiency technique previously described. In this system, one indication of the presence and strength of the interaction between two candidate proteins is the production of β-galactosidase. As such, β-galactosidase activity of the transformed yeast was assayed by two different methods: an agarose overlay assay and a liquid assay.

2.2.13.1 Assay of β-galactosidase Activity I: Agarose Overlay

The agarose overlay, a qualitative assay, entailed covering yeast growing on solid medium with a molten agarose solution (0.5% agarose (w/v) , 0.1% SDS (w/v), 0.5 M $NaPO_4$ buffer pH 7, 2% dimethylformamide (v/v), 0.05% X-gal (w/v)). Once the agarose had solidified, the plates were placed in a 30°C incubator and the appearance of blue colour, indicative of β-galactosidase activity, was monitored.

2.2.13.2 Assay of β-galactosidase Activity II: Liquid Assay

Quantitative determination of the strength of the interaction between two proteins in the yeast two hybrid system entailed measuring the β-galactosidase activity of a culture of yeast growing exponentially in liquid media (Ausubel et al., 1987). Briefly, 5 ml of prewarmed yeast media was inoculated with 500 µl of an overnight culture. These diluted cultures were incubated until the OD_{600} reached 0.5 at which point two 1 ml aliquots of each culture was transferred to microfuge tubes. These cells were pelleted and resuspended in 1 ml of Buffer Z (100 mM $NaPO_4$ buffer pH 7, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol). The OD_{600} was determined of the first suspension. The cells in the second suspension were broken open with four cycles of freezing in liquid N_2 followed by thawing in a 30°C water bath. 0.2 ml of 4 mg/ml *o*-nitrophenyl-β-D-galactoside was added to each crude lysate. Once yellow colour had developed, the reaction was stopped by the addition of 500 µl of 1 M Na_2CO_3. The cellular debris was pelleted by centrifugation and the OD_{420} and the OD_{550} of the supernatant was measured.

The β-galactosidase activity was calculated using the following formula:

$$\text{Units of } \beta\text{-galactosidase activity} = \frac{1000 \times [(OD_{420}) - (1.75 \times OD_{550})]}{(t) \times (v) \times (OD_{600})}$$

where
- t = time of reaction (min)
- v = volume of reaction (ml)
- OD_{600} = cell density
- OD_{420} = absorbance by *o*-nitrophenyl and light scattering by cell debris
- OD_{550} = light scattering by cell debris

2.2.14 Yeast Two-hybrid System II: PPARα-Interacting Protein Screen

A powerful application of the yeast two-hybrid system is as a genetic screen for interacting proteins (Fields and Song, 1989; Chien et al., 1991). A cDNA library fused to the coding sequence of the GAL4 AD is constructed from mRNA isolated from a desired source. This plasmid library is used to transform yeast that contain the expression vector of the GAL4 DBD fused to your target or bait protein. The transformed yeast are grown on SC(-Leu-Trp-His) plates. The lack of leucine and tryptophan select for yeast that contain both expression plasmids while the lack of histidine selects for those yeast that express the wild type HIS3 gene. This gene encodes an essential enzyme of the histidine synthetic pathway that is deleted from the genome. However, an artificially integrated HIS3 reporter gene regulated by the GAL4 enhancer element has been integrated into the genome of the *S. cerevisiae* strain HF7c. Thus, histidine selection results in a positive selection for functional reconstitution of GAL4 due to the interaction between the bait protein and a polypeptide encoded by the cDNA insert. The inhibitor of HIS3, 3-aminotriazole, can be added to increase the stringency of selection by selecting for yeast that produce higher levels of HIS3, indicative of a stronger interaction between the bait protein and the cDNA encoded polypeptide.

2.2.14.1 Screening of the Human HeLa S3 Cell cDNA Library

The first cDNA library screened was a human HeLa S3 cell Matchmaker library (Clontech). This library was transformed by electroporation (previously described) into HF7c yeast that contained pGBD-NΔmPPARα. Yeast that exhibited a His+ phenotype were analyzed as described below. The human liver X receptor was identified as a PPARα interacting protein in this screen.

2.2.14.2 Screening of the Human Liver cDNA Library

Since, PPARα is predominantly expressed in liver, a human liver Matchmaker library (Clontech) was obtained and used in two large scale screens.

The cDNA plasmid library was transformed into HF7c yeast that contained pGBD-NΔmPPAR by the previously described large scale method. 1.3×10^7 transformed yeast were plated onto SC(-Leu,-Trp,-His) plates and colonies of yeast that grew under these conditions were isolated. In total, 1400 His+ colonies of yeast were selected and subsequently replica plated onto SC(-Leu,-Trp,-His) plates that were supplemented with either 5 mM, 10 mM or 20 mM 3-aminotriazole. In total, 68 transformants grew on plates containing 5mM 3-aminotriazole, 40 transformants on 10 mM 3-aminotriazole and 36 transformants on 20 mM 3-aminotriazole. The transformants that grew in the presence of 15 mM and 20 mM 3-aminotriazole were selected for further analysis. Human RIP140 and human RXRα were identified as PPARα interacting proteins in this screen.

A second screen has been performed in a similar manner with the exception that the transformed yeast were plated directly onto selective media that contained 20 mM 3-aminotriazole. Of a total 1.8×10^7 transformed yeast, 33 transformants exhibited a His+ phenotype under these stringent conditions.

2.2.14.3 Isolation of Plasmids from His+ Yeast

The plasmids expressing the GAL4 AD-cDNA fusions were isolated from clonally purified yeast by preparing a total yeast DNA extract (Ausubel et al., 1987) and transforming *E. coli* to rescue the plasmids. Briefly, 1.5 ml of a saturated culture of yeast was pelleted in a microfuge tube and resuspended in 200 µl of Breaking Buffer (2% (v/v)

Triton X-100, 1% (v/v) sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). The cells were broken open and the plasmid DNA extracted by adding 0.3 g of sterile, acid washed glass beads (Sigma Chemical Company) and 200 µl of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol and vortexing this mixture for 2 min. The cellular debris was pelleted by centrifugation and 1 µl of the supernatant was used to transform DH10β *E. coli* by electroporation as previously described. The plasmid DNA was isolated from bacterial colonies that subsequently grew on 2YT+Amp plates. Restriction enzyme analysis of the plasmid DNA was carried out to identify the GAL4 AD-cDNA fusion containing plasmids.

2.2.14.4 Evaluation of Potential PPARα Interacting Proteins

GAL4 AD-cDNA fusions that potentially interacted with the GAL4 DBD-mPPARα fusion were evaluated by transforming the *S. cerevisiae* strain Y190 with all combinations of the relevant plasmids and controls. The transformed yeast were then assayed for β-galactosidase activity by the agarose overlay method previously described. The expression vector of a candidate PPARα interacting protein would exclusively demonstrate β-galactosidase activity in the presence of pGBD-NΔmPPARα, the PPARα expression plasmid. Additionally, no β-galactosidase activity was detected when the candidate interacting protein was expressed in yeast alone, or in combination with the GAL4 DBD or another unrelated GAL4 DBD fusion protein.

Once a candidate GAL4 AD-cDNA fusion expression plasmid was confirmed to encode a PPARα interacting protein, the cDNA insert was sequenced to determine the identity of the encoded polypeptide. Using the BLAST search engine (Altschul et al.,

1990), DNA and protein databases were searched to identify the encoded protein or to find homologous sequences.

2.2.15 Maintenance of Mammalian Cell Lines

2.2.15.1 Maintenance of BSC-40 Cells

BSC-40 cells (Brockman and Nathans, 1974) were cultured as a monolayer at 37°C / 5.0% CO_2 in Dulbecco's modified Eagle's medium. This medium was supplemented with 10% calf serum, 1% penicillin/streptomycin and 1% L-glutamine.

2.2.15.2 Maintenance of COS-1 cells

COS-1 cells (Gluzman, 1981) were cultured as a monolayer at 37°C / 5.0% CO_2 in Dulbecco's modified Eagle's medium. This was supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin cocktail and 1% L-glutamine.

2.2.15.3 Subculturing of Cells

Subculturing of confluent cell cultures involved washing the cells with sterile PBS (136.8 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4), detaching them by trypsinization (1 X trypsin-EDTA) and finally seeding new plates with an appropriate number of cells.

2.2.16 Introduction of DNA into Mammalian Cells

DNA was transfected into mammalian cells by the calcium phosphate co-precipitation method (Graham and van der Eb, 1998).

2.2.17 Overexpression of PPARs in COS-1 Cells

2.2.17.1 Transient Transfections of COS-1 Cells

In order to overexpress the various PPARs in mammalian cells, the PPAR expression plasmids were transfected into COS-1 cells. The calcium phosphate/DNA

coprecipitate was prepared by adding 60 μg of PPAR expression plasmid, 20 μg of sonicated salmon testes DNA and an appropriate amount of ddH_2O to give a final volume of 1.75 ml. 250 μl of 2 M $CaCl_2$ was added to the DNA suspension. Immediately, the DNA/$CaCl_2$ mixture was added dropwise to 2 ml of 2 X HBSS (0.28 mM NaCl, 50 mM HEPES pH 7.12, 1.5 m $Na_2HPO_4 \cdot 7H_2O$) while vortexing gently. The precipitate was incubated for 20 min at room temperature at which point 480 μl of the solution was added to each of four subconfluent 10 cm plates of COS-1 cells. Dimethyl sulphoxide was added to a final concentration of 0.5% (v/v). After 16 h, the cells were washed once with PBS and fresh media + 0.5% dimethyl sulphoxide was added. 48 h after the addition of the DNA precipitate to the cells were washed twice with cold PBS, harvested by scraping into 1.5 ml of cold PBS and transferred to a microfuge tube.

2.2.17.2 Preparation of COS-1 Cell Nuclear Extracts

In order to prepare an extract of nuclear proteins from the COS-1 cells that had been transfected with the PPAR expression plasmids, a method combining hypotonic cell lysis and high salt extraction of the nuclear proteins was used (Andrews and Faller, 1991). Harvested cells were washed with ice-cold PBS, resuspended in 400 μl of ice-cold Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and allowed to swell on ice for 10 min. The cells were vortexed for 10 s, pelleted, resuspended in 100 μl of cold Buffer C (20 mM Hepes pH 7.9, 25% glycerol (v/v), 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1.5 mM $MgCl_2$ and 0.5 mM DTT), and kept on ice for 20 min. Cellular debris was pelleted and the supernatant stored at -70°C after determination of the protein concentration.

2.2.18 Transient Transfections Assays

2.2.18.1 Transient Transfection of BSC-40 Cells

Transient transfection assays were performed to assess the *in vivo* effect of various factors on PPAR/RXR mediated signaling. Just before the preparation of the DNA precipitate (Graham and van der Eb, 1998), the cells were washed once with PBS and then provided with 5 ml of modified Dulbecco's modified Eagle's medium (no phenol red, 1% penicillin/streptomycin cocktail, 10% DEAE-dextran coated charcoal stripped fetal bovine serum, 4% L-glutamine). The cells were returned to the incubator to allow the temperature and pCO_2 of the media to equilibrate during the preparation of the calcium-phosphate/DNA coprecipitate.

Wy 14,643 was used in these studies as a model ligand/activator of PPARα. As such, the media was supplemented with Wy 14,643 to a final concentration of 100 μM from a 200 mM stock in dimethyl sulphoxide. The media of cells to which no drug had been added, was appropriately supplemented with dimethyl sulphoxide to a final concentration of 0.5% (v/v).

To prepare the precipitate, appropriate amounts of DNA were added along with adequate ddH_2O to give a final volume of 876 μl. This included the luciferase reporter plasmid and appropriate combinations of eukaryotic expression vectors. In order to control for variations in the amounts of plasmid DNA, the reactions were supplemented with compensatory amounts of empty expression vectors. Sonicated salmon testes DNA was used to maintain the total amount of DNA in each reaction at 60 μg. To this mixture was added 124 μl of 2 M $CaCl_2$. Immediately thereafter, this solution was added dropwise to 1 ml of 2 X HBSS while gently vortexing and the precipitate was incubated

at room temperature for 20 min. Subsequently, 480 µl of the precipitate was added to each subconfluent 60 mm plate of subconfluent BSC-40 cells. The precipitate was incubated on the cells for 16hours at which time the cells were washed once with PBS and supplemented with fresh modified Dulbecco's minimal essential media (+/- Wy 14,643). 48 h after the addition of the DNA precipitate, the cells were washed with PBS and cell lysates were prepared as described below.

2.2.18.2 Measurement of Luciferase Activity

Cell lysates were prepared by adding 400 µl of Reporter Lysis Buffer (Promega) to each 60 mm plate. The cells were immediately scraped off with a cell lifter, collected in microfuge tubes and stored on ice. This cell suspension was vortexed for 10 s and cellular debris pelleted by centrifugation at 12,000 x *g* for 2 min/4°C . Finally, 100 µl of the supernatant was saved for the determination of luciferase activity and protein concentration. If not immediately assayed, the lysates were stored at -70°C.

The measurement of the luciferase activity entailed the addition of 20 µl of cell lysate to 100 µl of luciferase reaction buffer (470 µM luciferin, 270 µM coenzyme A, 530 µM ATP, 33.3 µM DTT, 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA). The resulting fluorescence was measured in a Lumat LB9507 luminometer and normalized according to the protein concentration in each sample.

2.2.18.3 Treatment of Data from Transient Transfections

The data depicted in the transient transfections represents the combination of two separate experiments performed in duplicate. While the absolute values between experiments varies considerably, the relative levels of activity between the conditions

tested remained constant. In order to combine the data from separate experiments, within each experiment the activity achieved in the presence of the RXRα and PPARα expression vectors, the reporter construct and the PPARα agonist Wy 14,643 was arbitrarily set to a value of 100. The levels of activity achieved under all other conditions was adjusted appropriately. The data from the two separate experiments was then combined by determining the average of the four data points, two from each experiment. The error of the combined data was calculated by determining the standard deviation of the four values used to calculate the average level of activity.

RESULTS AND DISCUSSION

3. THE HETERODIMER OF PPARα/RXRα MEDIATES THE EFFECTS OF PEROXISOME PROLIFERATORS

Peroxisomes are cellular organelles predominantly found in the liver and the cortex of the kidneys (reviewed in Lazarow and Fujiki, 1985). They are defined by the presence of an oxidase and a catalase that generate and decompose hydrogen peroxide. Remarkably, in rodents, the number and size of these organelles is dramatically induced upon exposure to a high fat diet, starvation and the diverse class of xenobiotic compounds referred to as peroxisome proliferators. Many of the characterized peroxisomal enzymes are involved in either the catabolism or anabolism of fatty acids. Thus, the induction of peroxisomes was proposed to enhance the ability of an organism to metabolize fatty acids.

Peroxisomes contain a fatty acid β-oxidation pathway that is distinct from that of the mitochondria, and whose activity is induced by peroxisome proliferators (Lazarow and deDuve, 1976). The enzymes of the peroxisomal β-oxidation pathway include the acyl-CoA oxidase (AOx), the hydratase/dehydrogenase bifunctional enzyme (HD) and a ketoacyl-CoA thiolase (Osumi et al., 1987; Ishii et al., 1987; Hijikata et al., 1990; Bodnar and Rachubinski, 1991). The increase of β-oxidation activity by peroxisome proliferators was attributed to the induction of the transcription of these genes

(Lazarow et al., 1982; Furuta et al., 1982; Reddy et al., 1986). In order to understand the manner in which fatty acid metabolism was regulated at the level of transcription, the induction of the enzymes of the peroxisomal β-oxidation pathway by peroxisome proliferators provided an excellent model system.

Since the activation of transcription involves the binding of transcriptional activators to the promoters of target genes, the identification of DNA sequences that conferred responsiveness to peroxisome proliferators was the focus of a number of research groups.

B. Zhang, of our research group, successfully identified a 196 bp region from the promoter of the HD gene that, once inserted into the promoter of a heterologous gene, was necessary and sufficient to confer transcriptional responsiveness to peroxisome proliferators (Fig. 3.7, *Panel A*) (Zhang et al., 1992). This element was termed the HD peroxisome proliferator-responsive element (HD PPRE). The HD PPRE conferred responsiveness to peroxisome proliferators only in a hepatocyte cell line and not a kidney cell line. This liver-specific activity supported the localization of the effects of peroxisome proliferators primarily to the liver. Furthermore, it was shown that liver-specific factors from hepatocyte cellular extracts interacted with the HD PPRE but not from kidney nor epithelial cellular extracts. This supported the model that the HD PPRE bound a liver specific transcription factor(s) to mediate transcriptional activation by peroxisome proliferators.

The DNA elements that mediated the activation by peroxisome proliferators of the acyl-CoA oxidase gene (AOx) had also been identified and characterized (Fig. 3.7, *Panel A*) (Dreyer et al., 1992; Osumi et al., 1991; Tugwood et al., 1992). The AOx

PPRE functioned in a similar manner to the HD PPRE. Of interest was the fact that both of these elements contained sequences that were similar to repeats of the sequence TGACCT, the consensus binding site of nuclear receptors (Fig. 3.7, *Panel A* and Fig. 1.5). While the AOx PPRE is composed of two direct repeats of the TGACCT motif seperated by 1 nucleotide (DR1), the HD PPRE is more complex: it is composed of two DR1 elements seperated by two intervening bases. Further work by B. Zhang had demonstrated that factors from liver cell extract bound to these regions of the HD PPRE using Dnase I footprint analysis and methylation interference assay (Zhang et al., 1993). Furthermore, the mutation of residues in this region that abrogated binding by cellular factors correlated with the loss of the ability of the HD PPRE to mediate the transcriptional activation by peroxisome proliferators.

Since the integrity of these putative nuclear receptor binding sites was required for the binding of cellular factors and the ability to confer transcriptional responsiveness to peroxisome proliferators, nuclear receptors were candidate transcriptional activators to bind the HD and AOx PPREs and mediate the effects of peroxisome proliferators. This would be consistent with the role of ligand-activated nuclear receptors since their tissue expression pattern localizes the ligand responsiveness to these tissues. Thus, the pattern of tissues that are responsive to peroxisome proliferators could be due to the expression of a nuclear receptor in these cells that would bind to PPREs and, in response to peroxisome proliferators, activate the transcription of target genes.

The previous cloning of PPARα, a nuclear receptor that was activated by peroxisome proliferators and was expressed primarily in tissues in which peroxisome proliferators exerted their effects, provided a candidate nuclear receptor that could bind to

PPREs and mediate the effects of peroxisome proliferators (Issemann and Green, 1990; Dreyer et al., 1992). At this time, work by Tugwood et al., 1992, demonstrated that PPARα bound to the AOx PPRE *in vitro* and was sufficient to confer responsiveness to peroxisome proliferators *in vivo*. Since the AOx and HD PPREs were similar in structure and function, PPARα was a likely candidate nuclear receptor that would bind to the HD PPRE and mediate the transcriptional activation by peroxisome proliferators.

My involvement in this research project began with the goal of establishing that PPARs bound to the HD PPRE. This was followed by an investigation and characterization of the binding of PPARs to the HD and AOx PPREs. The results presented in this section detail the first demonstration that three PPAR isotypes, α, β and γ, bound to the HD PPRE (Marcus et al., 1993). These initial results were followed by the demonstration that PPARs bound to the AOx and HD PPREs cooperatively with RXRα. These results added PPAR to the growing list of nuclear receptors, such as RAR, VDR and TR, that dimerized with RXRα to bind their appurtenant DNA binding sites and contributed to the growing appreciation that RXRα acted as a promiscuous DNA binding partner for many nuclear receptors (Zhang et al., 1992a; Kliewer et al., 1992b). Although the binding of PPAR to DNA was necessary, complementary *in vivo* work by S. Marcus demonstrated that it was not sufficient for transcriptional activation. Thus, this entailed one of the first demonstrations that the DNA binding site could act as an allosteric modulator of the transcriptional activity of PPARs.

3.1 CHARACTERIZATION OF PPAR/RXR BINDING TO DNA

3.1.1 PPARs Expressed in COS-1 Cells Bind the HD and AOx PPREs

At the outset of this study, five PPARs had been characterized to date including the α, β and γ subtypes from *Xenopus laevis* (African clawed frog) and the α subtype from mouse and rat (Dreyer et al., 1992; Issemann and Green, 1990; Göttlicher et al., 1992). To determine if these PPARs bound to the AOx and HD PPREs, nuclear lysate from COS-1 cells transfected with expression plasmids for xPPARα, xPPARβ, xPPARγ and mPPARα (x, *X. laevis*; m, mouse) was used as a source of overexpressed PPAR. S. Marcus, a member of our research group, had demonstrated that PPARs expressed in COS-1 cells were able to mediate the activation of a target gene in response to peroxisome proliferators, an indication that the PPARs expressed in COS-1 cells were functional (Marcus et al., 1993).

The nuclear lysates containing the overexpressed PPARs were then utilized in electrophoretic mobility shift assay (EMSA) analysis to assess whether these PPARs were able to bind to dsDNA probes corresponding to the HD and AOx PPREs (Fig. 3.1). In this assay, the binding of proteins to the radiolabelled DNA probe results in the creation of a protein/DNA complex of slower mobility than that of the unbound DNA probe. This manifests itself as the appearance of radiolabelled DNA that does not migrate as far during the resolution of the DNA complexes by electrophoresis.

The results depicted in Figure 3.1 demonstrate that nuclear lysate prepared from COS-1 cells does not show binding to either the HD or AOx PPREs (lanes b and h). However, nuclear extracts from COS-1 cells expressing the various PPARs resulted in the formation of a protein-DNA complex on both the AOx and HD PPREs (lanes c-f,

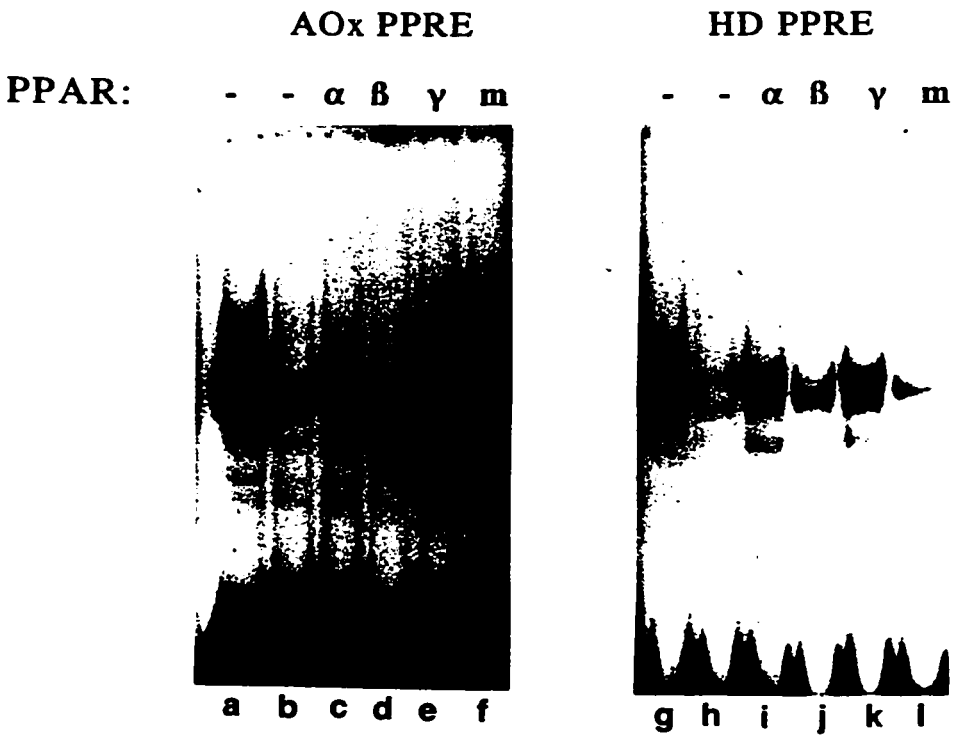


Figure 3.1: PPARs expressed *in vivo* bind to the AOx and HD PPREs.
Nuclear extracts prepared from COS-1 cells transfected with pSG5 (lanes b and h) or the pSG5 based expression vectors of *Xenopus laevis* PPARα (lanes c and i), PPARβ (lanes d and k), PPARγ (lanes e and k) and mouse PPARα (lanes f and l) were used in EMSA analysis with radiolabelled AOx PPRE probe (lanes b to f) and HD PPRE probe(lanes h to l). Cell extract from H4IIEC3 cells was incubated with the AOx and HD PPRE probes (lanes a and g respectively).

lanes i-l). The mobility of protein-DNA complexes were similar to the complex observed when the HD and AOx probes were incubated with nuclear lysate from H4IIEC3 cells (lanes a and g) , a peroxisome proliferator-responsive hepatocarcinoma cell line (Zhang et al., 1992; Zhang et al., 1993; Osumi et al., 1990). Therefore, the five PPARs that were overexpressed in COS-1 cells, including representatives of the α, β and γ subtypes, were all able to bind to the AOx and HD PPREs.

3.1.2 Factor in COS-1 Cell Extract Stimulates PPAR Binding to PPREs

To assess the ability of *in vitro* synthesized PPARs to bind to the HD PPRE, *in vitro* translated xPPARα, xPPARβ, xPPARγ, mPPARα and rPPARα (Fig. 3.2, *Panel A*) were utilized in EMSA with the radiolabelled HD PPRE (Fig. 3.2, *Panel B*). The addition of *in vitro* translated PPARs resulted in the formation of protein-DNA complexes (lanes c-g) while no complexes were detected with the addition of reticulocyte lysate (lane a).

In order to determine if cellular factors existed that could enhance the binding of PPARs to PPREs, nuclear extract from COS-1 cells was added to the EMSA reactions (lanes h-l). S. Marcus had demonstrated that the peroxisome proliferator mediated activation of a PPRE-controlled gene in COS-1 cells was dependent upon the exogenous addition of PPARs (Fig. 3.6) PPRE (Marcus et al., 1993). Thus, COS-1 cellular extract was chosen since these cells contained all the necessary factors for PPAR mediated induction of transcription. Complex formation in the presence of PPARs was enhanced by the supplementation of COS-1 nuclear extract (compare lanes c-g with lanes h-l) suggesting that this extract contained factors that acted cooperatively with all PPARs to bind DNA.

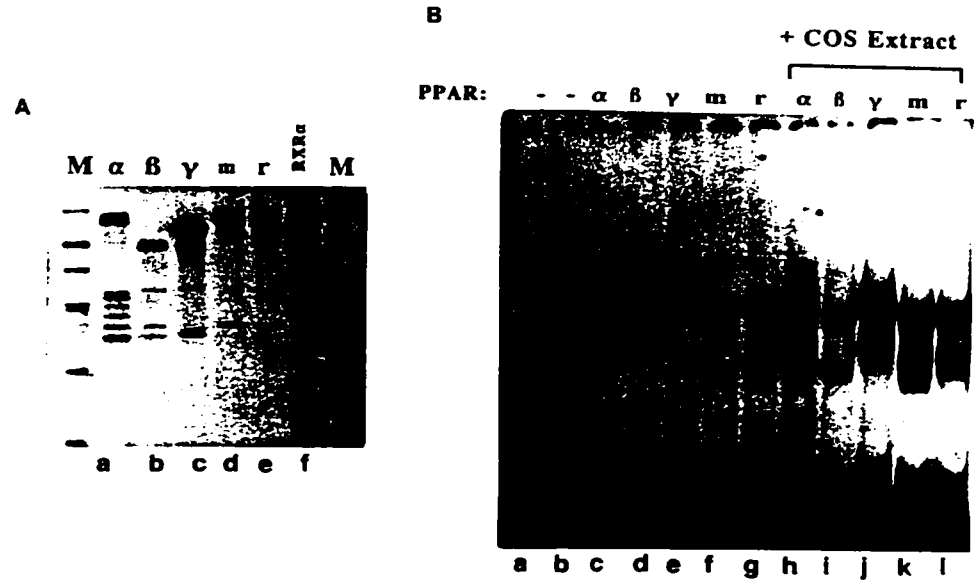


Figure 3.2: A cellular factor stimulates the binding of PPAR to DNA.
Panel A. The cDNAs of the Xenopus PPAR subtypes α, β and γ, mouse PPARα, rat PPARα and human RXRα (lanes a to f respectively) were transcribed and translated *in vitro* and the [^{35}S]methionine labeled products were analyzed by SDS-PAGE. The molecular weight markers (lane M) are expressed in kDa. *Panel B.* The various PPARs were synthesized in the absence of [^{35}S]methionine in parallel translation reactions with those depicted in *A*, and used in EMSA analysis with radiolabelled HD PPRE probe. *Xenopus laevis* PPAR α, PPARβ., PPARγ, mouse PPARα and rat PPARα were incubated with the HD PPRE probe in the absence (lanes c to g respectively) and in the presence of 0.2 μg of COS-1 cell nuclear extract (lanes h to l respectively). The HD PPRE probe was incubated alone or in the presence of 0.2 μg of COS-1 cell nuclear extract (lanes a and b respectively). The amount of reticulocyte lysate and protein was normalized by the appropriate addition of naïve reticulocyte lysate and bovine serum albumin.

3.1.3 PPARs Require RXRα to Bind AOx and HD PPREs

Since evidence pointed to the existence of a cellular factor that stimulated the binding of PPAR to PPREs, we set out to identify this factor. The recently discovered nuclear receptor, the retinoid X receptor α (RXRα), was one candidate (Mangelsdorf et al., 1990). At this time, this nuclear receptor had been shown to enhance the binding of the retinoic acid receptor (RAR), the vitamin D receptor (VDR) and the thyroid hormone receptor (TR) (Mangelsdorf et al., 1990; Zhang et al., 1992a; Kliewer et al., 1992b). It was proposed that RXRα formed heterodimers with TR, VDR and RAR to bind DNA. Furthermore, these heterodimers bound to a variety of nuclear receptor response elements composed of direct repeats (DRx) of the consensus half-site sequence TGACCT seperated by a number of nucleotides that varied from 1 to 5 (DR1 to DR5). The heterodimers of VDR/RXR, TR/RXR and RAR/RXR bound to DR3, DR4 and DR5 elements respectively. RXRα was capable of forming a homodimer in the presence of its agonist and binding to a DR1 response element (Zhang et al., 1992b). In summary, RXRα promiscuously interacted with nuclear receptors to bind DNA, recognized response elements of varying architecture and that RXR itself recognized a response element with similar architecture as the AOx and HD PPREs. Thus, RXRα was a candidate factor for stimulating the binding of PPAR to PPREs.

To test this hypothesis, the ability of *in vitro* translated RXRα to cooperatively bind with PPAR to the HD and AOx PPREs was assessed by EMSA analysis. As depicted in Figure 3.3, RXRα was unable to bind alone to either the HD or AOx PPREs (lane g). However, the binding of the three *X. laevis* PPAR subtypes and the α subtype

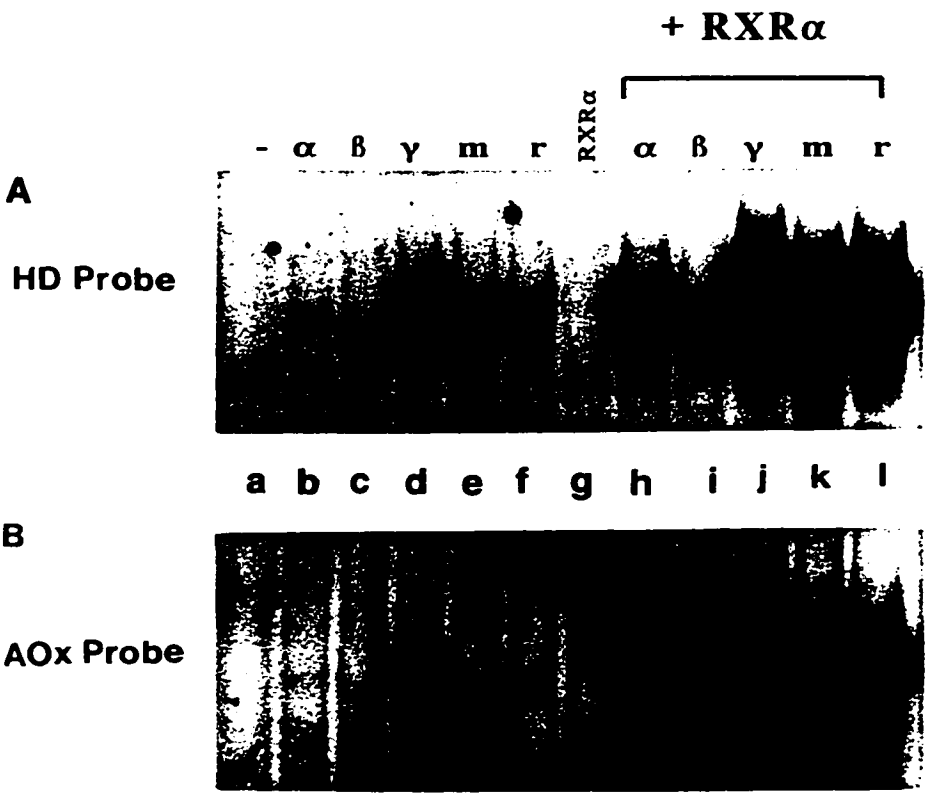


Figure 3.3: PPARs bind cooperatively with RXRα to the HD and AOx PPREs.
The various PPARs translated *in vitro* (Xenopus laevis PPARα (lanes b and h), PPARβ (lanes c and i), PPARγ (lanes d and j), mouse PPARα (lanes e and k) and rat PPARα (lanes f and l) were incubated with radiolabelled HD PPRE and AOx PPRE probes in EMSA analysis. In lanes a and g, no PPARs were added. These reactions were performed the absence (lanes a to f) or presence of *in vitro* translated human RXRα (lanes g to l). The amount of reticulocyte lysate added to each lane was normalized by the appropriate addition of naïve reticulocyte lysate.

of mouse and rat PPAR was greatly stimulated by the addition of RXRα (compare lanes b-f with lanes h-l). These results suggested that five PPARs assayed bound to PPREs cooperatively with RXRα.

3.1.4 RXRα is Present in the COS-1 and H4IIEC3 Cell Extract

While the cooperative binding of PPAR and RXRα to the HD and AOx PPREs was established, a number of issues remained to be resolved such as: the identity of the factor in COS-1 cells that enhanced PPARα binding; whether endogenous levels of RXRα in reticulocyte lysate were responsible for the low levels of PPAR binding in the absence of added RXRα (Fig. 3.3, lanes b-f); and the identity of the proteins in H4IIEC3 cell extracts that bound to the HD and AOx PPREs.

In order to ascertain whether RXRα was a component of the protein complex formed on the HD and AOx PPREs, EMSA analysis was performed in the presence of polyclonal antibody (Ab) directed against RXRα (Fig. 3.4). The addition of anti-RXRα Ab disrupted the protein-DNA complexes formed from H4IIEC3 nuclear lysate, *in vitro* translated PPARα alone, *in vitro* translated PPARα and RXRα, and *in vitro* translated PPARα with COS-1 nuclear lysate (compare lanes a-d with lanes e-h). In the case of the shifts produced by H4IIEC3 extract and *in vitro* translated RXRα/PPARα, a 'supershifted' complex of radiolabelled HD PPRE, RXRα, PPARα and anti-RXRα Ab, was produced (Fig. 3.4, lanes e and g, indicated by ►). The absence of a 'supershifted' complex with *in vitro* translated rPPARα or *in vitro* translated rPPARα plus COS-1 nuclear extract (lanes f and h) is probably the result of the initial low levels of protein-

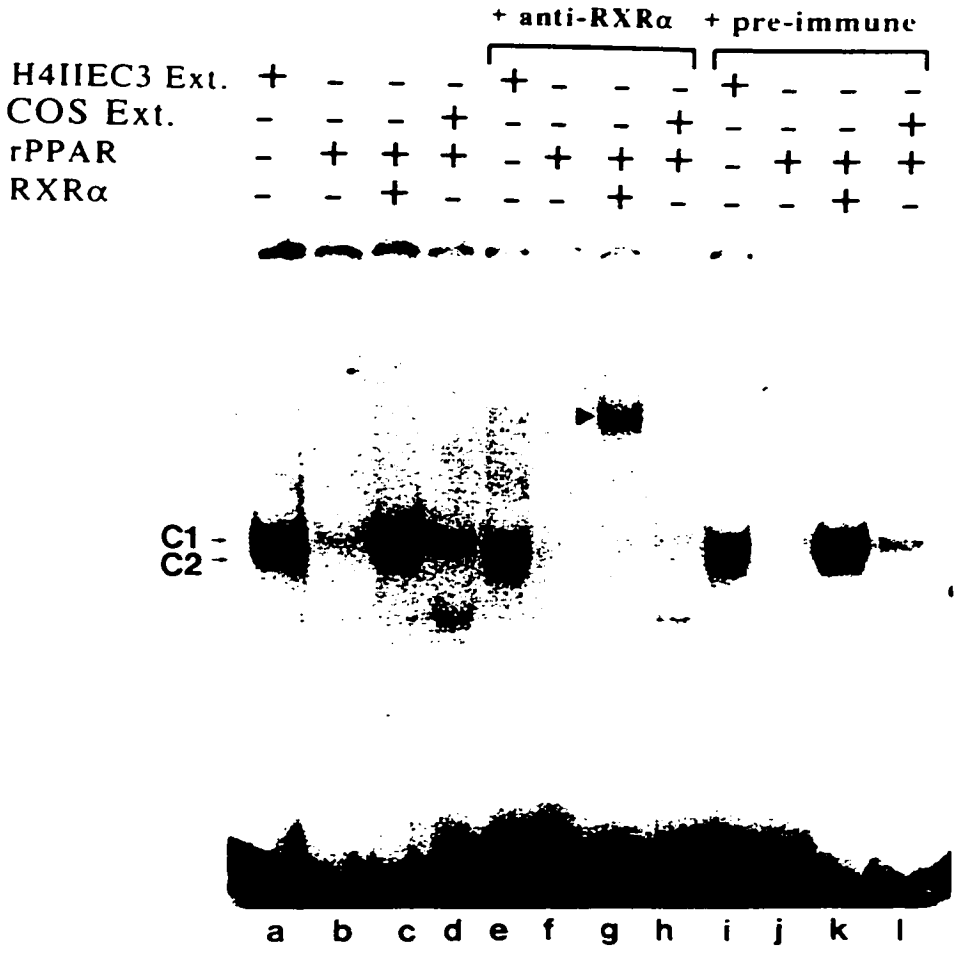


Figure 3.4: RXRα is present in complexes formed on the HD PPRE.
Radiolabelled HD PPRE was incubated in EMSA reactions with extract from H4IIEC3 cells (lanes a, e and i), *in vitro* translated rat PPARα (lanes b, f and j) supplemented with *in vitro* translated human RXRα (lanes c, g and k) or COS-1 cell nuclear extract (lanes d, h, and l). To these EMSA reactions was added 1 µl of polyclonal serum raised against human RXRα (lanes e to h) or preimmune serum (lanes i to l). C1 and C2 refer to the two protein DNA complexes formed on the HD PPRE when incubated with H4IIEC3 cell extract. The arrowhead corresponds to the 'supershifted' complex.

DNA complex formed. Therefore, RXRα is a component of all four protein-DNA complexes detected in this EMSA analysis.

Since the complex detected upon the addition of only *in vitro* translated PPARα contained RXRα (Fig. 3.4, compare lanes b, f and j), endogenous levels of RXRα in reticulocyte lysate supplied the cooperative binding partner for the *in vitro* translated PPARα. Furthermore, RXRα was the factor found in COS-1 cell extract that enhanced PPAR binding. Interestingly, the addition of H4IIEC3 nuclear lysate, resulted in the formation of two complexes (Fig. 3.3, lane a: C1 and C2). The addition of anti-RXRα antibody resulted in the disruption of the higher of the two complexes suggesting that the lower shift represented a complex of nuclear factor(s) bound to the HD PPRE of which RXRα was not a component (Fig 3.4, lanes a and e).

A number of other groups have demonstrated similar findings that PPAR cooperatively binds to PPREs with RXRα (Issemann et al., 1993; Kliewer et al., 1992c; Gearing et al., 1993; Bardot et al., 1993; Keller et al., 1993). In fact, additional factors are not required for the formation of the PPAR/RXR heterodimer since the addition of purified RXRα and PPARα was sufficient to form a protein-DNA complex with the HD PPRE (Chu et al., 1995).

3.1.5 Architecture of the PPAR/RXR Complex Bound to DNA

The presented findings of our work and that of others has established that PPAR and RXRα bind cooperatively to the HD and AOx PPREs (Marcus et al., 1993; Dreyer et al., 1992; Osumi et al., 1991; Tugwood et al., 1992). This work has since been expanded

upon by a number of groups that have focused on the binding of PPAR and RXR to PPREs.

The orientation of the PPAR/RXR heterodimer on the DR1 element (TGACCTxTGACCT) appears to be such that RXR binds to the 5' half site while PPAR binds to the 3' half site. This orientation is supported by studies that have demonstrated that PPAR was localized in the proximity of the 3' end of the PPRE and that PPAR recognized the 3' half-site resulting in the proposed orientation of RXR/PPAR depicted in Figure 3.5 (IJpenberg et al., 1997; DiRenzo et al., 1997; Palmer et al., 1995). Furthermore, the bases that flank the 3' half site influence the binding of the different PPAR subtypes to the PPRE while alterations in the region flanking the 5' half site had no effect (Palmer et al., 1995; Juge-Aubry et al., 1997; IJpenberg et al., 1997). Assuming that PPARs interact with this region of the PPRE, presumably through a putative carboxy-terminal extension of the DNA-binding domain (see Section 1.1.4.3), this provides further supporting evidence of the proposed orientation of the PPAR/RXR complex on the PPRE.

Finally, there exists only one documented determination of the affinity of the complex of PPAR/RXR for the AOx PPRE. This was determined to be K_d = 2.5 nmol, a value comparable to the K_d's determined for the complexes of TRα/RXRα, COUP-TF1, COUP-TFII and HNF4 binding to appurtenant response elements (Miyatmoto et al., 1997; Ladias et al., 1992).

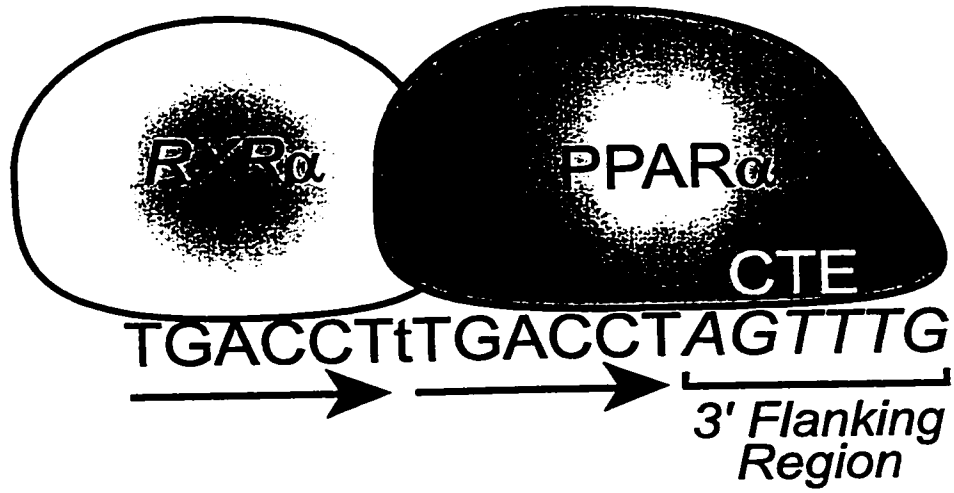


Figure 3.5: Schematic representation of PPARα/RXRα bound to the AOx PPRE. Based upon experimental evidence, it is proposed that RXRα recognizes the 5' TGACCT half site while PPAR binds to the 3' half site. The influence of the 3' flanking region of the PPRE on PPAR binding may be the result of the putative carboxy-terminal extension (CTE) interacting with this portion of the response element.

3.2 TRANSCRIPTIONAL ACTIVATION BY THE COMPLEX OF PPAR/RXR

3.2.1 PPAR and RXRα Cooperatively Activate Transcription

Since the PPAR bound cooperatively with RXRα to the HD and AOx PPREs, it was predicted that these two nuclear receptors would act cooperatively to mediate transcriptional activation. To demonstrate this cooperativity, transient transfection assays were used as a model system to evaluate PPAR activity. Firstly, a number of plasmids are introduced into a chosen cell line that does not express PPARs. These plasmids express either the genes of selected proteins (RXRα, PPARα) or a plasmid in which the transcription of a 'reporter' gene is under the control of the AOx PPRE located in the promoter region. The reporter gene used in these experiments was the luciferase gene, a gene that produces an enzyme with an easily measured activity (de Wet et al.,1987). The measured luciferase activity is taken to be indicative of the rate of transcription of the reporter gene. Finally, since this represents a model of peroxisome proliferator induced transcriptional activation, the activity of the reporter gene is measured in the presence and absence of the peroxisome proliferator and PPARα agonist, Wyeth 14,643 (Wy 14,643).

Figure 3.5 demonstrates that PPARα and RXRα act cooperatively to mediate the transcriptional activation by Wy 14,643 of the luciferase reporter gene under the control of the AOx PPRE. The addition of RXRα did not result in transcriptional activation that was significantly above that achieved with the reporter alone (AOx(X2)*luc*). The addition of PPARα alone did not demonstrate an appreciable increase in transcription in the absence of Wy 14,643. However, the addition of Wy 14,643 caused an increase in the luciferase activity, indicating a moderate induction in the transcription of the reporter

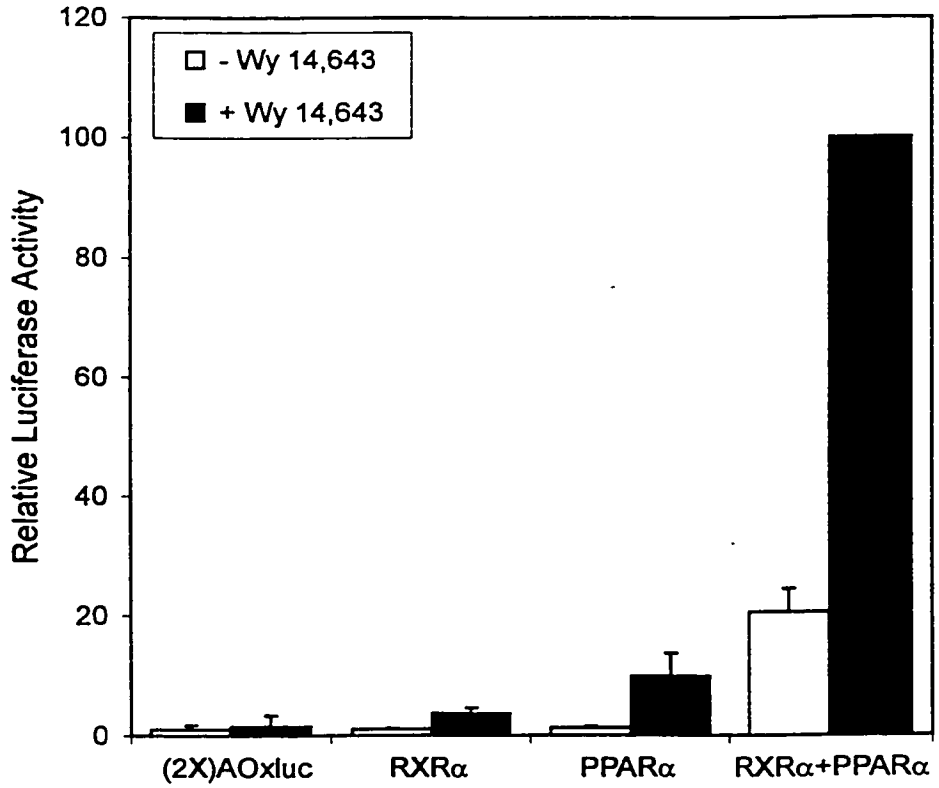


Figure 3.6: PPARα and RXRα cooperatively activate transcription.
BSC40 cells were transfected with the luciferase reporter plasmid pAOx(X2)*luc* (2 μg) and 0.5 μg of each of the expression vectors for PPARα and RXRα, and luciferase activity was measured. Where indicated, transfections were carried out in the presence of Wy 14,643 (100 μM final concentration). Values represent the average (± 1 S.D.) of three separate experiments carried out in duplicate and normalized to the activity observed with PPARα/RXRα + Wy 14,643, which was arbitrarily set to 100.

gene. Finally, the addition of both nuclear receptors resulted in a marked induction of both the levels of activity in the absence and presence of Wy 14,643. In fact, the induction of transcription was ~10X that achieved with the addition of PPARα alone. Thus, PPARα and RXRα acted cooperatively to mediate the activation of transcription by peroxisome proliferators.

The mechanism by which PPAR and RXRα cooperatively activated the transcription of the luciferase reporter gene was presumably a manifestation of the cooperative binding of PPAR and RXR to the AOx PPRE. The fact that the addition of exogenous PPARα alone resulted in only a modest level of activation was probably due to limiting amounts of RXRα present in the cells. However, upon the addition of exogenous RXRα, the levels of RXRα were no longer a limiting factor in the process of the formation of the complex of PPAR/RXRα bound to DNA and the subsequent activation of transcription.

3.2.2 PPRE as an Allosteric Modulator of PPAR Activity

3.2.2.1 DNA Binding is not Sufficient for Activation

While binding of PPARs to the AOx and HD PPREs was shown to require the requisite binding partner RXRα, complementary *in vivo* studies by S. Marcus demonstrated that this binding was not sufficient for activation (Fig. 3.7) (Marcus et al., 1993). These transient transfection assays measured the ability of the PPARs to activate the transcription of reporter genes under the control of the HD PPRE and the AOx PPRE (Fig. 3.7, *Panel A*) in response to the PPAR agonists Wy 14,643 and ciprofibrate. While xPPARα and mPPARα activated both reporter constructs, PPARβ was unable to activate either one, even in the presence of Wy 14,643 and ciprofibrate (Fig. 3.7, *Panel B*).

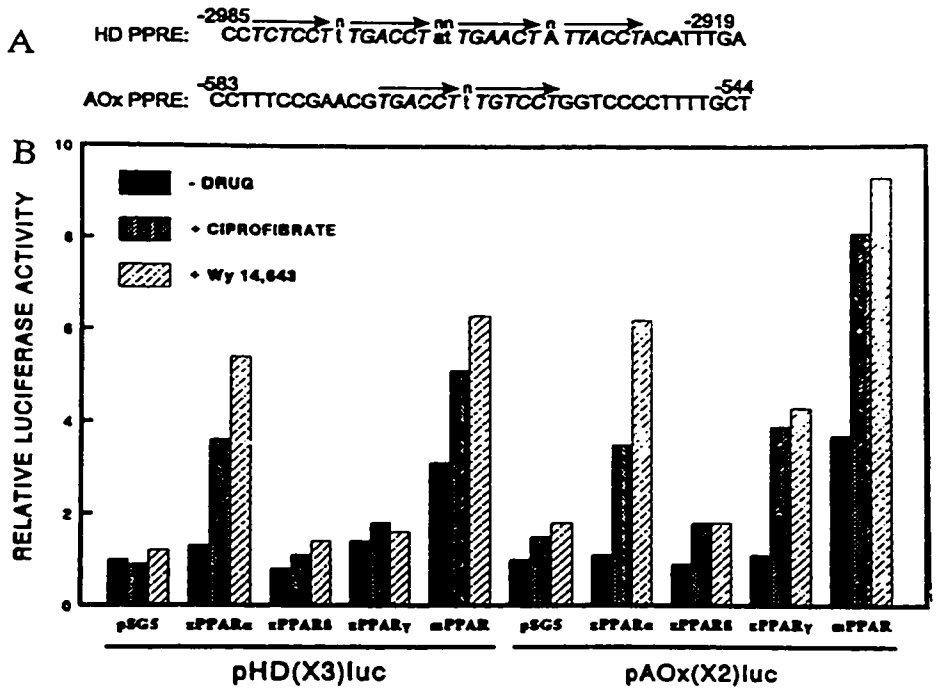


Figure 3.7: Differential activation by PPAR α, β and γ subtypes.
Panel A: Depiction of the sequences of the HD and AOx PPREs. The nucleotides are numbered with respect to the transcriptional start site of each gene. The direct repeats that are similar to the TGACCT consensus half site are indicated by arrows. The nucleotides that separate the direct repeats are denoted (n). *Panel B*: The luciferase reporter plasmids pAOx(X2)*luc* and pHD(X3)*luc* were cotransfected into COS-1 cells in combination with expression plasmids for *X. laevis* (x) PPARα, xPPARβ, xPPARγ and mouse (m) PPARα. Each combination of expression plasmids was transfected in the absence and presence of the peroxisome proliferators ciprofibrate (0.5 mM) and Wy 14,643 (0.1 mM). The luciferase activity was assayed from cell lysates and normalized to the luciferase activity obtained from the reporter construct alone in the absence of agonist. This level of activity had been arbitrarily set to 1. The presented data represents the average of two independent experiments done in duplicate. This figure represents data prepared by S. Marcus.

Interestingly, the AOx PPRE but not the HD PPRE mediated transcriptional activation by PPARγ in response to Wy 14,643 and ciprofibrate.

The inability of PPARβ to activate transcription through the AOx PPRE and HD PPRE is probably due to the fact that Wy 14,643 is not a potent agonist of this subtype (Kliewer et al., 1994; Jow and Mukherjee, 1995). Since only recently has a high affinity ligand and agonist of PPARβ been discovered, it remains to be seen if agonist activated PPARβ is capable of signalling through the HD and AOx PPREs (Brown et al., 1997).

The inability of PPARγ to utilize the HD PPRE may represent the fact that neither Wy 14,643 nor ciprofibrate are full agonists of this receptor. Wy 14,643 is only a partial agonist of PPARγ when compared to full PPARγ agonists such as 15d-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) or BRL49653 which were not available at the time of the study (Dreyer et al., 1992; Forman et al., 1995; Lehmann et al., 1995). However, subsequent studies have shown that PPARγ activated by PGJ2 is also unable to signal via the HD PPRE while the AOx element acts as a permissive element (Kassam et al., 1998).

Further characterization of the effect of PPARγ demonstrated that the addition of PPARγ antagonized PPARα mediated activation on the HD PPRE. The antagonism of PPARα mediated signalling by PPARγ (Marcus et al., 1993; Kliewer et al., 1994), probably represents the competition for binding to the PPRE by the two PPAR subtypes. These findings are supported by studies that show that this effect is not limited to the γ subtype since PPARβ is also able to antagonize PPARα mediated signalling (Jow and Mukherjee, 1995; Kliewer et al., 1994).

Although xPPARα, xPPARβ, xPPARγ and mPPARα all bound to the AOx and HD PPREs, this was not sufficient for transcriptional activation. In fact, the presence of agonist was required for significant activation of transcription of the reporter genes. Furthermore, the binding of PPARs to DNA and the presence of agonist was not sufficient for transcriptional activation as demonstrated by the differential activation of PPARγ on the AOx and HD PPREs.

3.2.2.2 HD PPRE Allosterically Regulates PPARγ

The inability of PPARγ to activate transcription in response to the presence of partial and full agonist presents the scenario in which the PPRE allosterically regulates the activity of the bound PPAR (Marcus et al., 1993; Kassam et al., 1998). According to the current model of transcriptional activation by nuclear receptors, the coactivation complex is recruited by DNA-bound, agonist-activated nuclear receptors (refer to Section 1.2.1). Studies have demonstrated that the DNA binding site can act as an allosteric regulator of bound receptors and influence their ability to recruit the coactivation complex in response to agonist (Kurokawa et al., 1995). When the complex of RAR and RXRα is bound to a DR4 response element, this complex is responsive to RAR agonist. However, when bound to a DR1 response element, this complex is unresponsive to agonist and unable to recruit coactivators (Kurokawa et al., 1995). Since PPARγ bound to the HD PPRE is not functional, it could be hypothesized that the HD PPRE allosterically modulates PPARγ such that it stabilizes the inactive form of the receptor and prevents the recruitment of coactivators. While it has been demonstrated that the complex of PPARγ/RXRα bound to the AOx PPRE recruits coactivators such as CBP and SRC-1 in presence of agonist (Schulman et al., 1998), it remains to be determined if

recruitment of coactivators by PPARγ bound to the HD PPRE is inhibited. Thus, the DNA binding site of PPAR may act as an allosteric inhibitor by stabilizing the inactive receptor conformation and preventing recruitment of coactivators in the presence of agonist.

In summary, the binding of PPAR to its appurtenant DNA binding site is necessary but not sufficient for mediating the transcriptional activation by agonists. Furthermore, the PPRE can act as an allosteric modulator of PPAR function in a subtype specific manner. Thus, the binding interface between PPAR and DNA contains determinants that not only result in regulation of the receptor activity but also determinants for a subtype specificity. Recent work described in the next section has identified the 3' flanking region of the PPRE as containing determinants that may play a role in this allosteric regulation (Fig. 3.5).

3.2.2.3 The 3' Flanking Region of the PPRE Modulates PPAR

All functional PPREs are composed of direct repeats of the consensus sequence TGACCT (Figs. 1.5 and 3.5). However, in contrast to nuclear receptors such as RXRα, the sequences that flank this core element on the 3' side are critical determinants for the binding of PPAR (Palmer et al., 1995; Juge-Aubry et al., 1997; IJpenberg et al., 1997). Alignment of characterized PPREs results in the following consensus sequence of the 3' flanking sequence (underlined): TGACCTa/tTGACCTAGTTTTG. The requirement for this region is highlighted by the fact that the complex of PPAR/RXR is unable to bind a DNA probe in which this region has been deleted (Palmer et al., 1995). Furthermore, altering the sequence of this region can greatly reduce the ability of PPAR/RXR to bind to the PPRE (Palmer et al., 1995). In fact, the affinity of PPAR for characterized PPREs

correlates more highly with conservation of the 3' flanking sequence than with conservation of the core DR1 repeats (Juge-Aubry et al., 1997; IJpenberg et al., 1997). The α subtype of PPAR is sensitive to these alterations while the γ subtype is more permissive of deviations from the consensus flanking sequence (Juge-Aubry et al., 1997).

Based upon the structure of TR/RXRα bound to DNA, the putative carboxy-terminal extension (CTE) of PPAR is a candidate domain that could form an interface with the 3' flanking region of the PPRE (refer to Section 1.1.4.3). If the putative CTE of PPAR formed extensive contacts with DNA similar to the CTE of TR, this could account for the important role this region plays in PPAR binding (Rastinejad et al., 1995).

Therefore, the 3' flanking sequence of the PPRE plays an essential role in determining whether PPAR is able to bind to this DNA site. In fact, it is of such importance that it should be considered as an integral part of the PPRE. Furthermore, this region is capable of acting as a subtype specific binding determinant and may be responsible for the ability of elements to mediate the activation of specific PPAR subtypes. This specificity may help to resolve the fact that while more than one PPAR subtype exists in a tissue, each subtype specific agonist elicits specific biological effects.

3.3 CONCLUSIONS

The data presented in this section comprises the first identification of the liver-specific factor that bound to the HD PPRE to mediate the transcriptional activation by peroxisome proliferators. This factor was the nuclear receptor PPAR. PPAR was found to bind to the HD PPRE cooperatively with RXRα, a nuclear receptor that acted as the requisite DNA binding partner of a number of nuclear receptors (Mangelsdorf et al., 1990; Zhang et al., 1992a; Kliewer et al., 1992b). The binding of PPAR/RXR to the HD and AOx PPREs did not require the presence of exogenous agonist. While the binding of PPAR to the PPRE was necessary, it was not sufficient for transcriptional activation. Firstly, the presence of agonist was required to achieve maximal levels of transcriptional activation and secondly, the PPRE itself acted as an allosteric modulator of the bound PPAR/RXR complex. While both the AOx and HD PPREs bound to the complex of PPARγ/RXRα, only the AOx PPRE acted as a permissive element, allowing PPARγ agonists to activate the transcription of target genes.

All three PPAR isotypes overlap in some degree in terms of tissue expression pattern and agonist sensitivity, however, they appear to play distinct roles in the cell (Limberger et al., 1996). How this specificity and insulation of signalling pathways of each PPAR subtype is manifested is poorly understood. Although evidence is presented in this section that all three isotypes of PPAR bind to response elements composed of the DR1 architecture, we have shown that this binding is not sufficient for transcriptional activation. In fact, the PPRE itself may act as an allosteric regulator of the bound PPARs, providing a possible mechanism by which the PPRE could insulate the regulation of its target gene to one specific PPAR subtype.

4. THE INTERACTION BETWEEN PPAR AND RXR

As detailed in the previous section, PPAR and RXRα were found to bind cooperatively to the AOx and HD PPREs. At the time of this discovery, the mechanism of this cooperative binding was unknown. One possibility was that PPAR and RXR interacted to form a heterodimer that could then recognize the AOx and HD PPREs. In order to determine if these nuclear receptors interacted *in vivo* in the absence of a DNA binding site and exogenous agonist, the yeast two-hybrid system was utilized.

The yeast two-hybrid system (Fig. 4.1) developed by S. Fields and coworkers is based upon the functional reconstitution of a transcriptional activator as a result of the interaction between two candidate proteins (Fields and Song, 1989). Taking advantage of the modular nature of the yeast transcriptional activator GAL4, the DNA binding domain (DBD: a.a. 1 to 147) and the transcriptional activation domain (AD: a.a. 768 to 881) are each appended to candidate interacting proteins (*Panel A*). Integrated within the *Saccharomyces cerevisiae* genome are reporter genes under the control of the GAL4 DNA binding sites (UAS_g: upstream activation sequence). Since in these yeast, the GAL4 gene has been disrupted, these reporter genes are inactive. The expression of the GAL4 DBD fused to the candidate protein results in binding of the construct to the UAS_g. No expression of the reporter gene results since this fusion lacks a transcriptional activation domain. The additional expression of the GAL4 AD fused to the second candidate protein will only result in activation of the reporter gene if the two

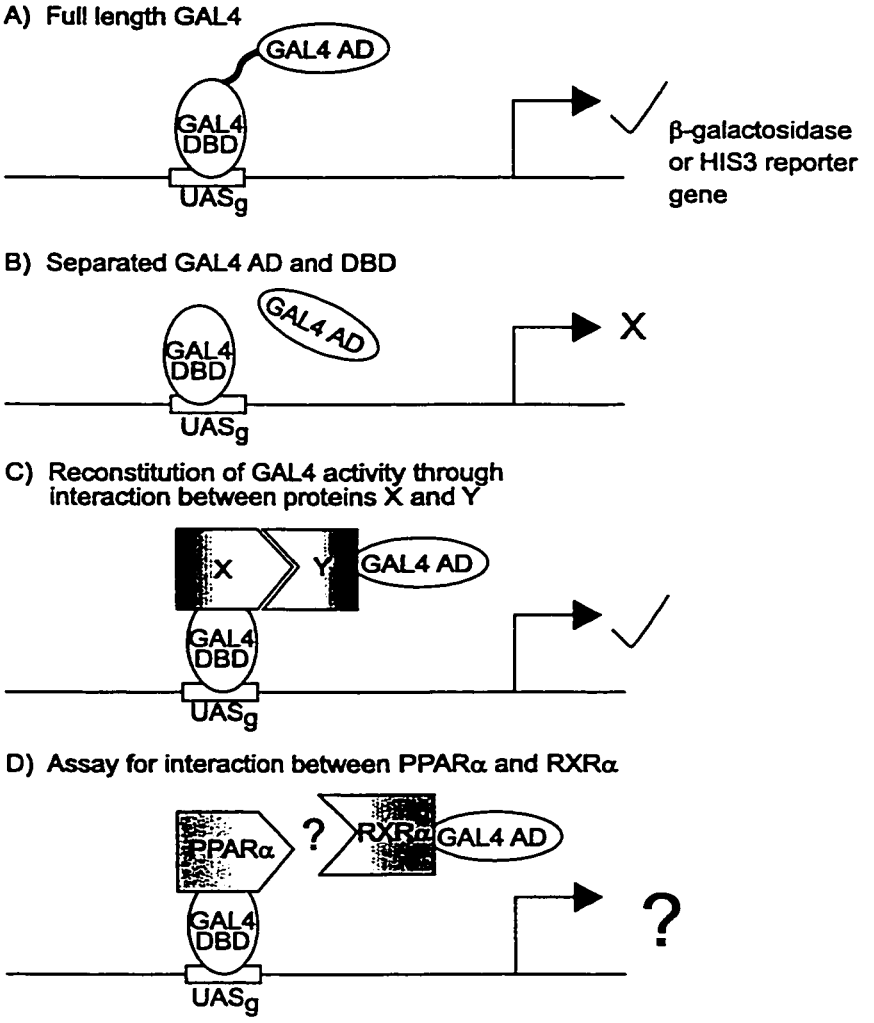


Figure 4.1: Schematic depiction of the yeast two-hybrid system.
The yeast two-hybrid system is based upon the yeast transcription factor GAL4. *Panel A.* GAL4 contains a DNA binding domain (DBD) and a transcriptional activation domain (AD). When bound to the GAL4 DNA binding site (UAS_g), the AD is localized in the proximity of the promoter of the reporter gene and transcriptional activation occurs. The two reporter genes used are the β-galactosidase gene and the HIS3 gene. *Panel B.* The GAL4 DBD and AD are functional modular domains. While the GAL4 DBD is able to bind the UASg, the AD is inactive since it is not localized to the promoter of the reporter gene. *Panel C.* The appending of two interacting proteins (X and Y) to the GAL4 DBD and AD results in the functional reconstitution of GAL4. The interaction between proteins X and Y result in the localization of the GAL4 AD in the proximity of the promoter of the reporter gene. *Panel D.* Depiction of the use of the yeast two-hybrid system to determine if PPARα and RXRα interact *in vivo*.

candidate proteins interact causing localization of the GAL4 AD in the proximity of the reporter gene promoter (*Panel C*). Therefore the functional reconstitution of GAL4 as a result of the interaction between the two candidate proteins forms the basis of this assay.

Two artificial reporter genes have been inserted into the genome of the *S. cerevisiae* strains utilized in the yeast two-hybrid assay. The first is the bacterial β-galactosidase gene. The detected activity of this enzyme is indicative of an interaction between candidate proteins. Furthermore, the level of β-galactosidase activity detected is also indicative of the strength of the interaction between the candidate interacting proteins. The stronger the interaction, the greater the functional reconstitution of GAL4 and hence the greater the transcriptional activation of the β-galactosidase reporter gene.

The second reporter gene is the *S. cerevisiae* HIS3 gene which encodes an essential enzyme of the histidine synthetic pathway in yeast. Since the genomic copy of the HIS3 gene has been disrupted, the expression of HIS3 is solely due that produced by the HIS3 reporter gene in response to the interaction between candidate proteins and results in the rescuing of the his^- auxotrophic yeast. The HIS3 reporter gene provides not only a qualitative confirmation of the results observed with the β-galactosidase reporter, but also provides a means of positive selection for only those yeast that express interacting GAL4 fusion proteins.

4.1 PPAR AND RXRα INTERACT *IN VIVO*

The β-galactosidase activity was measured of the *S. cerevisiae* yeast strain Y190 transformed with all combinations of the expression plasmids of the GAL4 DBD, GAL4 AD, and fusions of such with mPPARα and RXRα (Table 4.1). Significant β-galactosidase activity was only detected in the presence of the GAL4 DBD fusion of

Table 4.1: PPARα and RXRα interact in yeast two hybrid system.

The following represents the β-galactosidase activity of the *S. cerevisiae* strain Y190 transformed with various combinations of expression plasmids of the GAL4 DBD, GAL4 AD and fusion of these domains with PPARα and RXRα.

GAL4 DBD (GBD) Fusion[a]	GAL4 AD (GAD) Fusion[a]	β-galactosidase Activity (U)[b]
GBD		< 0.05
GBD-RXRα		< 0.05
GBD-mPPARα		0.11 ± 0.03
	GAD	< 0.05
	GAD-mPPARα	< 0.05
	GAD-RXRα	< 0.05
GBD	GAD	< 0.05
GBD	GAD-mPPARα	< 0.05
GBD	GAD-RXRα	< 0.05
GBD-RXRα	GAD	< 0.05
GBD-mPPARα	GAD	0.09 ± 0.01
GBD-RXRα	GAD-mPPARα	7.8 ± 1.1
GBD-RXRα	GAD-RXRα	< 0.05
GBD-mPPARα	GAD-mPPARα	0.11 ± 0.04
GBD-mPPARα	GAD-RXRα	6.9 ± 1.1
Full length GAL4		310

[a] Expression plasmids of the described GAL4 domains and GAL4 fusions are described in the Materials and Methods, Section 2.1.12.

[b] The β-galactosidase activity was assayed as described in Ausubel et al., 1990 and in the Materials and Methods, Section 2.2.13.2. The data presented represents the average activity of three independent yeast transformants measured in triplicate (± SEM).

mPPARα and the GAL4 AD fusion of RXRα as well as the converse. Thus, PPARα and RXRα interact *in vivo* in a specific manner irrespective of the identity of the appended GAL4 domain. Attesting to the validity of this interaction was the fact that the resultant β-galactosidase activity was comparable to the previously characterized interaction between p53 and the large T antigen (Miyata et al., 1994; Iwabuchi et al., 1993).

While dimerization between RXRα monomers requires the presence of the RXRα agonist, the interaction between PPARα and RXRα occurs in the absence of exogenously added PPARα or RXRα agonist (Zhang et al., 1992b). These results also demonstrate that DNA binding is not required for dimerization of PPARα and RXRα. Thus, the cooperative binding of PPAR and RXR to PPREs appears to be the result of the formation of a heterodimer of PPAR and RXR which then binds to the PPREs.

While a number of studies had detected an *in vitro* interaction between RXR and PPAR (Keller et al., 1993), the detection of this interaction in the yeast two hybrid system represented the first detection of this interaction *in vivo* (Juge-Aubry et al., 1995). In fact, this was the first demonstration of the interaction between two nuclear receptors in the yeast two-hybrid system.

4.2 PPARα DOES NOT HOMODIMERIZE

Since RXRα is capable of forming dimers in solution and binds to DNA in the presence of agonist (Zhang et al., 1992b), the ability of PPARα to form homodimers was assessed by transforming yeast with the GAL4 DBD and GAL4 AD fusions with mPPARα and assaying β-galactosidase activity (Table 4.1). Since level of activity produced was comparable to that attributed to the weak activation ability of the GAL4

DBD fusion of mPPARα alone, PPARα is unable to dimerize in the yeast two hybrid system. This provides further support that the complex formed in EMSA analysis upon the addition of only PPARα translated in reticulocyte lysate is not a homodimer of PPARα but a heterodimer of PPARα/RXRα (refer to Fig. 3.4, lane b).

4.3 BINDING INTERFACE OF PPARα AND RXRα

The N-terminal 82 amino acids of PPARα and the N-terminal 43 amino acids of RXRα are not required for dimerization since the GBD-NΔmPPARα was capable of interacting with GAD-RXRα (refer to Table 4.2). Comparing the interaction between GBD-NΔmPPARα and GBD-NΔmPPARα(Δ435) with RXRα demonstrates that a deletion of the last 33 amino acids of mPPARα results in a loss of interaction with RXRα. While a deletion of 33 amino acids from the C-terminus is sufficient to abrogate dimerization with RXRα, PPARα deleted of only 20 amino acids at the C-terminus retains the ability to interact with RXRα (Dowell et al., 1997a). The importance of this region is further supported by existence of a point mutant of mPPARα, L433R, in which the mutation of a leucine conserved amongst PPARs results in the abolition of dimerization with RXRα (Juge-Aubry et al., 1995). Taken together, these studies suggest that amino acids 435 to 458 are important in forming the binding interface with RXRα.

These amino acids are located with the putative Helix 10 of the PPARα ligand binding domain (Wurtz et al., 1996). The role of this region of the ligand binding domain in receptor dimerization is illustrated by the demonstration that a region that overlaps Helix 10 of RXR and RAR determines the identity of the heterodimeric partner (Mukherjee et al., 1997a). Furthermore, this region of the homodimer-forming COUP-

Table 4.2: PPARα has a detectable AF-1 activity in yeast.
The following represents the β-galactosidase activity of the *S. cerevisiae* strain Y190 transformed with the expression vector of the GAL4 DBD, the GAL4 DBD fusion of mPPARα, the N-terminal deleted NΔmPPARα, the N-terminal/C-terminal truncated NΔmPPARα(Δ435) and the GAL4 AD fusion of RXRα.

GAL4 DBD (GBD) Fusion[a]	GAL4 AD (GAD) Fusion[a]	Region of mPPAR Fused to GBD	β-galactosidase Activity (U)[b]
GBD		n/a	< 0.05
	GAD-RXRα	n/a	< 0.05
GBD-mPPARα		a.a. 26 to 468	0.11 ± 0.03
GBD-mPPARα	GAD-RXRα		6.9 ± 1.1
GBD-NΔmPPARα		a.a. 83 to 468	< 0.05
GBD-NΔmPPARα	GAD-RXRα		5.2 ± 1.2
GBD-NΔmPPARα(Δ435)		a.a. 83 to 435	< 0.05
GBD-NΔmPPARα(Δ435)	GAD-RXRα		< 0.05

[a] Expression plasmids of the described GAL4 domains and GAL4 fusions are described in the Materials and Methods, Section 2.1.12.

[b] The β-galactosidase activity was assayed as described in Ausubel et al., 1990 and in the Materials and Methods, Section 2.2.13.2. The data presented represents the average activity of three independent yeast transformants measured in triplicate (± SEM).

TF1, when transferred to RXRα, permitted the formation of RXR homodimers in the absence of agonist (Mukherjee et al., 1997a). The results of these studies are supported by the determination of the structure of a homodimer of the ligand binding domain of RXRα in which the dimerization interface between the RXRα monomers was primarily formed by Helix 10 of the ligand binding domain (Bourguet et al., 1995).

The interface between PPAR and RXR may act as more than an inert dimerization determinant. For example, the ability of RXR agonists to synergistically potentiate the action of PPAR agonists in a manner that does not involve the AF-2 domain of RXRα, would require the transmission of a signal through this interface to facilitate the activation of PPAR (Schulman et al., 1998). Furthermore, since RXR agonists activate the PPAR/RXR heterodimer, PPAR acts as a permissive RXR binding partner and the binding interface between PPAR and RXR permits the activation of the RXR ligand binding domain by agonist. (Lala et al., 1996; Mukherjee et al., 1997a; Kliewer et al., 1992c; Schulman et al., 1998). This is in contrast to the complexes of TR/RXR and RAR/RXR in which TR and RAR act as non-permissive binding partners, preventing RXRα agonists from activating the complex (Mukherjee et al., 1997a). Therefore the dimerization interface is required for not only the dimerization of RXR and PPAR but also determines the biological character of the DNA bound complex.

4.4 DELETION OF THE AF-1 ACTIVITY OF PPARα

The N-terminal region of nuclear receptors contains the AF-1 activation domain, an transcriptional activation function that does not require the presence of ligand. In fact studies have shown that the AF-1 and AF-2 domains compete for different factors indicating that these domains act separately. However, there is evidence that

coactivators, such as SRC-1, interact with both activation domains (Oñate et al., 1998). In the case of SRC-1, the interaction with the AF-1 domain is required for full transcriptional activation by the AF-2 in the presence of agonist (Oñate et al., 1998).

Since a minimal amount of β-galactosidase activity was detected in the *S. cerevisiae* strain Y190 transformed with the expression plasmid of GBD-mPPARα, this ability to activate transcription was attributed to the AF-1 function of mPPARα (Table 4.2). This agonist-independent activation presented a problem for the pursuit of a yeast two-hybrid screen for PPAR interacting proteins. In order to complete these studies, a construct of mPPARα that lacked this activity was required. To eliminate the AF-1 activity of GBD-mPPARα (a.a. 26 to 468 of mPPARα), a form of mPPARα was produced in which the N-terminus was further truncated: GBD-NΔmPPARα (a.a. 83 to 468). This construct was unable to activate the expression of the β-galactosidase reporter gene yet retained the ability to interact with the fusion of the GAL4 AD and RXRα (Table 5.2). Therefore, the AF-1 function of GBD-NΔmPPARα had been disrupted without affecting its ability to dimerize with RXRα.

Very little characterization of the AF-1 domain of PPARα has been completed to date. However, a survey of the constructs of PPAR utilized in yeast two-hybrid screens revealed that in all cases N-terminal truncations of PPAR were used, a suggestion that these research groups detected a similar AF-1 activity (Puigserver et al., 1998; Zhu et al., 1996; Zhu et al., 1997; Dowell et al., 1997a; Mizukami and Taniguchi, 1997). While the AF-1 domain of PPARγ has been demonstrated to been important in the biological

function of this receptor, the importance of this domain in PPARα remains to be determined (Werman et al., 1997; Tontonoz et al., 1995; Adams et al., 1997).

4.5 CONCLUSIONS

These studies comprise the first documentation of an *in vivo* interaction between PPARα and RXRα in the absence of appurtenant DNA binding sites and exogenously added agonists (Miyata et al., 1994). While RXRα requires agonist for both homodimerization and DNA binding (Zhang et al., 1992b), these results describe the formation of a heterodimer of PPARα and RXRα in the absence of agonist. Since neither the formation of the DNA binding complex nor binding to DNA requires agonist, it would be predicted that the complex of PPAR/RXR is constitutively bound to PPREs in the promoters of target genes. The predicted consequence of this constitutive binding of PPAR to the promoter depends upon the unresolved issue of whether the DNA bound complex of PPAR/RXR recruits corepressors (refer to Section 1.3.7.2). If in fact this complex recruits corepressors, the active repression of target gene transcription would occur. If, however, this complex does not recruit the corepression complex, the possible sampling of the active conformation and resultant recruitment of the coactivation complex (refer to Section 8.0) may result in the maintenance of a low level of transcriptional activation of target genes.

While the initial aim of utilizing the yeast two-hybrid system was to detect the interaction between RXRα and PPARα *in vivo*, the ultimate goal was to utilize this system as a genetic screen for novel PPARα interacting proteins. The fact that the interaction between PPARα and RXRα could be detected in this system, confirmed its ability to detect interactions between PPARα and relevant interacting proteins. However,

a fusion of the GAL4 DBD with mPPARα that did not constitutively activate transcription was required. Fortunately, an N-terminal deletion of mPPARα was characterized that retained the ability to interact with RXRα but was transcriptionally inactive. This fusion was utilized in the genetic screen for proteins that interacted with PPARα. Sections 6.0 and 7.0 describe the discovery and characterization of two such PPARα interacting proteins.

5. MODULATION OF PPAR ACTIVITY BY COUP-TF1 & HNF-4

Characterization of the HD PPRE by EMSA analysis demonstrated the presence of proteins in the nuclear extracts from HeLa cells and H4IIEC3 cells which bound to identical regions of the HD PPRE (Marcus et al., 1993; Zhang, 1995). While PPAR and RXR could be detected in complexes formed by proteins in H4IIEC3 nuclear extract (Fig. 3.4, lane a), PPAR and RXR could not be detected in complexes formed upon the addition of HeLa nuclear lysate (Zhang, 1995). This suggested that factor(s) present in HeLa extract recognized the same TGACCT direct repeats as the PPAR/RXR complex. Furthermore, the fact that, of the two complexes that bound to the HD PPRE from hepatocyte nuclear extract (Fig. 3.4, lane a C1 and C2), only the top one (C1) contained RXRα indicating that the bottom one (C2) could not contain PPARα and thus represented a factor that was neither RXRα nor PPARα. Finally, these factors recognized response elements composed of direct repeats of TGACCT, indicating that they could be members of the nuclear receptor superfamily.

The AOx and HD PPREs are composed of direct repeats of the nuclear receptor half-site motif TGACCT separated by a spacing of 1 and 2 nucleotides (notated as DR1 and DR2 elements). While the AOx PPRE is a simple DR1 element, the HD PPRE is more complex and composed of two DR1 elements separated by 2 nucleotides (Fig. 5.7). This spacing of 2 nucleotides between the DR1 elements creates a DR2 element.

The specificity of response element recognition is governed primarily by the

number of TGACCT repeats (e.g. 1 versus 2), the orientation of the TGACCT repeats (e.g. direct versus inverted) and the spacing between the TGACCT repeats (reviewed in Mangelsdorf and Evans, 1995). These criteria are primarily responsible for determining which nuclear receptors recognize and bind specific response element architectures. Other factors that affect specificity include the primary sequence of the half-sites and the composition of the flanking sequences (Mader et al., 1993; Palmer et al., 1995; Juge-Aubry et al., 1997; IJpenberg et al., 1997).

The existence of hundreds of nuclear receptors combined with the fact that it appears that there is a limited complement of utilized response element architectures means that multiple nuclear receptors will recognize the same response element architecture. In addition, nuclear receptors are capable of binding more than one response element (e.g. bind both DR1 and DR2 response elements) (Wang et al., 1989). Furthermore, nuclear receptors may bind response elements as homodimers and as heterodimers with each complex recognizing a different response element architecture. For example, RXRα binds to DR1 response elements as a homodimer, but recognizes DR1, DR3, and DR5 response elements as a heterodimer with PPARα, VDR, and RAR respectively (Zhang et al., 1992b; Marcus et al., 1993; Kliewer et al., 1992b).

Since one response element is not necessarily the exclusive binding site of one nuclear receptor, the binding of other nuclear receptors to the HD and AOx PPREs was possible. Therefore, the factors that B. Zhang detected binding to these PPREs were quite likely nuclear receptors (B. Zhang, 1995). We set out to identify candidate nuclear receptors that could bind PPREs and characterize their effects on PPARα/RXRα mediated signalling.

Candidate nuclear receptors that would bind to the PPREs and modulate the activity of PPAR would firstly have to be expressed in the same tissues to permit the convergence of their signalling pathways. These nuclear receptors would also have to recognize and bind to response elements that shared the same architecture as the PPREs (DR1, DR2). Since the additional factors that bound to the HD and AOx PPREs did so without RXRα or PPAR, these nuclear receptors would have to be capable of binding DNA as a monomers, forming DNA binding homodimers or forming heterodimers that did not include PPARα or RXRα.

We chose to investigate the role of two nuclear receptors that met the above criteria for their ability to bind to PPREs and modulate the activity of PPAR. The first was the chicken ovalbumin upstream promoter – transcription factor 1 (COUP-TF1), a transcriptional repressor in mammalian cells (Wang et al., 1989). The second was the hepatocyte nuclear factor-4 (HNF-4), a constitutive transcriptional activator (Sladek et al., 1990). The following section details the characterization of the binding of these factors to PPREs and the effects that these auxiliary factors have on PPAR/RXR mediated signalling. This entailed the first identification and characterization of nuclear receptors other than PPAR and RXR that bound to the PPREs and modulated the activity of the complex of PPARα/RXRα.

5.1 COUP-TF1 ANTAGONIZES THE ACTION OF PPAR

COUP-TF1 is a nuclear receptor that was originally identified as an activator of the chicken ovalbumin gene (Kliewer et al., 1992a). To date, no agonist has been identified for COUP-TF1 and this nuclear receptor appears to act as a repressor of transcription in mammalian systems (Miyata et al., 1993; Kliewer et al., 1992a; Klinge et al., 1997). COUP-TF1 was selected as a potential modulator of PPARα activity since it binds with highest affinity to direct repeats of TGACCT separated by 1 or 2 nucleotides and both the AOx and HD PPREs contain response elements of this architecture (Wang et al., 1989; Kliewer et al., 1992a). Furthermore, COUP-TF1 and PPARα are coexpressed in the liver permitting the convergence of their signalling pathways (Kliewer et al., 1992a; Issemann and Green, 1990). Finally, B. Zhang identified COUP-TF-like factors from the nuclear lysate of the peroxisome proliferator-responsive hepatocyte cell line H4IIEC3, that bound to the AOx and HD PPREs (Winrow et al., 1994). To investigate whether COUP-TF1 and PPAR signalling converged, my goal was to establish that COUP-TF1 did indeed bind to the AOx and HD PPREs.

5.1.1 COUP-TF1 Binds to the HD and AOx PPREs

In order to assess whether COUP-TF1 recognized and bound to the AOx PPRE and the HD PPRE, EMSA analysis was performed (Figs. 5.1 and 5.2). The addition of *in vitro* translated COUP-TF1 with radiolabelled HD and AOx PPREs resulted in the formation of a specific protein-DNA complex indicating that COUP-TF1 bound to both the AOx and HD PPREs (Fig. 5.1 and 5.2: lane d). While the addition of COUP-TF1 resulted in the formation of a major complex with the AOx PPRE, a number of other complexes of differing mobility were detected (Fig. 5.1: lane d). It is possible that this

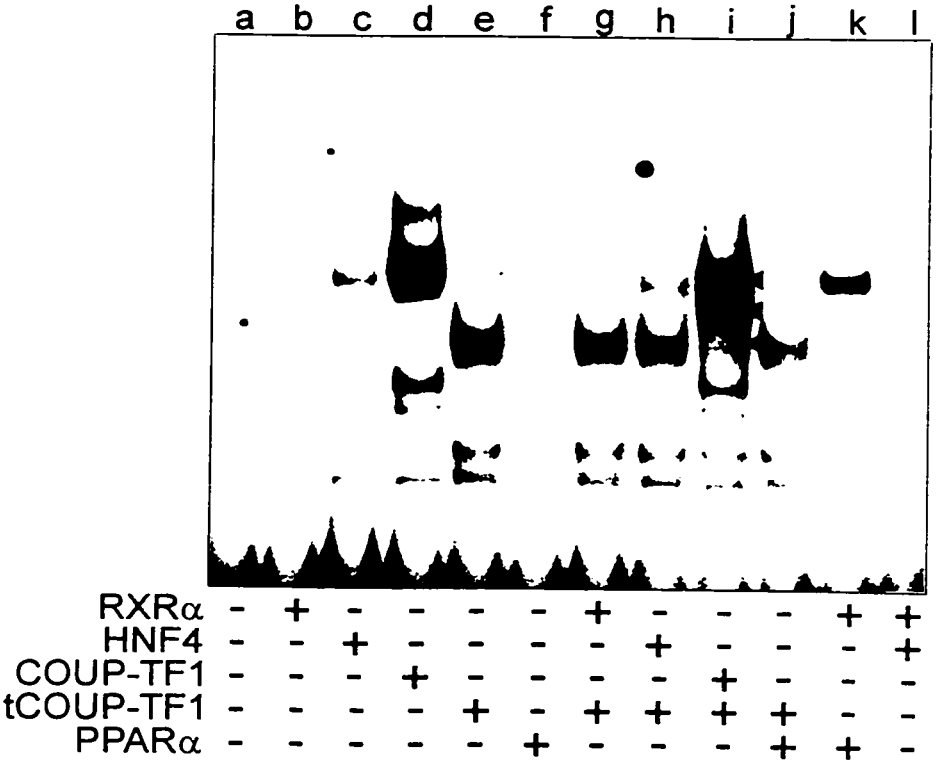


Figure 5.1: COUP-TF1 binds to the AOx PPRE as a homodimer.
In vitro translated RXRα, rat PPARα, COUP-TF1 and tCOUP-TF1 (N-terminal truncated form: a.a. 51 to 413) were incubated with radiolabelled AOx PPRE in EMSA analysis. The complexes formed by the COUP-TF1 homodimer, COUP-TF1/tCOUP-TF1 heterodimer and the tCOUP-TF1 homodimer are indicated by the top, middle and bottom arrows (◂) respectively.

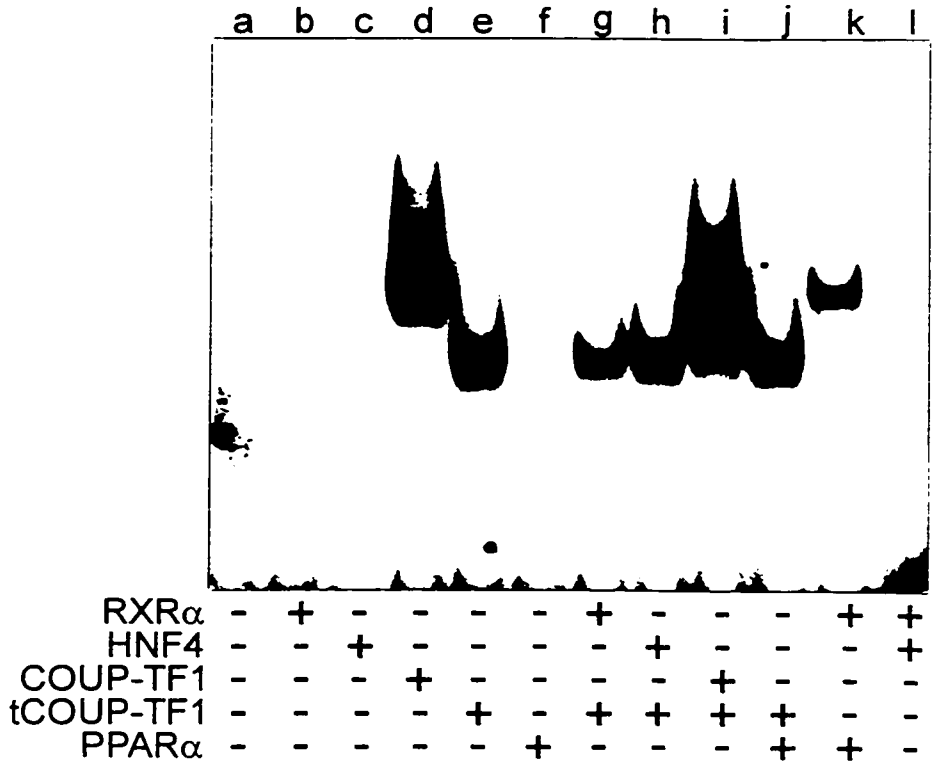


Figure 5.2: COUP-TF1 binds to the HD PPRE as a homodimer.
In vitro translated RXRα, rat PPARα, COUP-TF1 and tCOUP-TF1 (N-terminal truncated form: a.a. 51 to 413) were incubated with radiolabelled HD PPRE in EMSA analysis. The complexes formed by the COUP-TF1 homodimer, COUP-TF1/tCOUP-TF1 heterodimer and the tCOUP-TF1 homodimer are indicated by the top, middle and bottom arrows (◂) respectively.

represents the binding of monomeric COUP-TF1 to the AOx PPRE (refer to Section 5.1.3). Therefore, COUP-TF1 binds to the AOx and HD PPREs *in vitro*.

5.1.2 COUP-TF1 Binds to the AOx and HD PPREs as a Homodimer

Since the complex formed upon the addition of COUP-TF1 migrated with similar mobility to that of PPARα/RXRα, it was probable that COUP-TF1 bound to the AOx and HD PPREs as a homodimer (Fig. 5.1 and 5.2: compare lane d with lane k). To demonstrate that COUP-TF1 bound to the PPREs as a homodimer in EMSA analysis, an N-terminal truncated form of COUP-TF1 was engineered: t-COUP-TF1 (a.a.: 51 to 413). The addition of tCOUP-TF1 in EMSA analysis with radiolabelled HD and AOx PPREs demonstrated the formation of specific protein-DNA complexes that migrated with a faster mobility as would be predicted (Fig. 5.1 and 5.2: lane e). Both the full length and truncated forms of COUP-TF1 were added to EMSA binding reactions. The result was the formation of three complexes (Figs. 5.1 and 5.2: lane i; indicated by the arrows). The slowest migrating complex migrated with the same mobility as the complex formed by the addition of only COUP-TF1, presumably a COUP-TF1 homodimer. The fastest migrating complex corresponded to the complex formed by the tCOUP-TF1 homodimer. The appearance of a complex of intermediate mobility suggested the formation of a complex of intermediate mass and thus a heterodimer of COUP-TF1/tCOUP-TF1. Therefore, COUP-TF1 binds to the AOx and HD PPREs as a homodimer.

The fact that the truncated form of COUP-TF1 was able to bind DNA and dimerize with the full length COUP-TF1 indicates that the first 50 amino acids are not required for either DNA binding or formation of the dimerization interface.

These results have identified COUP-TF1 as an additional nuclear receptor that binds to the AOx and HD PPREs and have confirmed the studies by B. Zhang that detected a COUP-TF1-like complex bound to both the AOx and HD PPREs from hepatocyte nuclear lysate (Zhang, 1995; Miyata et al., 1993). In fact this entails the first demonstration of the binding of another nuclear receptor other than PPAR and RXR to a PPRE.

5.1.3 COUP-TF1 Binds to the AOx PPRE as a Monomer

In EMSA analysis, the addition of COUP-TF1 and tCOUP-TF1 resulted in the formation of a protein-DNA complex with the AOx PPRE that migrated faster that the most abundant species (Fig. 5.1: lane d). This complex was not detected in comparable experiments with the HD PPRE (Fig. 5.2: lane d). Studies of the related protein COUP-TFII, revealed a similar distribution of protein-DNA complexes in EMSA analysis with the AOx PPRE but not the HD PPRE (Palmer et al., 1995). Since purified proteins were used in this EMSA analysis, the authors suggested that the faster migrating species corresponded to the binding of a COUP-TFII monomer to the AOx PPRE (Palmer et al., 1995). It is therefore proposed that the lower complex observed upon the addition of COUP-TF1 (Fig. 4.2) corresponds to a monomer binding to the AOx PPRE. Furthermore, the use of the smaller tCOUP-TF1 results an increase in the mobility of the proposed monomer-DNA complex, attesting to the presence of tCOUP-TF1 in this complex. However, it cannot be ruled out that other factors supplied by reticulocyte lysate are present in this complex. The similarities between the complexes formed by purified COUP-TFII and the *in vitro* translated COUP-TF1 as well as the PPRE specific complex formation suggest that COUP-TF1 also binds as a monomer to the AOx PPRE.

5.1.4 Architecture of COUP-TF1 Binding to the HD PPRE

Analysis of COUP-TF1 binding to the HD PPRE by B. Zhang demonstrated that the RXR/PPAR heterodimer bound the second DR1 response element since disruption of the second TGACCT half-site had no effect on binding (Zhang, 1995; Miyata et al., 1993). However, the binding of COUP-TF1 was abrogated by the disruption of the second and the third half-sites but not the fourth half-site. Since COUP-TF1 recognizes DR2 response elements such as that formed by the second and third half-sites, COUP-TF1 may compete with RXR/PPAR for binding to overlapping response elements (refer to Fig. 5.7) (Kliewer et al., 1992a). The recent identification of the first half site as an integral part of the HD PPRE raises the possibility that COUP-TF1 may bind to the DR1 element formed by the first two half-sites (Chu et al., 1995).

5.1.5 COUP-TF1 Antagonizes PPAR/RXR Mediated Signalling

The effect of COUP-TF1 on PPAR/RXR mediated transcriptional activation was assessed by B. Zhang in transient transfection assays (Miyata et al., 1993; Zhang, 1995). Utilizing a reporter gene under the regulatory control of the HD PPRE as a model PPAR/RXR inducible gene, it was shown that COUP-TF1 antagonized PPAR/RXR mediated transcriptional activation in a dose-dependent manner (Fig. 5.3). These results are supported by studies that demonstrate COUP-TF1 antagonism of PPAR/RXR mediated activation of the malic enzyme promoter, (Baes et al., 1995; Liu and Chiu, 1994). In addition, COUP-TF1 antagonizes the signalling of HNF-4 and RXRα mediated by response elements to which COUP-TF1 can also bind (Kliewer et al., 1992a; Ladias et al., 1992; Nakshatri and Bhat-Nakshatri, 1998; Liu and Chiu, 1994).

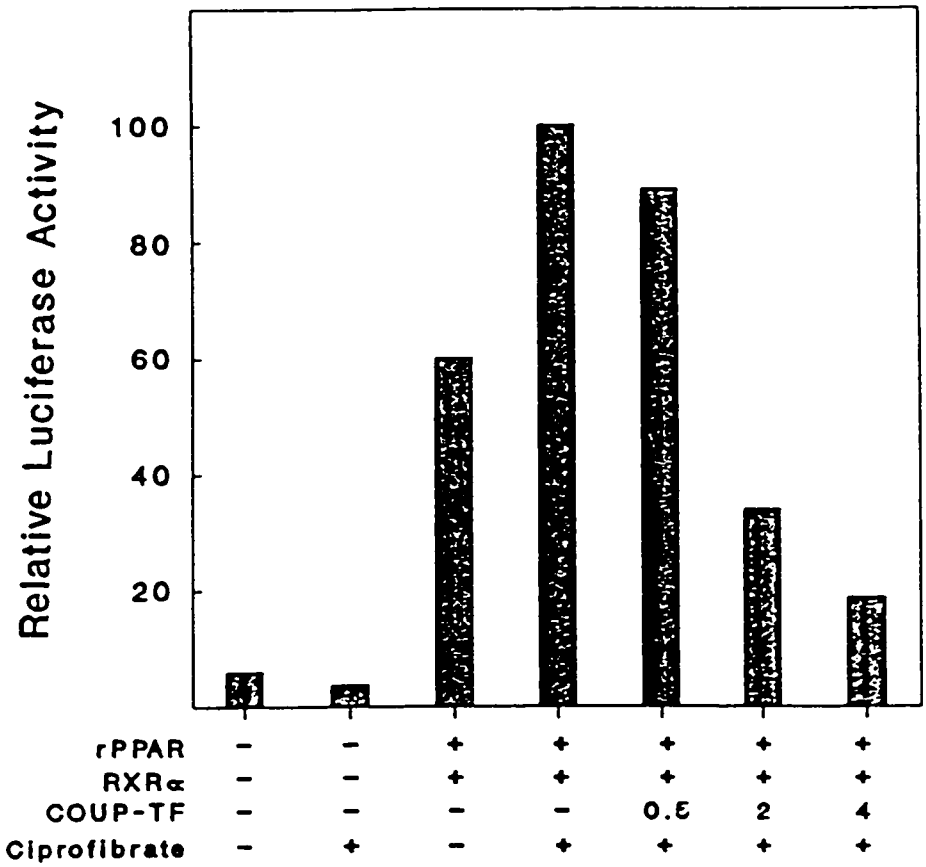


Figure 5.3: COUP-TF1 antagonizes signalling by PPARα/RXRα
BSC40 cells were transfected with reporter plasmid pHD(X3)luc (5 μg), 2 μg of each of the expression vectors for PPARα, RXRα and varying amounts of the COUP-TF1 expression plasmid (0, 0.5, 2, 4 μg). Where indicated, transfections were carried out in the presence of Wy 14,643 (100 μM final concentration). Values represent the average of two separate experiments carried out in duplicate and normalized to the activity observed with PPARα/RXRα + Wy 14,643, which was set to 100. This figure represents the work of B. Zhang.

The antagonism of transcriptional activation of PPAR/RXR mediated signalling may be the result of three mechanisms. The first is competition for binding to the PPRE with the PPAR/RXR heterodimer. This would result in the prevention of PPAR/RXR from binding to the PPRE and mediating activation by agonists such as peroxisome proliferators. In order for COUP-TF1 to act as a regulator of PPAR/RXR binding, the affinity of COUP-TF1 for the PPRE or the levels of COUP-TF1 must be dynamic. In fact, phosphorylation of COUP-TF1 is correlated with an increase in binding of COUP-TF1 to the PPRE (Brodie et al., 1996). This increase in phosphorylation of COUP-TF1 was elicited by compounds which inhibit adipocyte differentiation, a process in which PPARγ plays an integral role (Brodie et al., 1996; Kliewer et al., 1995). These studies suggest that binding of COUP-TF1 to a PPRE is dynamically regulated by the phosphorylation status of COUP-TF1. Furthermore, this dynamic regulation of COUP-TF1 may manifest itself in the biological process of adipocyte differentiation, a process in which PPARγ plays an integral role.

The second proposed mechanism of COUP-TF1 antagonism of RXR/PPAR mediated signalling involves the ability of COUP-TF1 to interact with RXRα (Kliewer et al., 1992a). The interaction between RXRα and COUP-TF1 is proposed to result in the sequestration of RXRα from the formation of productive RXRα heterodimers (Kliewer et al., 1992a). However, the addition of molar excesses of RXRα in EMSA analysis did not antagonize the binding of either COUP-TF1 or tCOUP-TF1 to the HD PPRE, bringing into question the relevance of this mechanism in PPAR/RXR mediated signalling (Fig. 5.4: compare lane b with lanes c to f and lane g with lanes h to k).

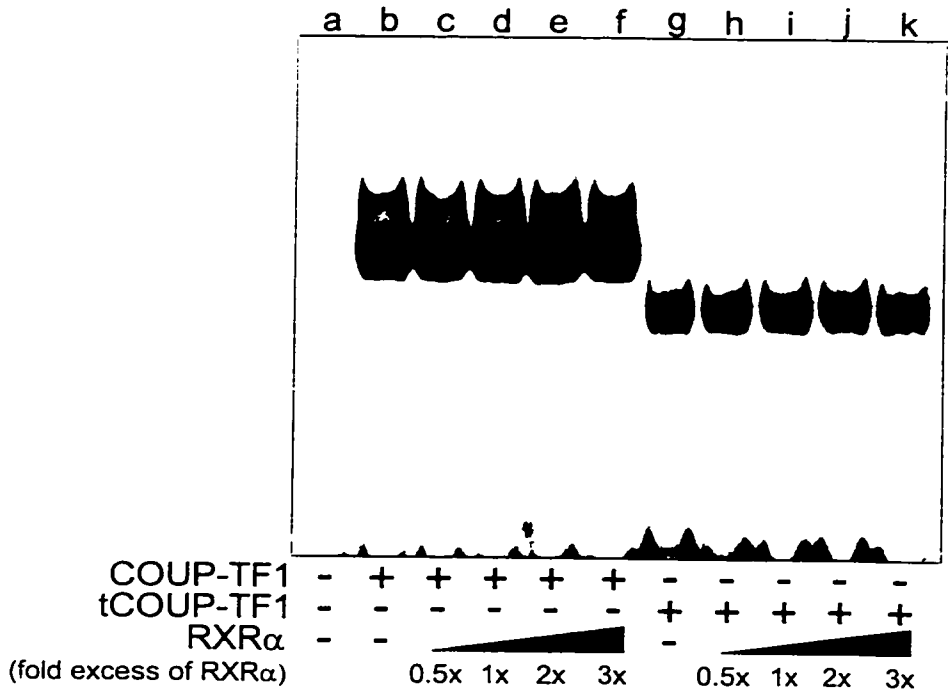


Figure 5.4: RXRα does not antagonize the binding of COUP-TF1 to the HD PPRE. *In vitro* translated COUP-TF1 and truncated (t) COUP-TF1 and RXRα were incubated with radiolabelled HD PPRE in EMSA analysis. Equimolar amounts of COUP-TF1 and tCOUP-TF1 were added as indicated. Excess RXRα was added as indicated. The fold excess of RXRα refers to the molar excess of RXRα.

Finally, COUP-TF1 interacts with the corepressors N-CoR and SMRT (Hirotaka et al., 1997). Thus the binding of COUP-TF1 to response elements results in the active repression of transcription through the recruited coactivation complex. Therefore, not only would the binding of COUP-TF1 to a PPRE result in inhibition of PPAR/RXR forming a productive DNA bound complex but also result in active repression of transcription of PPAR regulated genes.

5.1.6 Summary

These results demonstrated that COUP-TF1 binds to the AOx and HD PPREs and entailed the first characterization of a nuclear receptor other that PPAR/RXR binding to the AOx and HD PPREs. While COUP-TF1 binds to the HD PPRE as a homodimer, COUP-TF1 may bind to the AOx PPRE as both a homodimer and monomer. The determination of the *in vivo* effects of COUP-TF1 on PPAR mediated signalling demonstrated an antagonism of PPAR signalling mediated by the HD PPRE. A result consistent with the role of COUP-TF1 as a repressor in mammalian cells. Taken together, these results suggest that the antagonism by COUP-TF1 is due to the competition for binding to the HD PPRE with PPAR/RXR and the recruitment of the corepression complex once bound to the PPRE. The net result would be that PPAR/RXR is prevented from binding to the PPRE and activating transcription in response to peroxisome proliferators and that COUP-TF1 bound to the PPRE actively represses the transcriptional activity of the target gene.

5.2 HNF-4 MODULATES PPAR MEDIATED SIGNALLING

HNF-4 is a nuclear receptor that regulates a number of genes involved in lipid metabolism (Jiang et al., 1997). HNF-4 appears to act as a transcriptional activator that requires an intact AF-2 domain and interacts with the coactivator CBP (Hadzopoulou-Cladaras et al., 1997; Yoshida et al., 1997; Nakshatri and Bhat-Nakshatri, 1998). To date, no HNF-4 agonist has been identified. In fact, the activation function of HNF-4 appears to be constitutively active and potentiated by the removal of an inhibitory domain, a feature that is unique to HNF-4 amongst nuclear receptors (Hadzopoulou-Cladaras et al., 1997).

A number of aspects of HNF-4 mediated signalling identify it as having the potential to converge with PPARα mediated signalling. In addition to regulating the transcription of genes involved in lipid metabolism, HNF-4 binds as a homodimer to response elements that are homologous to DR1 response elements such as PPREs (Jiang et al., 1997; Sladek et al., 1990; Nakshatri and Bhat-Nakshatri, 1998). The homology is sufficient that HNF-4 can both recognize and activate the transcription of target genes through these response elements (Nakshatri and Bhat-Nakshatri, 1998). A comparison of the binding sites of HNF-4 with the AOx PPRE (Fig. 5.5, *Panel B*), demonstrates that the HNF-4 binding site matches very closely to the AOx PPRE (10/11 possible matches). The alignment of the HNF-4 binding site with the HD PPRE demonstrates three potential binding sites that overlap with both DR1 elements of the HD PPRE (7/11 or 8/11 possible matches). Furthermore, these two signalling pathways are both localized to the liver since both of these nuclear receptors are expressed in this tissue (Sladek et al., 1990, Issemann and Green, 1990). Finally, B. Zhang identified HNF-4-like factors from the

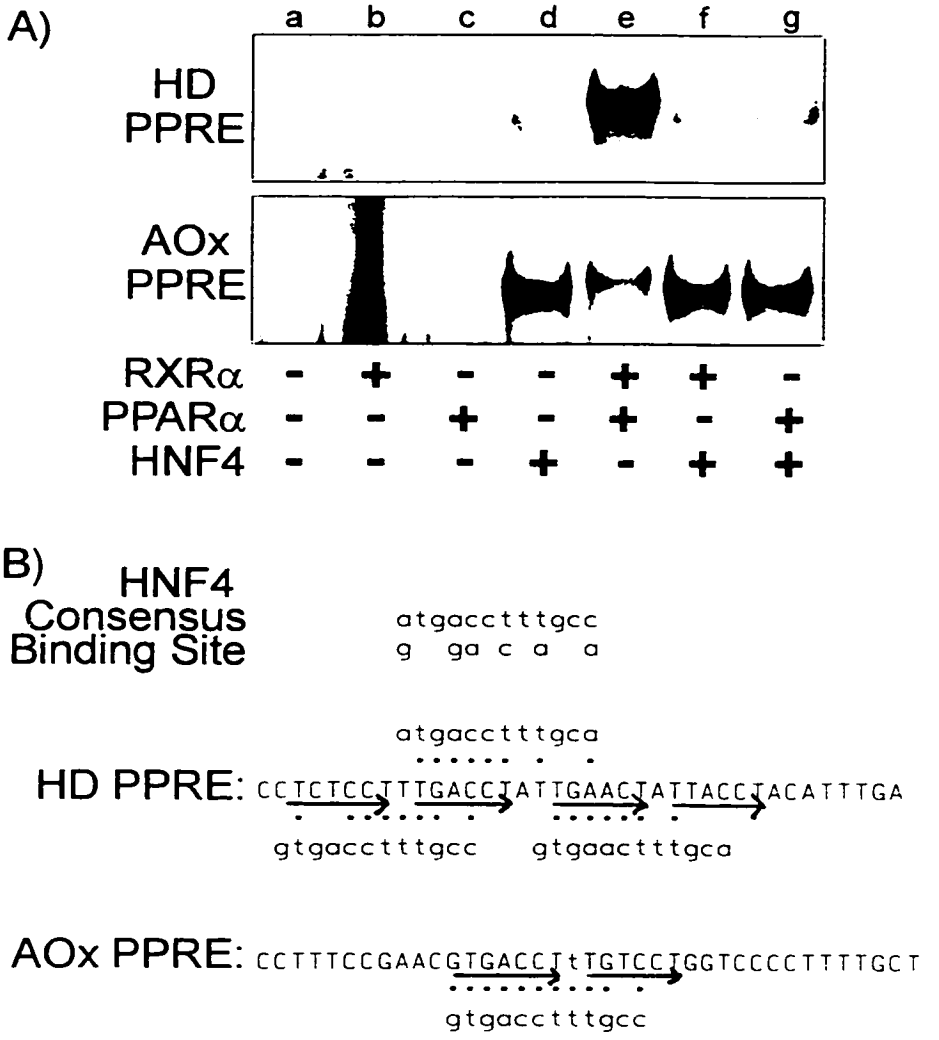


Figure 5.5: HNF-4 binds to the AOx and HD PPREs.
Panel A. In vitro translated RXRα, rat PPARα and HNF-4 were incubated with radiolabelled AOx and HD PPREs in EMSA analysis. *Panel B.* Alignment of the consensus binding site of HNF-4 (Sladek et al., 1990) with the HD and AOx PPREs. The direct repeats of the consensus sequence TGACCT are indicated with arrows.

peroxisome proliferator-responsive hepatocyte cell line H4IIEC3, that bound to the AOx and HD PPREs (Winrow et al., 1994). To investigate whether HNF-4 and PPAR signalling converged, my goal was to establish that HNF-4 did indeed bind to the AOx and HD PPREs.

5.2.1 HNF-4 Binds to the AOx and the HD PPREs

In order to assess whether HNF-4 recognized and bound to the AOx PPRE and the HD PPRE, EMSA analysis was performed (Figs. 5.5, *Panel A*). The addition of *in vitro* translated HNF-4 in EMSA with radiolabelled HD and AOx PPREs resulted in the formation of a specific protein-DNA complex, indicating that HNF-4 bound to both the AOx and HD PPREs (lane d). The mobility of this shift was similar to that of PPARα/RXRα bound to these PPREs indicating that HNF4 probably bound to these DNA binding sites as a dimer (compare lanes d and e). While the binding of HNF-4 to the AOx PPRE was easily detected, the binding of HNF-4 to the HD PPRE was minimal. This reduced affinity of HNF-4 for the HD PPRE (lane d), as compared to the binding of PPARα/RXRα (lane e), could be a result of the AOx PPRE being a better match to the ideal HNF-4 binding site (Fig. 5.5, *Panel B*).

EMSA analysis was performed to assess whether the binding of HNF-4 to the AOx PPRE and, in particular, the HD PPRE could be enhanced by the addition of RXRα or PPARα, (Winrow et al., 1994). Neither PPARα nor RXRα augmented the binding of HNF-4 to either the HD PPRE or the AOx PPRE (Fig. 5.5, *Panel A*: compare lane d with lanes f and g).

The binding of HNF-4 to the HD PPRE was shown by B. Zhang to require the second TGACCT repeat of the composite HD PPRE (refer to Fig. 5.7) (Zhang, 1995;

Winrow et al., 1994). While binding of PPAR/RXR required the third and fourth TGACCT repeats that compose the second DR1 element, the PPAR/RXR complex did not require the second TGACCT repeat for binding to the HD PPRE (Zhang, 1995; Miyata et al., 1993). Since the consensus HNF-4 binding site overlaps both the first and second half-sites as well as the second and third half-sites, it is possible that HNF-4 may recognize and bind to both regions. Consequently, HNF4 may bind to both the exclusion or inclusion of PPAR/RXR to the HD PPRE.

These results have identified HNF-4 as an additional nuclear receptor that bound to the AOx and HD PPREs and confirmed the studies by B. Zhang that detected an HNF-4-like complex bound to both the AOx and HD PPREs from hepatocyte nuclear lysate (Zhang, 1995; Winrow et al., 1994).

5.2.2 HNF-4 Antagonizes PPAR Signalling Through the AOx PPRE

Complementary studies by C. Winrow demonstrated that HNF4 antagonized PPARα/RXRα mediated signalling mediated by the AOx PPRE (Fig. 5.6, *Panel A*) (Winrow et al., 1994). Transient transfection analysis revealed that the transcriptional activation by PPARα/RXRα of a reporter gene under the control of the AOx PPRE was antagonized by HNF4 in a dose-dependent manner. In fact, HNF4 antagonized both agonist-independent and agonist-dependent activation of the reporter gene.

5.2.3 HNF-4 Potentiates PPAR Signalling Through the HD PPRE

In contrast to the effect of HNF-4 on PPAR signalling mediated by the AOx PPRE, C. Winrow demonstrated that HNF-4 potentiated agonist-activated PPAR signalling mediated by the HD PPRE (Fig. 5.7, *Panel B*) (Winrow et al., 1994). While

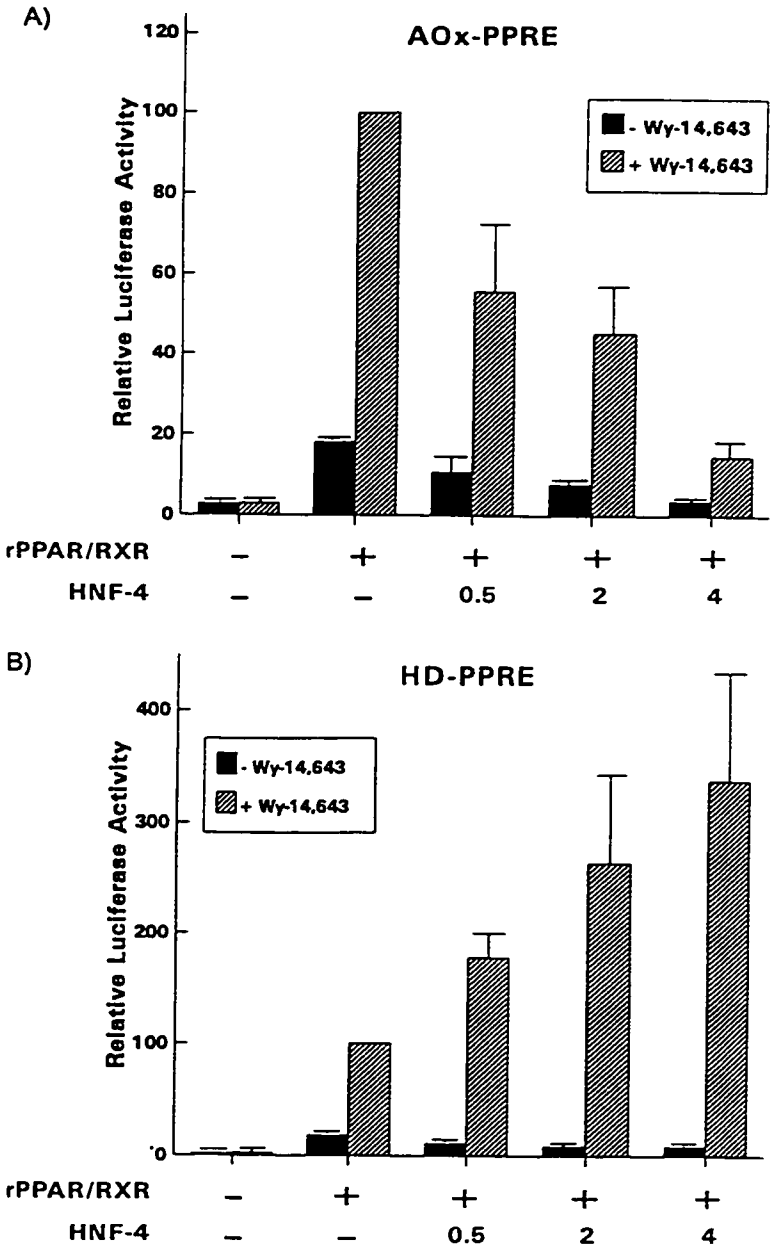


Figure 5.6: HNF4 modulates PPAR/RXR signaling in a PPRE-dependent manner. *Panel A*, HNF-4 antagonizes PPAR/RXR signaling mediated by the AOx PPRE. BSC40 cells were transfected with reporter plasmid pAOx(X2)luc (5 μg), 2 μg of each of the expression vectors for PPARα, RXRα and varying amounts of the HNF-4 expression plasmid (0, 0.5, 2, 4 μg). Where indicated, transfections were carried out in the presence of Wy 14,643 (100 μM final concentration). Values represent the average (± 1 S.D.) of two separate experiments carried out in duplicate and normalized to the activity observed with PPARα/RXRα + Wy 14,643, which was set to 100. *Panel B*, HNF-4 potentiates PPARα/RXRα signaling mediated by the HD PPRE. Cells were transfected as described above with the exception that the pHD(X3)*luc* reporter plasmid was used. This figure represents the work of C. Winrow.

the agonist-independent activation by PPAR was antagonized in a similar manner to that seen with the AOx PPRE, the agonist-dependent activation was potentiated in a dose-dependent manner by HNF-4.

5.2.4 PPRE Dependent Modulation of PPAR Activity by HNF-4

The *in vivo* results depict HNF-4 as a repressor of agonist-independent and dependent transcriptional activation by PPARα/RXRα of the luciferase reporter gene under the control of the AOx PPRE. This repressive activity of HNF-4 is difficult to reconcile with the general role of HNF-4 as a constitutive transcriptional activator (Sladek, 1993; Nakshatri and Bhat-Nakshatri, 1998; Ladias et al., 1992; Ladias, 1994). In further support of HNF-4 as a transcriptional activator, a recent study has shown that the coactivator CBP binds to HNF-4 and potentiates transcriptional activation by HNF-4 in the absence of agonist (Yoshida et al., 1997). These studies suggest that HNF-4 acts as a constitutive activator once bound to DNA in the promoter region of target genes.

In light of these studies, the antagonism of PPAR signalling on the AOx PPRE contradicts the proposed role of HNF-4 as a transcriptional activator. If HNF-4 competes for binding to the AOx PPRE with PPAR/RXR, the addition of increasing amounts of HNF-4 should result in an increase in transcription of the reporter gene in the absence of PPAR agonist. In fact, the addition of HNF-4 alone did not result in activation of either the AOx PPRE or HD PPRE regulated reporter genes (Winrow et al., 1994). Furthermore, a dose-dependent decrease is observed with not only the agonist-independent activation but also the agonist-dependent activation by PPAR. Similarly, HNF-4 antagonizes PPAR/RXR activity mediated by the HD PPRE in the absence of PPARα agonist.

A recent study suggests that the activity of HNF-4 is dependent upon the basal promoter utilized in the construction of reporter genes. While confirming the binding of HNF-4 to the AOx PPRE, the authors demonstrate that binding was not sufficient for activation of a reporter gene regulated by the AOx PPRE. In fact, the ability of HNF-4 to activate transcription of the reporter gene was dependent upon the basal promoter appended to the reporter construct. The use of the basal promoter from the thymidine kinase gene was permissive for activation by HNF-4 while the basal promoter from the CRBPII gene resulted in a loss of activation by HNF-4 (Nakshatri and Bhat-Nakshatri, 1998). Unfortunately, the influence of HNF-4 on PPAR mediated activation on either reporter construct was not assayed. The observation that HNF-4 bound to both the AOx and HD PPREs but failed to activate the reporter constructs utilized in this study (pAOx(X2)*luc* and pHD(X3)*luc*) suggests that the basal promoter, that of the carbamyl phosphate synthetase gene (Howell et al., 1989), was not permissive for HNF-4 activation.

Thus, in order to understand the consequence of HNF-4 binding to the AOx PPRE, one must examine the *in vivo* effects of the addition of HNF-4 on a luciferase reporter construct that is regulated by the whole promoter of the AOx gene and not just the PPRE. As suggested by the work of Nakshatri and Bhat-Nakshatri, 1998, this would permit the definition of the AOx promoter as permissive or non-permissive for HNF-4 signalling. Once this has been accomplished, the investigation of the effects of HNF-4 on PPAR/RXRα mediated signalling could be carried out in the context the natural promoter of the AOx PPRE.

In light of these arguments, the potentiation by HNF-4 of PPAR/RXR signalling mediated by the HD PPRE presents an interesting challenge to the proposed model (Winrow et al., 1994). While these results would suggest that this reporter construct is permissive for HNF-4 mediated signalling, the fact that HNF-4 by itself is unable to activate this reporter gene invalidates this argument. It is likely that the ability of HNF-4 to potentiate PPAR/RXR mediated signalling may be a result of the complex and composite nature of the HD PPRE. Although the binding of HNF-4 to the HD PPRE is weak, the possibility exists that a complex of HNF-4/HNF-4 and PPAR/RXR may be able to simultaneously bind the HD PPRE. Mutation of the second direct repeat resulted in a loss of the ability of HNF-4 to bind the HD PPRE while the binding of PPAR/RXR was unaffected (Winrow et al., 1994). It is possible that a dimer of HNF4 could occupy the 5' DR1 element while PPAR/RXR could occupy the 3' DR1 element (Fig. 5.7). This arrangement is not without precedence since two heterodimers of PPARα/RXRα can occupy both DR1 elements at the same time (Chu et al., 1995). Therefore, the potentiation of RXR/PPAR signalling by HNF-4 could be a result of the close apposition of the two dimers and a potentiation of transcriptional activation only after the PPAR agonist-dependent recruitment of the coactivation complex. In this role, HNF-4 may serve to stabilize/facilitate the interaction between agonist-activated PPAR and the coactivation complex.

Therefore, assuming that the basal promoter of the carbamyl phosphate synthetase gene is non-permissive for HNF-4 signalling, the competition between HNF-4 and PPARα/RXRα for binding to the AOx PPRE results in antagonism of PPAR mediated signalling. Using the same assumption, the potentiation of PPAR/RXR signalling by

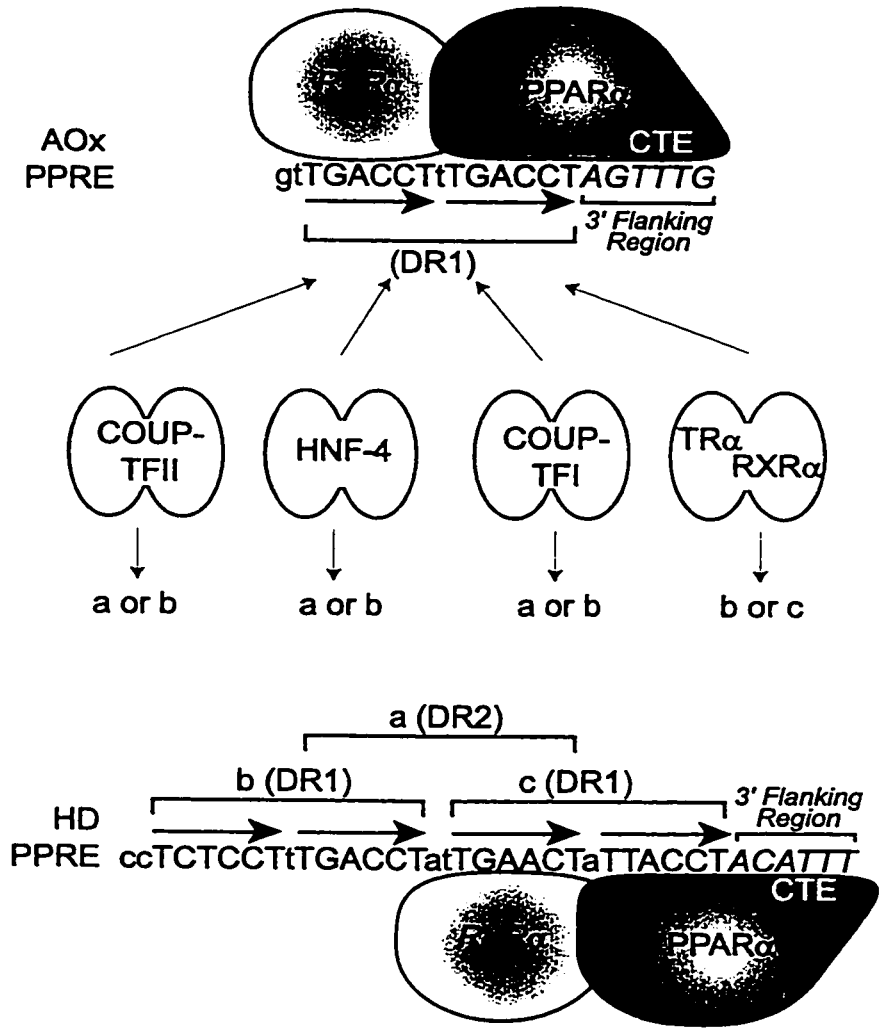


Figure 5.7: Schematic of auxiliary factors binding to the AOx and HD PPREs.
Schematic depiction of the binding of PPAR, RXRα, COUP-TF1, HNF4, COUP-TFII and TRα with the AOx and HD PPREs. The TGACCT consensus half-sites are highlighted with arrow. PPAR and RXR are depicted bound to each PPRE with the carboxy-terminal extension (CTE) of PPARα in contact with the 3' flanking region of the PPREs. Note the composition of the HD PPRE: two DR1 response elements that are separated by two nucleotides.

HNF-4 could be due to the effects of the binding of both dimers to this complex PPRE. Finally, in order to understand the role HNF-4 plays in the regulation of target genes, one must study these effects in the context of the natural promoter of the target gene.

Taken together, these results have important implications for the role of HNF-4 in transcriptional regulation. Firstly, the dependence of HNF-4 activity upon determinants present in the promoter region of target genes suggests that binding to a promoter is not sufficient for activation of that gene. This implies that the ability of HNF-4 to recruit the coactivation complex is not constitutive or that the recruited coactivation complex is not compatible with all promoters. Secondly, the dependence of HNF-4 activity on the basal promoter suggests that a specific complement of factor(s) is required for HNF-4 to act as a transcriptional activator. It is possible that these factors may interact and activate HNF-4, permitting the recruitment the coactivation complex. Conversely, these *cis*-acting factors may act as allosteric inhibitors of HNF-4 function.

Therefore, gene promoters that contain HNF-4 binding sites may be of two types: permissive and non-permissive (Nakshatri and Bhat-Nakshatri, 1998). The binding of HNF-4 to a response element located in a permissive promoter of a target gene will result in constitutive activation by HNF-4. However, the binding of HNF-4 to a response element of a non-permissive gene will have no effect. That is unless other factors such as PPAR/RXR also bind to this response element. In this case, HNF-4 would antagonize PPAR/RXR signalling by competing for binding to the response element. While the binding of HNF-4 to the simple AOx element presents a scenario of competition for the DNA binding site, the complex nature of the HD PPRE presents an interesting situation in which both HNF-4 and RXRα/PPARα could bind simultaneously. While HNF-4 can

bind to PPREs and activate transcription of model reporter genes, it remains unknown if the genes regulated by the AOx and HD PPREs are permissive for HNF-4 signalling.

5.2.5 Summary

These results entail the first demonstration that HNF-4 binds to both the AOx and HD PPREs, although the binding to the HD PPRE is minimal. The determination of the *in vivo* effects of HNF-4 on PPAR mediated signalling suggested an antagonism of PPAR signalling mediated by the AOx PPRE while HNF-4 appeared to potentiate PPAR signalling mediated by the HD PPRE. These results suggested differential regulation of the AOx and HD genes of the peroxisomal β-oxidation pathway. However, recent studies that have characterized the requirements of HNF-4 mediated transcriptional activation necessitate a re-evaluation of the differential effects of HNF-4 on transcriptional activation mediated by the AOx and HD PPREs. Significant issues that must be resolved include the evaluation of the activation of AOx PPRE and HD PPRE regulated reporter constructs that utilize an HNF-4 permissive basal promoter. Furthermore, the determination of whether a complex of an HNF-4 homodimer and a PPAR/RXR heterodimer are able to bind to the HD PPRE would suggest an alternative explanation to the differential results obtained between the AOx and HD PPREs. Finally, an evaluation of whether HNF-4 bound to a PPRE is able to recruit coactivators would identify these elements as capable of binding a form of HNF4 competent to recruit coactivators and thus activate transcription.

5.3 OTHER MODULATORS OF PPAR/RXR MEDIATED SIGNALLING

The number of factors that are able to interact with PPREs continues to grow. For instance, the nuclear receptor TRα has been demonstrated to interact with the AOx PPRE (Fig. 5.7) (Winrow et al., 1996; Miyatmoto et al., 1997). In fact, both a monomer of TRα and the heterodimer of TRα/RXRα competed for binding to the AOx PPRE with PPAR/RXR. The study by Winrow et al, 1994, describes the AOx PPRE functioning as a negative thyroid response element. These response elements are activated by TR in an agonist-independent manner while the addition of agonist results in repression of target gene transcription (Tagami and Jameson, 1998). The effect of TRα signalling on PPAR/RXR mediated signalling followed a similar trend (Winrow et al., 1996). While TRα in the absence of thyroid hormone potentiated PPAR/RXR mediated signaling in the presence and absence of PPAR agonist, TRα in the presence of thyroid hormone antagonized PPAR/RXR mediated transcriptional activation in the presence and absence of PPAR agonist. Although a study by Miyamoto et al., 1997, describes a similar antagonism by TRα of PPAR signalling mediated by the AOx PPRE, they do not describe any difference in the effect of TR agonist. However, they do demonstrate that the DNA binding ability of TRα is required for antagonism of PPAR signalling. Since the affinities of both TRα/RXR and PPAR/RXR is similar for the AOx PPRE (K_d ~ 2 nmol) (Miyatmoto et al., 1997), TRα probably competes for binding to the AOx PPRE with PPAR/RXR. Once TRα/RXRα is bound, the AOx PPRE may act as a negative thyroid hormone receptor response element. This would provide a possible mechanism that could account for the fact that thyroid hormone inhibits expression of the acyl-CoA oxidase gene (Bendik and Pfahl, 1995).

Another factor that interacts with the HD PPRE is the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (Marcus et al., 1996). COUP-TFII binds the HD PPRE and antagonizes signalling mediated by PPAR/RXR (Fig. 5.7). However, in the presence of the orphan receptor coactivator (ORCA), COUP-TFII activates transcription of a reporter gene under the control of the HD PPRE (Marcus et al., 1996). Furthermore, COUP-TFII activated by ORCA potentiates PPAR/RXR mediated signalling. Of interest is the fact that ORCA is a ligand of the tyrosine kinase $p56^{lck}$, suggesting integration of tyrosine kinase signalling pathways with the activation of orphan nuclear receptors and activation of genes under the control of PPREs.

5.4 CONCLUSIONS

These studies were the first to demonstrate that nuclear receptors other than PPAR and RXRα bound to PPREs. It was found that the transcriptional repressor COUP-TF1 and the transcriptional activator HNF-4 both bound to both the AOx and HD PPREs. COUP-TF1 bound to both the AOx and HD PPREs as a homodimer and possibly to the AOx PPRE as a monomer. Complementary work by B. Zhang demonstrated that COUP-TF1 antagonized the PPAR/RXR mediated activation of a reporter gene under the control of the HD PPRE. The mechanism of this antagonism was shown to not involve the sequestration of RXRα by COUP-TF1. Therefore it is proposed that COUP-TF1 competes for binding to the PPRE with PPAR/RXR and consequently recruits the corepression complex to actively repress the expression of the target gene.

HNF-4 was shown to bind to both the HD and AOx PPREs. Complementary work by C. Winrow demonstrated that HNF-4 antagonized PPAR/RXR signalling mediated by the AOx PPRE, presumably through the competition with PPAR/RXR for binding to the PPRE. However, HNF-4 potentiated agonist-dependent signalling by PPAR/RXR mediated by the HD PPRE. It is proposed that this may occur through the formation of a complex of PPAR/RXR/HNF4 bound to the HD PPRE. Since the promoter of the reporter construct is non-permissive for HNF-4 signalling, further study is required to determine the effects of HNF-4 on PPAR/RXR activity in the context of a reporter gene controlled by an HNF-4 permissive promoter. Furthermore, the promoters of the AOx and HD genes must be assessed for their ability to permit transcriptional activation by HNF-4.

These studies were the first to demonstrate the binding of other nuclear receptors to the AOx and HD PPREs. Furthermore, the binding of these nuclear receptors results in the modulation of PPAR/RXR mediated signalling. A mechanistic understanding of the role auxiliary factors play in modulating PPAR/RXR mediated signalling requires not only the identification of the mechanism of regulation but also the relevance of the mechanism. This involves achieving an appreciation of whether these factors play a static role or and a dynamic role in the regulation of PPAR. The first scenario which involves a closed, static system, an equilibrium is established in which the activation of the target gene is the net result of the association of various competing factors that bind to the response element and exert their biological effects (Fig. 5.7). This would include constitutive activators such as HNF-4, and repressors such as COUP-TF1, as well as the neutral PPAR/RXR complex.

The inclusion of a dynamic element to the system can be broken into two components. The first involves an alteration in the biological activity of a factor in the previously described scenario. For example, the activation of one component by agonist, such as PPARα, would result in the increase in the transcription of the target gene. The second component of dynamic regulation is an alteration in the amount of an factor or an alteration in the affinity of the factor for a response element.

Finally, an appreciation of how these regulatory mechanisms are manifested as biological phenomena requires an appreciation of not only the net effect of all factors, including PPAR and RXR, that bind to the PPRE, but also the influence of the full complement of factors bound by the promoter.

6. LXRα ANTAGONIZES PPARα MEDIATED SIGNALLING

The function of PPARα is dependent upon not only the protein-DNA interactions detailed in Section 3.0 and 5.0, but also protein-protein interactions such as the interaction with its heterodimerization partner RXRα. A full understanding of the mechanism of PPAR mediated transcriptional activation of target genes will only be possible once the complete complement of modulatory and effector proteins that interact with PPAR have been identified. Therefore, to identify protein-protein interactions involved in mediating PPARα activity, the yeast two-hybrid system was used to screen for novel PPARα interacting proteins.

This screen was successfully used to discover a previously undescribed nuclear receptor that interacted with mPPARα (Miyata et al., 1996). This novel receptor was predominantly expressed in the liver and hence named the liver X receptor (LXRα) (Willy et al., 1995). LXRα is implicated in the regulation of enzymes involved in the metabolism of cholesterol since it is activated by hydroxylated derivatives of cholesterol and LXRα null mice demonstrate an impaired ability to regulate cholesterol levels (Peet et al., 1998; Janowski et al., 1996). The following section details the discovery and isolation of LXRα, the characterization of its interaction with PPARα and RXRα and the identification of a novel modulator of PPAR/RXR mediated signalling.

6.1 INTERACTION CLONING OF LXRα

The yeast two-hybrid system can be utilized as a genetic screen for discovering novel protein-protein interactions (Fields and Song, 1989; Chien et al., 1991). As depicted in Fig. 4.1, the functional reconstitution of GAL4 occurs when two interacting proteins are appended to the GAL4 DNA binding domain (DBD) and activation domain (AD). Based on this scheme, the genetic screen entails fusing your target or "bait" protein to the GAL4 DBD and a cDNA library to the GAL4 AD. The expression plasmids for these constructs are cotransformed into yeast that contain the HIS3 reporter gene. Yeast that contain a cDNA encoded polypeptide that interacts with your protein of interest, will functionally reconstitute the activity of GAL4 and produce HIS3 permitting growth on plates that lack histidine. These his+ yeast are selected for further analysis to validate and characterize the interacting polypeptide (refer to Section 2.2.1.4 for more information).

To this end, the N-terminal deleted mPPARα was fused to the GAL4 DBD (a.a. 83-468, pGBD-NΔmPPARα) and used as 'bait' to screen a Matchmaker human HeLa cell cDNA library fused to the GAL4 AD (cloned into pGADGH (Clontech)). These expression plasmids were introduced into the *S. cerevisiae* strain HF7c since it contains both HIS3 and β-galactosidase reporter genes under the control of the GAL4 DNA binding site (Fig. 4.1: UASg). A total of nine yeast transformants grew on plates lacking histidine out of a total of 5×10^5 transformants. Plasmids expressing the cDNA-GAL4 AD fusions were isolated from the nine his+ positive colonies. To test for 'false positives', each GAL4 AD-cDNA fusion was tested for specificity with PPARα baits, empty GBD bait vectors, and irrelevant baits by cotransformation into the *S. cerevisiae*

strain Y190 and measuring β-galactosidase activity by the agarose overlay assay. All nine GAD-cDNA fusion expression plasmids conferred a his+, β-gal+ phenotype exclusively in the presence of pGBD-NΔmPPARα demonstrating that they interacted with PPARα in a specific manner.

Sequence analysis showed that two of these GAD-cDNA constructs (KM1 and SM1) contained overlapping sequences of a novel member of the nuclear receptor superfamily (Fig. 6.1). Of interest was the identification of the same clone in a two-hybrid screen for RXRα interacting factors, demonstrating that this novel nuclear receptor also interacted with RXRα (Miyata et al., 1996). A 1.7 kbp cDNA was isolated from a λgt11 human liver cDNA library using GAD-SM1 as a probe by R. Rachubinski. The open reading frame of this cDNA encoded a protein of 447 a.a.. The GAL4 AD-cDNA fusion isolated from the yeast two-hybrid screen corresponded to a.a. 61 to 447 of this open reading frame.

The identification of this novel nuclear receptor and the nucleotide sequence of the cDNA confirmed the work of another group that cloned the same novel nuclear receptor and named it the liver X receptor (LXRα) (Willy et al., 1995). This protein was shown to interact with RXRα confirming our identification of GAD-SM1 as an RXRα interacting protein (Miyata et al., 1996).

6.2 LXRα IS COEXPRESSED WITH PPARα IN THE LIVER

To determine the tissue expression pattern of LXRα mRNA, H. Patel performed Northern analysis. Using the radiolabelled cDNA of GAD-SM1 as a probe, a

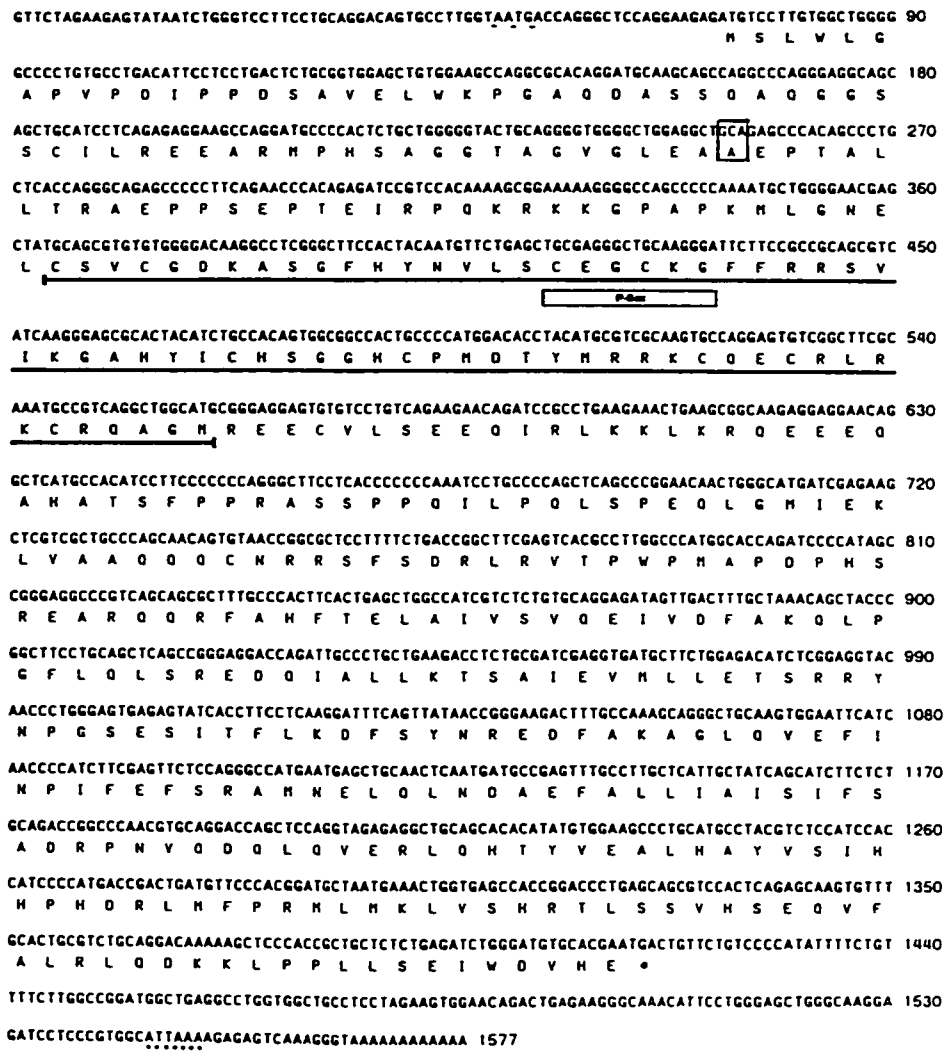


Figure 6.1: cDNA sequence of the novel nuclear receptor LXRα.
Nucleotide and predicted protein sequence of the cDNA of LXRα isolated from the λgt11 library by R. Rachubinski. The DNA binding domain is underlined. The P-Box, the region of the DNA binding domain that makes direct contact with the DNA binding site is noted. The first amino acid of the GAL4 AD-cDNA fusion SM1 is boxed (Ala 33). Of note is that SM1 encoded the remaining portion of LXRα (a.a. 33 to 447).

Northern blot of mRNA from representative human tissues (Clontech) was probed for expression of LXRα mRNA (Fig. 6.2). A specific mRNA species that corresponded to the predicted size of 1.7 kbp was detected predominantly in liver. Expression of LXRα mRNA was also detected in skeletal muscle, heart and at very low levels in the pancreas, kidney, lung and placenta. LXRα mRNA could not be detected in the brain. Of note is that a larger form of LXRα mRNA is expressed in skeletal and cardiac muscle. The origin and significance of this variant remains unknown.

Since PPARα is expressed in the liver (Issemann and Green, 1990), these results demonstrate that LXRα and PPARα are coexpressed in the liver, providing support that the interaction between of LXRα and PPARα represents a biologically relevant interaction.

6.3 LXRα INTERACTS WITH PPARα AND RXRα *IN VIVO*

The yeast two-hybrid system was used to characterize the *in vivo* interaction between the fusion of LXRα and the GAL4 AD isolated in the yeast two hybrid screen, with the GAL4 DBD fusions of RXRα and PPARα. The vector expressing the fusion of the GAL4 AD and LXRα (GAD-SM1: a.a. 61 to 447 of LXRα) was transformed into Y190 yeast alone and in combination with the vectors that expressed fusions of the GAL4 DBD with NΔmPPARα (a.a. 83-468 of mouse PPARα), and RXRα (a.a. 25 to 462 of human RXRα) (Table 6.1). The interaction between GBD-NΔmPPARα or GBD-RXRα was specific as reporter gene activity was only detected when these fusions were coexpressed with the GAD-SM1 (Table 6.1). However, the interaction between PPARα and LXRα was much weaker than that of RXRα and LXRα as demonstrated

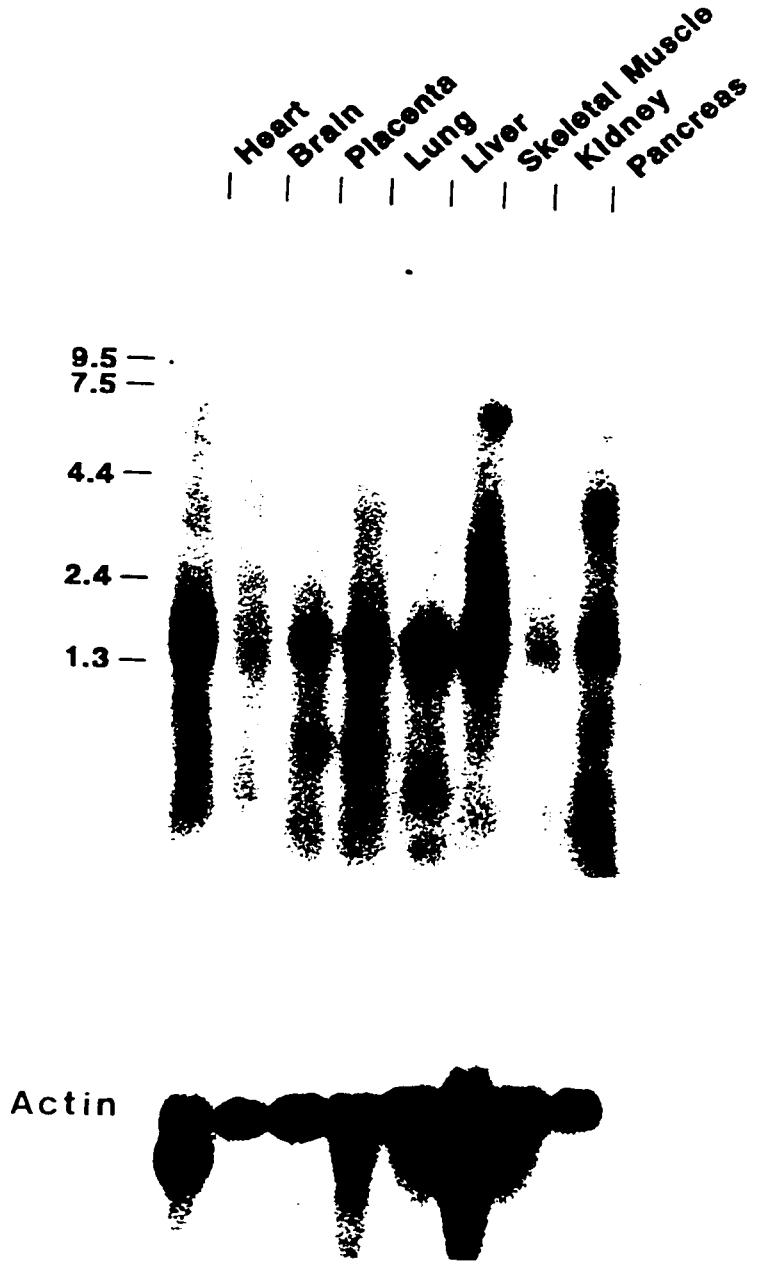


Figure 6.2: Tissue expression pattern of LXRα mRNA.
Northern analysis of the tissue expression pattern of LXRα. A Northern blot of mRNA from representative human tissues (Clontech) was probed with the radiolabelled SM1 cDNA. The units of molecular weight ladder is in kilobasepairs. The blot was stripped and probed for actin expression in order to normalize for differences in the amount of mRNA loaded in each lane. This data was prepared by H. Patel.

Table 6.1: LXRα interacts with PPARα and RXRα in the yeast two-hybrid system.

GAL4 DBD (GBD) Fusion	GAL4 AD (GAD) Fusion	β-galactosidase Activity[a]
GBD		white
GBD-NΔmPPARα		white
GBD-RXRα		white
	GAD	white
	GAD-SM1[b]	white
	GAD-RXRα	white
GBD	GAD	white
GBD-NΔmPPARα	GAD	white
GBD-RXRα	GAD	white
GBD	GAD	white
GBD	GAD-SM1	white
GBD	GAD-RXRα	white
GBD-NΔmPPARα	GAD-SM1	light blue
GBD-NΔmPPARα	GAD-RXRα	blue
GBD-RXRα	GAD-SM1	blue
GBD-RXRα	GAD-RXRα	white

[a] β-galactosidase activity was determined by agarose overlay using *S. cerevisiae* strain Y190 harboring expression vectors of the GAL4 DBD and GAL4 AD fusions. Refer to Materials and Methods, Section 2.2.13.1.

[b] GAD-SM1 refers to a GAL4 AD fusion of LXRα encoding a.a. 61-447.

qualitatively by the differences in the levels of β-galactosidase activity detected in the agarose overlay assay. Therefore, LXRα interacts with RXRα and PPARα *in vivo*. These interactions occurred in the absence of either exogenous agonist or DNA binding.

6.4 LXRα INTERACTS WITH PPARα AND RXRα *IN VITRO*

In order to verify a physical interaction between PPARα, RXRα and LXRα, protein binding assays using immobilized maltose binding protein (MBP) fusion proteins of PPARα and RXRα and *in vitro* translated, ^{35}[S]-methionine-labeled full-length LXRα or an N-terminal truncated form: NΔRIP1 (a.a. 61 to 447) synthesized *in vitro*). Full length LXRα bound to MBP-PPARα but not the control, MBP coupled beads (Fig. 6.3, *Panel A*, compare lanes 2 and 4). By comparison, a greater proportion of both LXRα and NΔRIP1 added to the binding reactions bound to MBP-RXRα (Fig. 6.3, *Panel B*, compare lanes 2,3 with 5,6). In fact, the proportion of LXRα added to the binding reaction that bound to MBP-RXRα was approximately 10 fold greater than the proportion of LXRα that bound to MBP-PPARα (Miyata et al., 1996). Thus, both PPARα and RXRα interact with LXRα *in vitro* although the interaction of PPARα with LXRα appears to be weaker than that of RXRα and LXRα.

6.5 LXRα BINDS TO DNA COOPERATIVELY WITH RXRα

It was postulated that the interaction between LXRα and PPARα or RXRα represented the formation of heterodimers that could bind DNA. In order to test this hypothesis, EMSA analysis was performed with *in vitro* translated LXRα, PPARα and RXRα and a representative selection of synthetic radiolabelled response elements. These

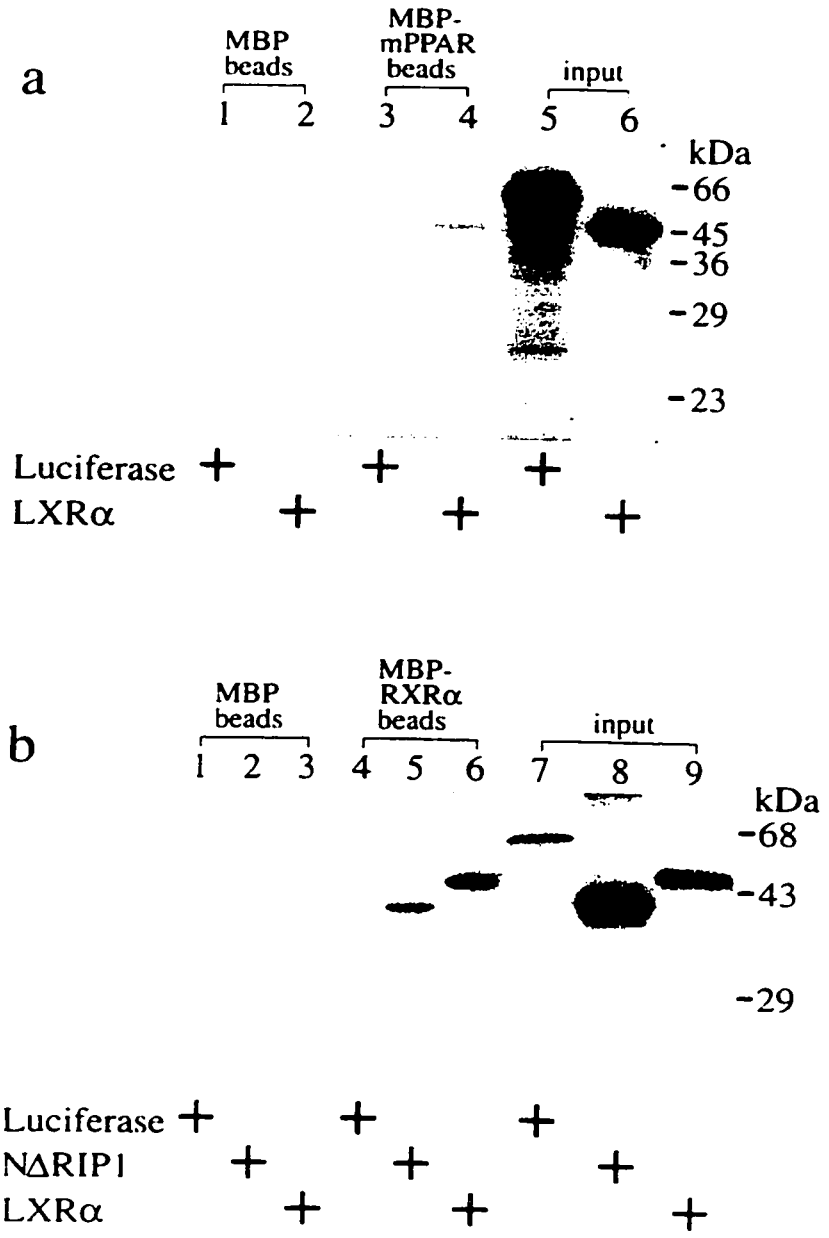


Figure 6.3: LXRα Interacts with the mPPARα and RXRα *In Vitro*
Panel A, [^{35}S]Methionine-labeled full-length LXRα or luciferase synthesized *in vitro* was incubated with immobilized MBP-PPARα or MBP alone, as indicated, and the bound radiolabelled protein was analyzed by polyacrylamide gel electrophoresis. Lane 1 represents 10% of the labeled material added to each of the binding assays. *Panel B*, [^{35}S]Methionine-labeled full-length LXRα or luciferase synthesized *in vitro* was incubated with immobilized MBP-RXRα or MBP alone, as indicated, and the bound radiolabelled protein was analyzed by polyacrylamide gel electrophoresis. Lane 1 represents 10% of the labeled material added to each of the binding assays.

response elements corresponded to the consensus binding sites of nuclear receptors in which direct repeats of the TGACCT half-site were separated by 0 to 5 nucleotides (DR0, DR1, DR2, DR3, DR4, DR5). The spacing of these elements by 0 to 5 nucleotides represents the known range of spacing of characterized nuclear receptor response elements.

The ability of PPARα, RXRα and LXRα to bind natural response elements was also assayed. To determine if the interaction of PPARα with LXRα represented the formation of a complex that could bind to PPREs, the ability of these proteins to bind the AOx and HD PPREs was assessed *in vitro* by EMSA. Work by Willy et al., 1995, had identified a response element in the mouse mammary tumour virus (MMTV) promoter to which LXRα bound and activated transcription. This 'natural' LXR response element was also assayed as a binding site for PPARα/LXRα dimers.

LXRα was unable to bind to any of the tested response elements on its own (Fig. 6.4, *Panel A*, lane 4). However, LXRα did bind cooperatively with RXRα to the DR4 response element and minimally to the DR5 response element (Fig. 6.4, *Panel A*, lane 7). Furthermore, the cooperative binding of LXRα and RXRα was detected with the MMTV LXRE, a natural DR4 response element as previously seen (Willy et al., 1995).

6.6 LXRα DOES NOT BIND TO DNA WITH PPARα

The assessment by EMSA of the binding of LXRα to various response elements did not detect the binding of LXRα/PPAR heterodimers to any of the synthetic response elements tested (DR0 – DR5), the HD PPRE, the AOx PPRE or the MMTV LXRE (Fig 6.4, lane 6). Although a complex can be detected binding to the AOx and HD PPREs in the presence of both PPARα and RXRα, this complex is attributed to the binding of

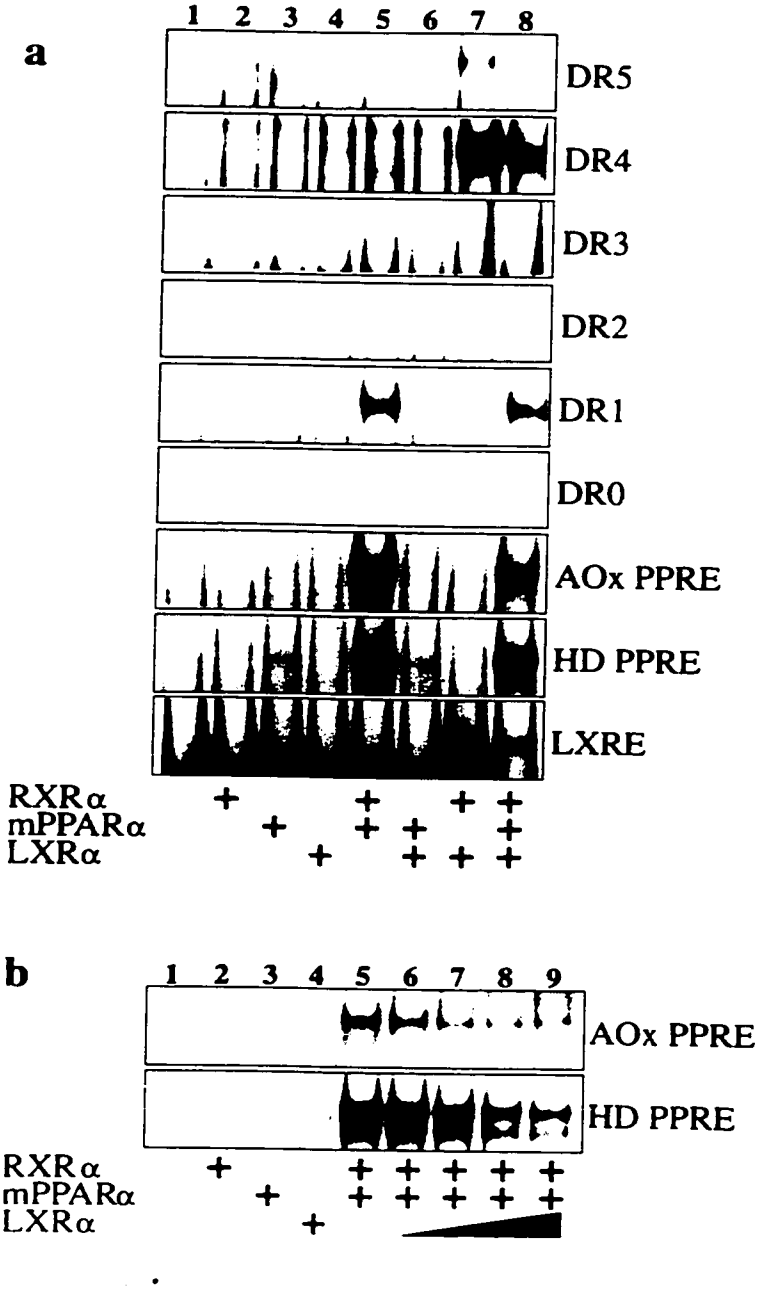


Figure 6.4: LXRα binds with RXRα but not PPARα to synthetic response elements. *Panel A.* The ability of *in vitro* translated RXRα, PPARα and LXRα to bind to various radiolabelled response elements was evaluated by EMSA analysis. The response elements tested include direct repeats of TGACCT separated by 0 to 5 nucleotides (DR0–DR5), the AOx PPRE, the HD PPRE and the LXR response element from the mouse mammary tumor virus promoter (LXRE). *Panel B.* Radiolabelled AOx PPRE and HD PPRE was incubated with a constant amount of *in vitro* translated RXRα and PPARα and an increasing amount of *in vitro* translated LXRα. Protein concentration in each reaction was normalized by the appropriate addition of naïve reticulocyte lysate.

PPARα with the endogenous RXRα present in reticulocyte lysate (Fig. 6.4 *Panel A*, compare with lane 6 with lane 3 for AOx and HD PPREs) (Marcus et al., 1993).

While a heterodimer of PPAR and LXR could not be detected in this assay, the possibility remains that PPAR and LXR may heterodimerize to bind atypical DNA binding sites. Furthermore, the binding of a PPAR/RXR heterodimer may require determinants that are not present with the DNA elements tested, the presence of PPAR/LXR agonists and even the presence of other cofactors. Even though the interaction between PPAR and LXR does not confer the ability to form DNA binding heterodimer in these assays, the surprising finding that PPAR and TRβ bind as a heterodimer to a DR2 response element serves as a reminder that PPAR can bind to response elements other than DR1 elements (Bogazzi et al., 1994).

6.7 LXRα ANTAGONIZES THE BINDING OF PPARα/RXRα TO PPRES

EMSA analysis of the *in vitro* binding of the PPARα/RXRα heterodimer to the AOx PPRE, HD PPRE and the synthetic DR1 response element revealed that protein-DNA complex formation was decreased upon the addition of LXRα (Fig. 6.4, *Panel A*, compare lanes 5 and 8). In fact, the addition of increasing amounts of LXRα while maintaining the amounts of PPARα and RXRα constant, resulted in a progressive decrease in the complex of PPARα/RXRα bound to the AOx PPRE and HD PPRE (Fig. 6.4, *Panel B*). In a similar manner, the presence of PPARα resulted in the decrease of RXRα/LXRα binding to the MMTV LXRE, and the synthetic DR4 and DR5 response elements (Fig. 6.4, *Panel A*, compare lanes 7 and 8).

Since PPARα, RXRα and LXRα are able to heterodimerize in solution, the antagonism of binding of PPARα/RXRα to PPREs by the presence of LXRα could be a

result of the competition of between the formation of PPARα/RXRα heterodimers that are capable of binding PPREs with the formation of PPARα/LXRα dimers that do not bind DNA and RXRα/LXRα dimers that do not recognize DR1 response elements such as PPREs. Conversely, the presence of PPARα antagonizes the binding of LXRα/RXRα to DR4 response elements through the formation of PPARα/LXRα and PPARα/RXRα dimers that do not recognize these response elements.

While the formation of PPARα/LXRα dimers would contribute to the antagonism described above, the weak affinity of PPARα for LXRα as compared to the affinity of PPARα for RXRα and LXRα for RXRα means that it is most likely that the formation of PPARα/RXRα and LXRα/RXRα dimers are responsible for the binding antagonism.

The competition observed between RXRα, PPARα and LXRα for the creation of productive DNA binding complexes is also observed between TRα, PPARα and RXRα (Juge-Aubry et al., 1995). In this case, the dimer of TRα and RXRα binds to DR4 response elements and activates transcription in the presence of TRα agonist. However, the introduction of PPARα results in an antagonism of TRα/RXRα binding to the TRE. Since PPARα/RXRα does not bind to the TRE, a DR4 response element, the formation of the PPAR/RXRα dimer inhibits the formation of a productive TRα/RXRα complex. This was further supported by the fact that a mutant PPARα that was incapable of dimerizing with RXRα was unable to antagonize the binding of TRα/RXRα to DNA and the resultant agonist-induced transcriptional activation.

Therefore, binding of nuclear receptor dimers to response elements is antagonized by the presence of other nuclear receptors that are capable of complexing the involved

receptors into inactive complexes. These inactive complexes may be incapable of binding DNA as is proposed of the PPARα/LXRα dimer and result in a general repression of signalling mediated by PPAR or LXR. Furthermore, while these complexes may not recognize one type of response element, they may function through different response elements. This is evidenced in the competition of PPARα and LXRα for dimerization with RXRα, resulting in a decrease in signalling through one pathway while potentiating signalling through another.

6.8 LXRα SIGNALS THROUGH DR4 RESPONSE ELEMENTS

LXRα binds to DNA response elements with a DR4 architecture as a heterodimer with RXRα (Fig. 6.4, lane 7) (Miyata et al., 1996, Willy et al., 1995). Response elements of this architecture have been shown to be sufficient to confer LXRα mediated transcriptional regulation to a reporter gene (Willy et al., 1995, Miyata et al., 1996). In fact, the first natural LXRα response element to be identified has DR4 architecture (Lehmann et al., 1997). Interestingly, the complex of LXRα bound to DNA is responsive to both RXRα and LXRα agonists (9-*cis* retinoic acid and 22-hydroxycholesterol respectively) indicating that LXRα is a permissive heterodimerization partner of RXRα (Willy et al., 1995; Miyata et al., 1996).

6.9 LXRα ANTAGONIZES PPARα/RXRα MEDIATED SIGNALLING

Since LXRα does not bind to DR1 response elements with either RXRα or PPARα, it would be expected that LXRα would be unable to utilize these response elements to activate the transcription of target genes. This was in fact the case, even in

the presence of RXRα (Miyata et al., 1996). While LXRα could not directly influence signalling through the PPREs, an indirect influence was possible.

Since the presence of LXRα inhibited the binding of PPARα/RXRα to the AOx and HD PPREs, it was hypothesized that the LXRα would antagonize PPARα/RXRα signalling by reducing the levels of PPARα and RXRα available to bind PPREs. The *in vivo* effect of LXRα on PPAR/RXR mediated signalling was assessed by H. Patel in transient transfection assays that made use of a luciferase reporter gene under the control of the AOx PPRE as a model PPARα target gene (Miyata et al., 1996). The expression plasmids of PPARα, RXRα and LXRα were transfected in all combinations along with the luciferase reporter construct pAOx(X2)*luc* into BSC40 cells. The addition of PPARα and RXRα expression plasmids (0.5 μg each) in the presence of the PPARα agonist Wy 14,643 resulted in an agonist induced increase in the transcription of the luciferase reporter gene (Fig. 6.5, *Panel A*). The addition of each receptor individually or the combination of LXRα/RXRα and LXRα/PPARα had little effect on the activity of the luciferase reporter gene. However, LXRα strongly inhibited the agonist-dependent and independent activity of the combination of PPARα/RXRα. This occurred in a dose dependent manner (Fig. 6.5, *Panel B*).

Therefore, LXRα antagonizes PPARα/RXRα mediated signalling *in vivo*. Presumably this antagonism is a manifestation of the competition between PPARα, RXRα and LXRα to form heterodimers that are either capable (PPARα/RXRα) or incapable (LXRα/RXRα and LXRα/PPARα) of signalling through PPREs.

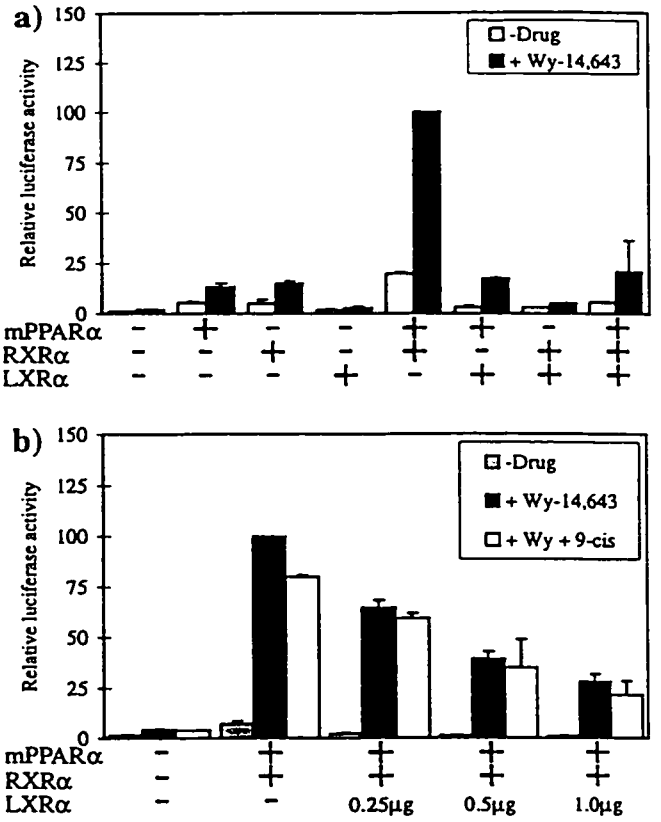


Figure 6.5: LXRα antagonizes PPARα mediated transcriptional activation.
Panel A: The pAOx(X2)*luc* reporter plasmid was transfected into BSC40 cells in combination with the expression plasmids of PPARα, RXRα and LXRα (0.5 μg each) in the absence or presence of the peroxisome proliferator Wy 14,643, as indicated. The luciferase activity of each condition was then measured. *Panel B*: The effect of increasing amounts of LXRα on PPARα/RXRα signalling was determined by measuring the luciferase activity of the pAOx(X2)*luc* in the presence of constant amounts of the PPARα/RXRα expression plasmids (0.5 μg) while adding increasing amounts of the LXRα expression plasmid (0.25 to 1 μg). This data is the average of three independent transfections carried out in duplicate. The luciferase activity is normalized to that obtained in the presence of PPARα, RXRα and Wy 14,643. This value was arbitrarily set to 100%. The error is ± 1 S.D. This figure represents data produced by H. Patel.

6.10 CONCLUSIONS

This section has detailed the discovery of a previously unknown nuclear receptor, LXRα, that interacts with both PPARα and RXRα. Although the interaction of LXRα with PPARα is weaker than with RXRα, both interactions are specific and can be detected *in vivo* and *in vitro*. LXRα binds to nuclear receptor binding sites cooperatively with RXRα with a preference for elements of a DR4 architecture. The heterodimer of PPARα/LXRα does not bind to any of the synthetic response elements tested (DR0-DR5) nor the AOx or HD PPREs. Interestingly, the presence of LXRα antagonized the binding of PPARα/RXRα heterodimers to PPREs, presumably through the competition for RXRα due to the formation of LXRα/RXRα heterodimers. This binding antagonism of PPARα/RXRα heterodimers to PPREs by LXRα was manifested *in vitro* as a dose-dependent inhibition of PPAR mediated signalling by LXRα. Therefore, the competition for shared components between signalling pathways influences the activity of each pathway involved, especially if there is a dynamic component to the system such as a in the activity or quantity of a competing factor.

Since LXRα and PPARα are key regulators of metabolic pathways, cholesterol and free fatty acids respectively, this presents the intriguing possibility that the interplay between PPARα and LXRα mediated signalling is manifested biologically as a coordination of these metabolic pathways at the level of transcription.

7. RIP140 MODULATES PPARα MEDIATED SIGNALLING

The specificity of transcriptional regulation by PPARs, like other members of the nuclear receptor superfamily, likely involves interaction with multiple auxiliary cofactors that link receptors to the basal transcriptional machinery and/or that participate in chromatin remodeling. A number of such factors, which are recruited into multimeric coactivator/corepressor complexes by nuclear receptors, includes coactivators such as SRC-1, p300/CBP, p/CIP, p/CAF and corepressors such as N-CoR, SMRT (Shibata et al., 1997; Torchia et al., 1997; Korzus et al., 1998). While factors such as SRC-1 appear to be exclusively recruited by nuclear receptors, factors such as p300/CBP are utilized by other cellular signalling pathways (Korzus et al., 1998; Kamei et al., 1996). Evidence is accumulating that both common and receptor-specific interacting cofactors play a role in PPAR function. SRC-1, p300, and PBP have been shown to bind to and enhance transactivation by PPAR (Zhu et al., 1996; Dowell et al., 1997a; Zhu et al., 1997). More recently, PGC-1, a novel cell-specific coactivator was identified which binds to PPARγ and links this receptor to the process of adaptive thermogenesis (Puigserver et al., 1998).

Understanding the molecular mechanisms and specificity of gene regulation by PPAR requires the identification of the full spectrum of factors that bind to or modulate PPAR activity. The three PPAR subtypes so far identified (α, β and γ) differ in expression pattern, tissue specificity, and ligand sensitivity and appear to play distinct

roles in the cell (Limberger et al., 1996). However, how this specificity and selectivity is manifested is poorly understood since PPAR subtypes are coexpressed in many cell types, respond in varying degrees to an overlapping spectrum of agonists, and regulate similar target genes. Towards this end, the yeast-two hybrid system was used to identify novel factors that interact with mouse PPARα.

The receptor interacting protein 140 (RIP140) was discovered to be a PPARα interacting protein. RIP140, a putative nuclear receptor coactivator, was demonstrated previously to interact in a agonist-dependent manner with ER, RAR, TR, and RXRα (Cavaillès et al., 1995; L'Horset et al., 1996) and moderately stimulate ER activity (Cavaillès et al., 1995). Interestingly, RIP140 bound to PPARα in the absence of agonist *in vitro* and *in vivo* and repressed PPARα-mediated transactivation *in vivo*. These findings identify RIP140 as a novel coregulator of PPARα signalling and furthermore, indicates that RIP140 can also antagonize the activity of nuclear receptors.

7.1 INTERACTION CLONING OF RIP140

A fusion of the GAL4 DBD with the N-terminal deleted mPPARα (a.a. 83-468), pGBD-NΔmPPARα, was used as bait to screen a Matchmaker human liver cDNA library cloned into pGAD10 (Clontech) in *S. cerevisiae* strain HF7c (total of 1.2×10^7 independent transformants). 33 yeast transformants grew on plates containing 20 mM 3-aminotriazole and lacking histidine, leucine, tryptophan. Plasmids expressing the GAD-cDNA fusions were isolated from the 33 positive colonies and tested for specificity with PPARα baits, empty GBD bait vectors, and irrelevant baits by cotransformation into *S. cerevisiae* strain Y190 and measuring β-galactosidase activity by the agarose overlay assay. Four GAD-cDNA fusion expression plasmids conferred a his+, β-gal+ phenotype

exclusively in the presence of pGBD-NΔmPPARα. Sequence analysis showed that two of the plasmids (KM390 and KM406) contained partial cDNAs encoding human RIP140 (amino acids 164-869) and were chosen for further study. The remaining two plasmids contained cDNA encoding human RXRα and an unknown protein.

RIP140 is an 1158 amino acid long protein initially identified based on its agonist-dependent interaction with and ability to stimulate the activity of the ER (Cavaillès et al., 1995). Sequence analysis of the cDNA inserts from both clones revealed that they contained sequences encoding amino acids 164-869 of human RIP140 Fig. 7.1, *Panel A: GAD-RIP140*), although they arose from independent cloning events. This region of RIP140 overlaps previously identified nuclear receptor-interacting domains (Fig 7.1 *A*: Site 1 and Site 2) and contains 5 out of the 9 copies of the LXXLL motif that individually or in combination, have been shown to mediate the agonist-dependent interaction of RIP140 and the coactivator SRC-1 with nuclear receptors (refer to Section 1.2.4.3)(Heery et al., 1997; L'Horset et al., 1996).

7.2 RIP140 INTERACTS WITH PPARα *IN VIVO*

The yeast two-hybrid system was used to characterize the *in vivo* interaction between PPARα and RIP140 (Fig. 7.1, *Panel B*). The vectors expressing the GAL4 AD-RIP140 fusions were transformed into Y190 yeast alone and in combination with the vectors that expressed fusions of the GAL4 DBD with NΔmPPARα(amino acids 83-468 of mouse PPARα), and RXRα (amino acids 25 to 462 of human RXRα). To assess whether the AF-2 domain of mPPARα was required for interaction with RIP140, a GAL4 DBD fusion with a deleted form of mPPARα missing the carboxyl terminal 33 amino

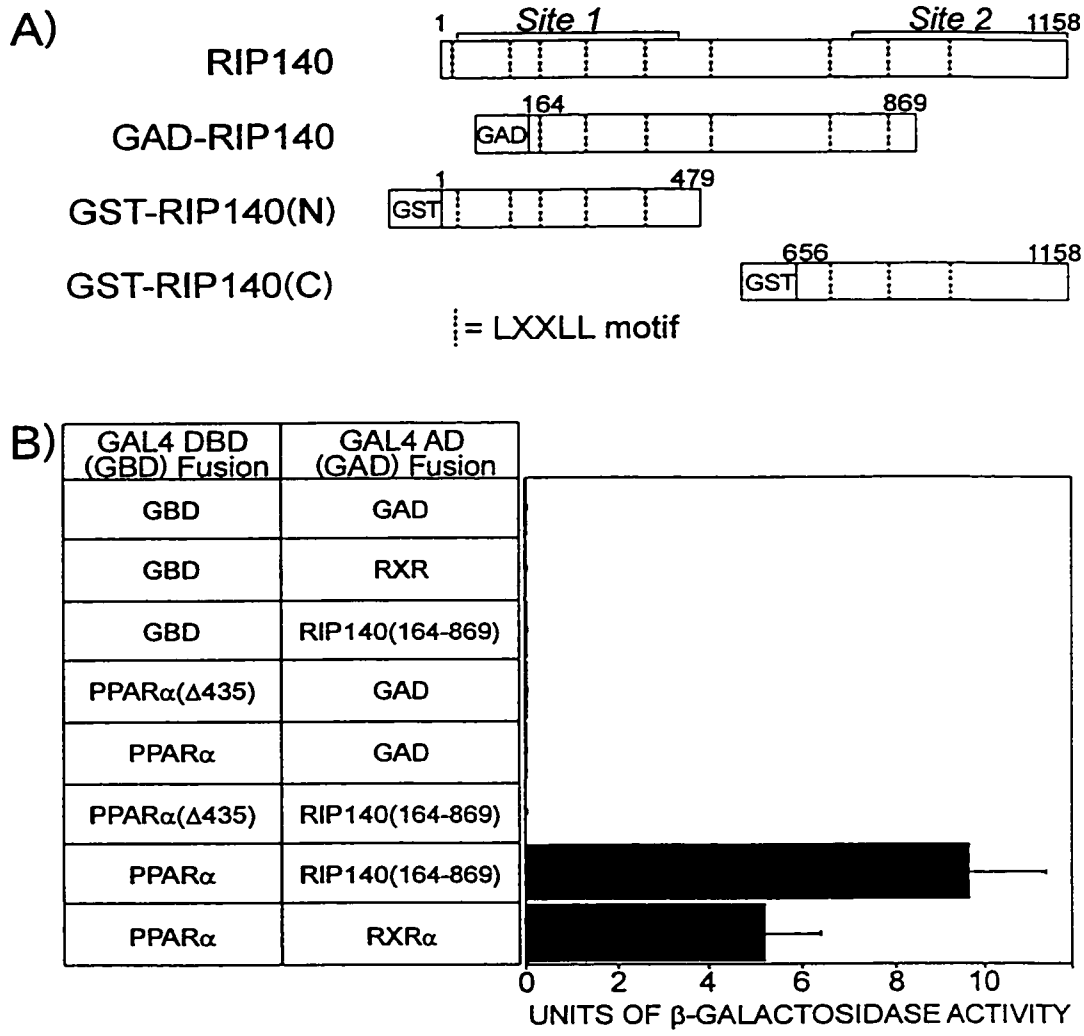


Figure 7.1: The *in vivo* interaction of RIP140 and PPARα requires the AF-2 domain. *Panel A,* Schematic depiction of full-length RIP140, the region of RIP140 (residues 164 to 869) contained in the PPARα-interacting clones isolated in the yeast two-hybrid screen (GAD-RIP140), and the RIP140 amino-terminal and carboxyl-terminal GST fusion proteins used in the *in vitro* binding studies (GST-RIP140(N), GST-RIP140(C)). The LXXLL motifs and nuclear receptor binding domains (site 1 and site 2) as previously described are indicated (Heery et al., 1997; L'Horset et al., 1996). *Panel B,* Interaction between RIP140 and PPARα in the yeast two-hybrid system. The *S. cerevisiae* strain Y190 was transformed with various combinations of vectors that expressed either GBD, GBD-ΔNmPPARα (residues 83 to 469), the C-terminal truncated GBD-ΔNmPPARα(Δ435) (residues 83 to 435), GAD, GAD-RXRα and GAD-RIP140, and β-galactosidase activity was determined from liquid cultures. The data presented represents the average activity of three independent yeast transformants measured in triplicate (± SEM).

acids was included. This region encompasses the AF-2 domain which is required for agonist-dependent activation (Wurtz et al., 1996; Dowell et al., 1997b).

The interaction between RIP140 and PPARα in yeast was specific as reporter gene activity was detected only when GBD-ΔNmPPARα was coexpressed with the GAD-RIP140 fusion protein (Fig. 7.1, *Panel B*). Interestingly, β-galactosidase activity arising from the PPARα/RIP140 interaction was two-fold greater than that obtained with the interaction of PPARα and its heterodimerization partner RXRα. RIP140 did not demonstrate a detectable interaction with GBD-RXRα in this assay. This can be explained by the absence of the RXRα agonist, 9-*cis* retinoic acid, which is required for interaction between these two proteins (L'Horset et al., 1996). GBD-ΔNmPPARα is missing the first 83 amino acids of PPARα, indicating that the extreme amino terminal region is not essential for binding to RIP140 *in vivo*. However, GAD-RIP140 was unable to interact with GBD-ΔNmPPARα(Δ435), the derivative that lacked the extreme carboxyl terminus of PPARα (Fig. 7.1, *Panel B*) whose expression in yeast was confirmed by Western analysis (J. Knez, personal communication). This region contains the putative helix 12 required for agonist-dependent AF-2 mediated transactivation (Wurtz et al., 1996; Dowell et al., 1997b). In fact, Treuter *et al.,* 1998, demonstrated that point mutations that disrupted the PPARγ AF-2 domain eliminated the interaction between RIP140 and PPARα (Treuter et al., 1998). This is consistent with other studies that have shown that the integrity of the AF-2 domain is essential for agonist-dependent interaction of RIP140 with nuclear receptors (Cavaillès et al., 1995; L'Horset et al., 1996; Masuyama et al., 1997). In the case of PPARα however, while the integrity of the AF-2

domain is required, an *in vivo* interaction was observed in the absence of exogenous PPARα agonist, although it remains possible that yeast contain endogenous agonists for this receptor.

Three recent studies have also investigated the interaction between PPARα and RIP140 *in vivo* (Treuter et al., 1998; Miyata et al., 1998; Yan *et al.*, 1998). Treuter *et al.*, 1998, used the yeast two-hybrid system to demonstrate that PPARα and RIP140 interact in the absence of agonist and that the addition of various agonists had no effect on binding. Yan *et al.*, 1998, however showed that in a mammalian two-hybrid system, the interaction was strongly enhanced by the addition of ligand suggesting that the interaction between PPARα and RIP140 was agonist-enhanced. These results must be questioned since in this study, the activity of the reporter gene was a result of not only an interaction between PPARα and RIP140 but also by agonist activation of PPARα itself.

7.3 RIP140 INTERACTS WITH PPARα AND RXRα *IN VITRO*

In order to verify a physical interaction between PPARα and RIP140 and to directly examine the effects of agonist, H. Patel conducted protein binding assays using immobilized glutathione S-transferase (GST) fusion proteins of PPARα and RXRα and full-length, ^{35}[S]-methionine-labeled RIP140 synthesized *in vitro* (Fig. 7.2, *Panel A*). Full length RIP140 bound to GST-PPARα but not the control, GST coupled beads (compare lanes 2 and 8). In fact, approximately 8%-10% of the input radioactivity bound to GST-PPARα. Addition of the PPARα agonist Wy 14,643 (or vehicle) had no effect on the binding efficiency (compare lanes 2 and 4). A similar agonist-independent interaction between these two proteins was observed in co-immunoprecipitation experiments with anti-PPARα antibody and equimolar amounts of RIP140 and PPARα

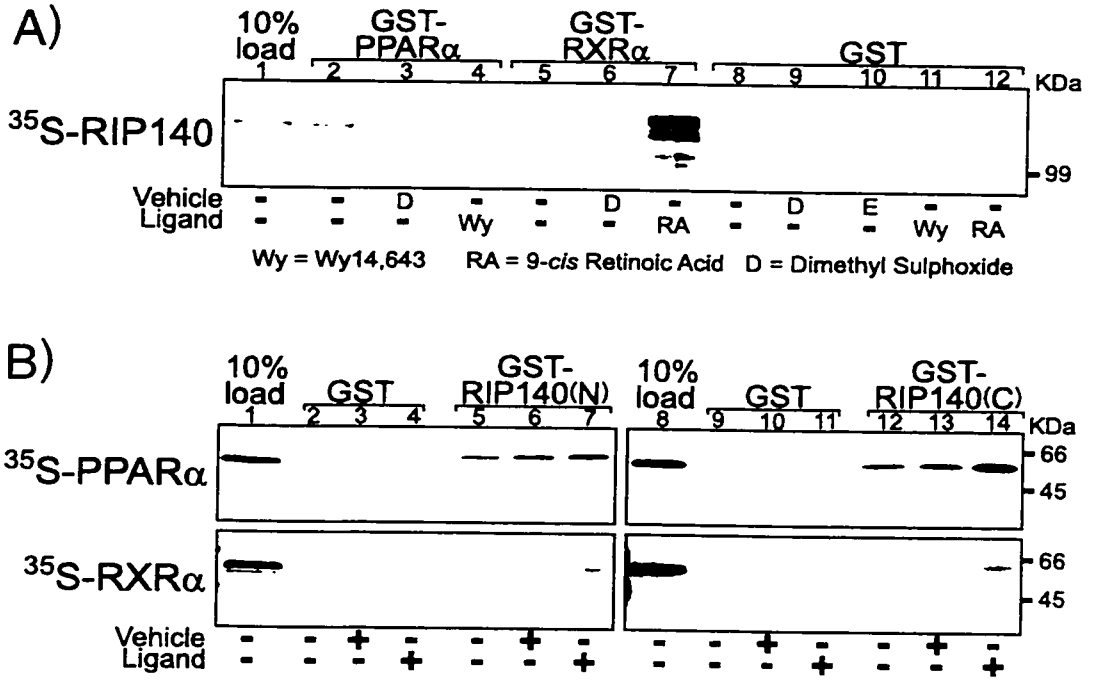


Figure 7.2: RIP140 interacts with PPARα in an agonist-independent manner.
Panel A, [^{35}S]Methionine-labeled full-length RIP140 synthesized *in vitro* was incubated with immobilized GST-PPARα, GST-RXRα or GST alone, as indicated, and the bound radiolabeled protein was analyzed by polyacrylamide gel electrophoresis. Lane 1 represents 10% of the labeled material added to each of the binding assays. Where indicated, binding reactions were carried out in the presence of a final concentration of 100 μM Wy14,643 (Wy), 1 μM 9-*cis* retinoic acid (RA) or the equivalent volume of vehicle (dimethyl sulphoxide (D), as indicated. *Panel B*, PPARα and LXRα interact with amino-terminal and carboxyl-terminal domains of RIP140. [^{35}S]Methionine-labeled PPARα, RXRα and LXRα synthesized *in vitro* were incubated with GST-RIP140(N) or GST-RIP140(C) and the bound material was analyzed as above. Lanes 1 and 8 represent 10% of each receptor added to the binding assays. Addition of appropriate ligand or vehicle was as previously described. This figure represents the work of H. Patel.

(L. Meertens, personal communication). By comparison, binding of RIP140 to GST-RXRα was marginal in the absence of the RXRα agonist 9-*cis* retinoic acid (0.3% of input radioactivity) but increased significantly (35% of input radioactivity) in the presence of agonist (compare lanes 5 and 7). The above results indicate that RIP140 interacts efficiently with PPARα in the absence of agonist. The results of a recent study support the existence of an agonist-independent interaction between PPARα and RIP140 *in vitro* (Treuter et al., 1998). However, the addition of some PPAR agonists resulted in a dose dependent increase in binding while other agonists had no effect.

Reciprocal experiments performed by H. Patel using immobilized GST-RIP140 fusion proteins were used to further characterize the interaction with PPARα and RXRα (Fig. 7.2, *Panel B*). In this case, GST fusion proteins were used that contained separate amino- and carboxyl-terminal domains of RIP140 (Fig. 7.1 *Panel A*), corresponding to amino acid residues 1-479 (GST-RIP140(N)) and 656-1158 (GST-RIP140(C)), that have been shown to be capable of independently binding to nuclear receptors (L'Horset et al., 1996). PPARα bound to both GST-RIP140(N) and GST-RIP140(C) with equal efficiencies (approximately 3% of input) but not to GST coupled beads (compare lanes 2 and 5, 9 and 12). Interestingly, inclusion of Wy 14,643 moderately enhanced binding of PPARα (6% and 9% of input bound to GST-RIP140(N) and GST-RIP140(C) respectively) (compare lanes 5 and 7, 12 and 14). In contrast, efficient binding of labeled RXRα to both fusion proteins was dependent on the presence of 9-*cis* retinoic acid (compare lanes 5 and 7, 12 and 14). These findings indicate that PPARα can bind to separable domains of RIP140 in a agonist-enhanced manner *in vitro*.

7.4 RIP140 ANTAGONIZES PPAR/RXR MEDIATED SIGNALLING

To examine the effect of the interaction between RIP140 and PPAR on transactivation mediated by PPARα *in vivo*, transient transfection assays were carried out using pAOx(X2)luc, a luciferase reporter gene that contains a PPAR response-element from the rat acyl-CoA oxidase gene. As shown in Fig 7.3 *Panel A*, cotransfection of PPARα and RXRα expression plasmids (0.5 μg each) led to an 18 fold and 100 fold induction in reporter gene activity in the absence and presence of Wy 14,643, respectively. Both agonist-independent and agonist-dependent activity were reduced by approximately 50% by the inclusion of an equivalent amount of the RIP140 expression plasmid. RIP140 had no effect on reporter gene activity on its own or when cotransfected with the individual receptor expression plasmids.

Previous reports have indicated that RIP140 stimulates transcriptional activation by nuclear receptors. However, this stimulation was modest and was only observed when low levels of RIP140 expression plasmids were used (Cavaillès et al., 1995). We therefore carried out transfections using a wide range of concentrations of the RIP140 expression plasmid (0.01 ng to 5 μg) while keeping the amounts of PPARα and RXRα expression plasmids constant. As shown in Fig. 7.3 *Panel B*, RIP140 inhibited PPARα/RXRα mediated transactivation at all concentrations tested in a dose-dependent manner. Activation was completely repressed at the highest concentration of RIP140 plasmid used (5 μg). Thus the addition of RIP140 over a broad range resulted in a dramatic antagonism of agonist-dependent and independent PPARα/RXRα mediated transcriptional activation. This is in contrast to previous investigations in which RIP140

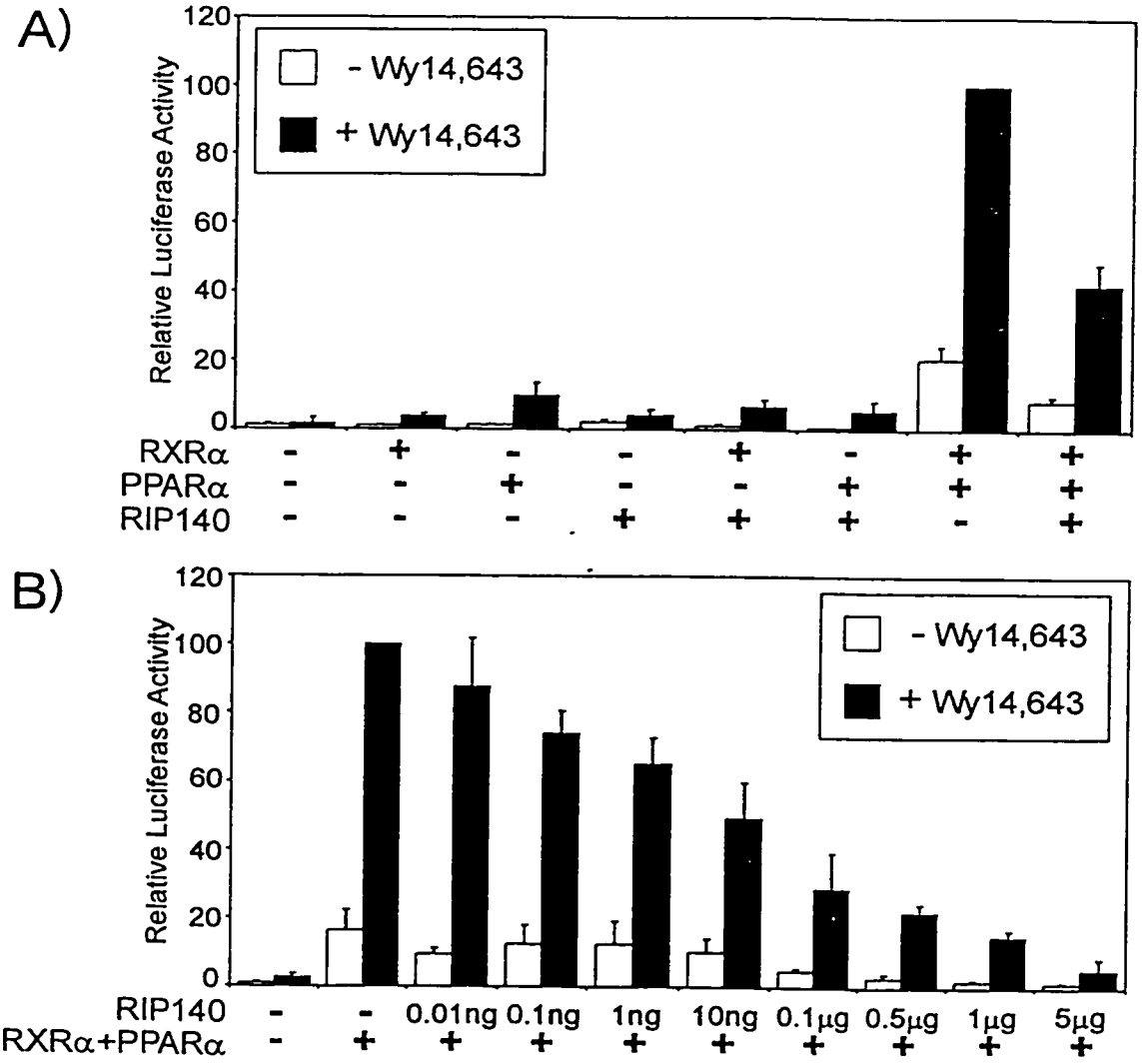


Figure 7.3: RIP140 antagonizes transactivation mediated by PPARα/RXRα *in vivo*. *Panel A*, BSC40 cells were transfected with reporter plasmid pAOx(X2)luc (2 μg) and 0.5 μg of each of the expression vectors for PPARα, RXRα and RIP140 as indicated, and luciferase activity was measured. Where indicated, transfections were carried out in the presence of Wy 14,643 (100 μM final concentration). *Panel B*, RIP140 represses PPARα/RXRα activity in a dose-dependent manner. Cells were transfected with PPARα and RXRα expression plasmids (0.5 μg each) as above with increasing amounts of RIP140 expression plasmid (0.01 ng to 5 μg), in the presence or absence of Wy 14,643, as indicated. Values represent the average (± 1 S.D.) of three separate experiments carried out in duplicate and normalized to the activity observed with PPARα/RXRα + Wy 14,643, which was set to 100.

potentiated ER and RAR mediated signalling at low levels of added RIP140 expression plasmid but antagonized at higher amounts (Cavaillès et al., 1995). Furthermore, RIP140 appears to potentiate signalling by the androgen receptor, although it was not determined if RIP140 bound directly to the androgen receptor (Ikonen et al., 1997). The antagonism of RIP140 of PPARα mediated signalling implicates RIP140 as a modulator of PPARα signalling. These findings indicate that RIP140 acts as an antagonist of the signalling of a nuclear receptors and contributes to the functional ambiguity/complexity of the role of RIP140.

7.5 AGONIST-INDEPENDENT INTERACTION OF RIP140 AND PPARα

While RXRα, the heterodimerization partner of PPARα, requires agonist to interact with RIP140, SRC-1 (L'Horset et al., 1996; Cavaillès et al., 1995; Dowell et al., 1997a; DiRenzo et al., 1997; Takeshita et al., 1996; Jeyakumar et al., 1997; Chakravarti et al., 1996; Kamei et al., 1996), the agonist-independent interaction between RIP140 and PPARα is atypical. Interestingly, evidence exists that PPARα interacts in an agonist-independent manner with these same coactivators. Dowell *et al.*, 1997a have demonstrated that in the absence of added agonist, a segment of the CBP homologue p300 (a.a. 39-221) interacts with PPARα in the yeast two-hybrid system and bind to the PPAR./RXR complex bound to DNA. A fragment of SRC-1 (a.a. 579 to 932) also bound to PPARα in a agonist-independent manner in the yeast two-hybrid system while interaction with RXRα required agonist. Finally, *in vitro* binding assays identified a larger portion of p300 (a.a. 1 to 450) that interacted with PPARα in the absence of agonist (Dowell et al., 1997a).

Further evidence of an agonist independent interaction between other nuclear receptors and coactivators. For instance CBP forms a complex with the DNA bound RXRα/PPARγ in the absence of PPARγ agonists while requiring the presence of the AF-2 domain of PPARγ (Schulman et al., 1998). In addition, SRC-1 was isolated in a yeast two-hybrid screen in the absence of agonist as a protein that interacted with the γ subtype of PPAR and *in vitro* binding assays confirmed the nature of this interaction (Zhu et al., 1996). In a similar study, CBP was identified as a protein that interacted with hepatocyte nuclear factor 4, a nuclear receptor for which no known agonist has been identified (Yoshida et al., 1997). Furthermore, recent evidence from our lab demonstrated that human LXRα, mouse PPARγ2 and mouse PPARβ (FAAR) interact with RIP140 in the absence of agonist (H. Patel, personal communication).

Admittedly, the presence of endogenous agonist could account for the apparent agonist-independence of the interaction between RIP140 and PPARα, PPARδ, PPARγ2 or LXRα. However, the demonstration of this binding in *in vivo* and *in vitro* systems combined with the observed nature of the interactions between PPARγ and SRC-1 (Zhu et al., 1996) and HNF4 and CBP (Yoshida et al., 1997) make it less likely that the presence of endogenous agonists can account for the binding activity of all five of these nuclear receptors. Additionally, two studies have demonstrated that the interaction between GST-fusions of the ligand binding domains of the *Xenopus laevis* PPARs with SRC-1 is agonist induced (Krey et al., 1997) and the *in vitro* interaction between PPARγ and a β-gal fusion of SRC-1 is also agonist-dependent. It is unclear why these discrepancies exist, although they may be the result of the partial or fusion constructs used.

Based on biochemical and structural studies, the currently hypothesized mechanism of agonist-dependent recruitment of coactivators by nuclear receptors involves the stabilization of the ligand binding domain in an active conformation upon binding of agonist (Wagner et al., 1995; Renaud et al., 1995; Bourguet et al., 1995; Wurtz et al., 1996). The conformational change between the inactive and active conformations results in the creation of a coactivator binding interface that requires helix 12/AF-2 domain of the ligand binding domain (Hörlein et al., 1995; Renaud et al., 1995; Wurtz et al., 1996). Recently, a leucine rich LXXLL motif present in coactivators recruited by the binding of agonist to nuclear receptors, was shown to be necessary and sufficient for agonist dependent interaction between the ER and RIP140 or SRC-1 (Heery et al., 1997).

Paradoxically, the agonist independent interaction between RIP140 and PPARα requires the C-terminal region that contains the AF-2 domain and the predicted Helix 12 (Wurtz et al., 1996; Dowell et al., 1997b; Treuter et al., 1998), evidence that is consistent with the proposed model of agonist induced coactivator binding. One could reconcile the observed data with an alternative explanation that other binding determinants of RIP140 mediate this interaction. However, the GST-fusion binding assays demonstrate that similar degraded/truncated forms of RIP140 bind to both GST-PPARα and GST-RXRα (H. Patel, personal communication) suggesting similar RIP140 binding determinants are involved but with different requirements for agonist.

A recent study has demonstrated that the interaction of RIP140 with the ternary complex of PPARγ/RXRα bound to DNA requires the presence of the RXRα ligand 9-*cis* retinoic acid and the AF-2 domain of RXRα (Treuter et al., 1998). Since upon the addition of PPARγ agonists, RIP140 binding could not be detected, it appears that

RIP140 preferentially binds to the RXRα subunit of the DNA bound heterodimer. In contrast, RIP140 interacts in a ligand-dependent manner with both TR and RXR when this complex is bound to DNA (Treuter et al., 1998). The relevance of the binding specificity of RIP140 to the components of the PPARγ/RXRα heterodimer remains unknown.

One could reconcile the differences between the presented data and the current coactivator binding model by proposing a modified mechanism of coactivator recruitment by PPARα. Firstly, in the absence of agonist, the ligand binding domain exists in a conformation in which the coactivator binding interface is in the active conformation but a bound corepressor inhibits binding of coactivators. Upon binding of agonist, the resulting conformational change (Dowell et al., 1997b) displaces the corepressor and recruitment of the coactivation complex occurs. This is similar to the interaction between RAR and p/CAF in which agonist displaces N-CoR and permits binding of p/CAF although this association involves the DNA binding domain of RAR and does not require AF-2 domain (Korzus et al., 1998; Blanco et al., 1998). Therefore, the observed agonist independent binding of PPARα with RIP140, SRC-1 and p300 may be a consequence of a modified manner in which PPARα recruits coactivators and the absence of a relevant corepressor in the binding assays.

7.6 ROLE OF RIP140 IN NUCLEAR RECEPTOR SIGNALLING

The role of RIP140 in nuclear receptor mediated signalling is unclear and ambiguous. RIP140 may contain an intrinsic transcriptional activation ability, associates with nuclear receptors in a AF-2 domain/agonist dependent manner, moderately stimulates the activity of the ER and even strongly potentiates signalling by the androgen

receptor (Cavaillès et al., 1995; L'Horset et al., 1996; Heery et al., 1997; Ikonen et al., 1997; Treuter et al., 1998). For this reason some researchers have referred to RIP140 as a coactivator. However, RIP140 interacts with PPARs in a agonist-independent manner and antagonizes signalling by ER and PPARα (Cavaillès et al., 1995). While, it is clear that RIP140 is not a typical coactivator such as SRC-1 or a cointegrator such as CBP, its opposing functional characteristics are difficult to reconcile.

One possible role for RIP140, that addresses some of its inherent inconsistencies, is as a regulator of the limited cellular supply of components of the coactivator complex such as CBP. For example, the presence of only one functional copy of the CBP gene results in the developmental abnormalities that characterize Rubinstein-Taybi syndrome (Petrij et al., 1995). Furthermore, it has been documented that different nuclear receptors compete for limiting amounts of common coactivators required for agonist dependent activation (Zhang et al., 1996; Kamei et al., 1996). Recently, competition for limiting amounts of the cointegrator CBP, resulted in nuclear receptor and AP-1 (Fos/Jun) signalling pathways impinging upon each other (Kamei et al., 1996). RIP140 could conceivably prevent the sequestration of limiting coactivators such as CBP by acting as an attenuator of nuclear receptor signalling or as a "sink" for agonist bound AF2 domains. Each of these roles would reconcile some of the functional ambiguities of RIP140.

7.6.1 An Attenuator of Nuclear Receptor Signalling

RIP140 may act to antagonize signalling by nuclear receptors in a global manner. This would allow the cell to attenuate nuclear receptor signalling by competing with the coactivator complex for binding to agonist bound, activated AF2 domains. As a result,

this would provide the cell with a mechanism to inhibit nuclear receptor mediated transcriptional activation and divert limiting amounts of shared components of the coactivation complex into other cellular signalling pathways. This would reconcile the agonist-dependent interaction between RIP140 and agonist activated nuclear receptors with its strong antagonistic action on signalling. This hypothetical role can be easily tested by assessing whether coactivators such as SRC-1, CBP, p/CAF and p/CIP can relieve RIP140 antagonism of PPAR/RXR mediated signalling.

7.6.2 A Nuclear Receptor 'Sink'

Alternatively, RIP140 may act as a 'sink' or reservoir for nuclear receptors that exist in a conformation capable of recruiting the coactivation complex. The conception of this idea arose from the need to reconcile the apparent limiting amounts of coactivators that exist in the cell (Kamei et al., 1996) with the findings that in the absence of exogenous agonists, the three PPAR subtypes and HNF-4 are apparently capable of recruiting coactivators such as SRC-1, CBP and p300 (Dowell et al., 1997a; Zhu et al., 1996; Yoshida et al., 1997). Any of these receptors, in the absence of agonist, would presumably be prevented from recruiting the coactivator complex by a bound corepressor. In order to ensure that these receptors which are not complexed to corepressors, do not recruit coactivators, RIP140 could sequester these free receptors.

Evidence that supports RIP140 in the role of an activated nuclear receptor 'sink' comes from studies of the ER mediated repression of the apoAI (apolipoprotein AI) enhancer element (Harnish et al., 1998). In this case, the activation of this enhancer element by nuclear receptors is antagonized by the ER in the presence of estrogen. This repression of signalling is attributed to the competition for a limiting quantity of

coactivators recruited by the receptors involved in the two different signalling pathways. RIP140 also antagonized the activation by the apoAI enhancer in a dose-dependent manner. One would predict that if RIP140 acted as an attenuator of nuclear receptor signalling, the combination of RIP140 with agonist bound ER would result in greater repression above that seen with only ER and estrogen. However, the opposite was true. In fact, RIP140 relieved the repression by the ER and estrogen, a result that would support RIP140 as a nuclear receptor 'sink', working in this case to prevent excess agonist-activated ER from sequestering essential coactivators required by the apoAI element.

7.7 CONCLUSIONS

These studies have added the putative coactivator RIP140 to the growing list of modulators of PPARα mediated transcriptional activation. RIP140 was shown to interact *in vitro* and *in vivo* with PPARα. The interaction *in vivo* required the presence of the region of PPARα that contained the AF-2 domain, a result consistent with the current model of coactivator recruitment by nuclear receptors (refer to Section 1.2.4.3 and Fig.1.4). However, this interaction occurred in the absence of exogenous agonist *in vivo* and was moderately enhanced by agonist *in vitro*. In comparison with the interaction between RIP140 and RXRα which requires agonist, the interaction between RIP140 and PPARα is atypical. Although these results may indicate that PPARα recruits coactivators in a modified manner from the proposed model of nuclear receptor coactivator recruitment, alternative explanations exist (refer to Section 8.0). The findings that RIP140 strongly antagonizes PPARα mediated signalling contributes to the functional ambiguity of RIP140. The immediately determination of whether coactivators are able to relieve RIP140 repression would greatly further our understanding of the function of RIP140 and help to integrate RIP140 into the current model of nuclear receptor mediated transcriptional activation.

Even though the transcriptional regulatory complexes utilized by various cellular signalling pathways share components; functional complexes require the differential recruitment of additional requisite factors (Korzus et al., 1998). In light of the agonist-independent recruitment of RIP140 by PPARα and the resulting antagonism, nuclear receptors may themselves differentially recruit effectors of transcriptional activation adding to the mechanistic complexity of signalling and functional consequences.

8. A NOVEL MODEL OF NUCLEAR RECEPTOR ACTION

PPAR displays an 'atypical' agonist-independent interaction in solution with not only RIP140 but also SRC-1 and CBP (Miyata et al., 1998; Dowell et al., 1997a; Schulman et al., 1998; Zhu et al., 1996). This challenged the assumption that the activation of PPAR by agonist conforms to the standard model of agonist-induced rearrangement of the AF-2 domain, creation of a coactivator binding surface and subsequent recruitment of the coactivation complex (Fig. 1.4). In an attempt to reconcile the atypical behavior of PPAR with the proposed mechanism of action of nuclear receptors, the mechanistic aspects of nuclear receptor activation were integrated with a pharmacological model of the action of a cell surface receptor. This produced a more advanced model of nuclear receptor activation that generated a number of hypothetical explanations of the atypical behavior of PPAR.

Many of the basic mechanisms of nuclear receptor action can be better understood by viewing them as receptors. While much interest in nuclear receptors is focused on their activity as transcriptional activators, the fact that these proteins act as classical receptors is integral to fully understanding their mechanism of action. It is well appreciated that the action of nuclear receptors involves a tripartite system of ligand, receptor and biological effector (reviewed in Katzenellenbogen et al., 1996). The allosteric ternary complex model, developed by R. Lefkowitz, was conceived to explain the action of the β-adrenergic receptor, a seven trans-membrane helix, heterotrimeric G-protein coupled receptor (De Lean et al., 1980; Samama et al., 1993). In many ways, this

receptor is similar to nuclear receptors since they both bind agonistic and antagonistic ligands and require interaction with another factor to effect the biological response. In light of these similarities and the recent characterization of coactivators and corepressors, the allosteric ternary complex model was applied to the action of nuclear receptors. The result is an advanced model of nuclear receptor action that will now accommodate a number of phenomena such as receptor activity in the absence of agonist, constitutively active nuclear receptors and the biological activity of the corepression complex. The application of this model to nuclear receptors provides a comprehensive pharmacological appreciation of the mechanism of action of nuclear receptors.

8.1 ALLOSTERIC TERNARY COMPLEX MODEL

The allosteric ternary complex model (Fig 8.1) of receptor activation centers around the assumptions that receptors isomerize between an active and inactive state (R vs. R*) and that the effectors of biological activity are only recruited by the R* state (Samama et al., 1993). Therefore, the process of effecting a biological response involves two separate events, the isomerization to the R* state and the recruitment of the effector to the R* conformation. In the absence of ligand, the proportion of receptors in the R* state is governed by the isomerization constant J. Thus receptors are able to exert a biological response, albeit minor, in the absence of ligand.

Therefore, an agonist preferentially binds to the R* state, influencing the isomerization of R to R* and stabilizing the complex of LRE. An antagonist has an equal affinity for the two states and does not elicit a biological response but will inhibit the action of an agonist by competing for binding to the receptor. Finally, a negative

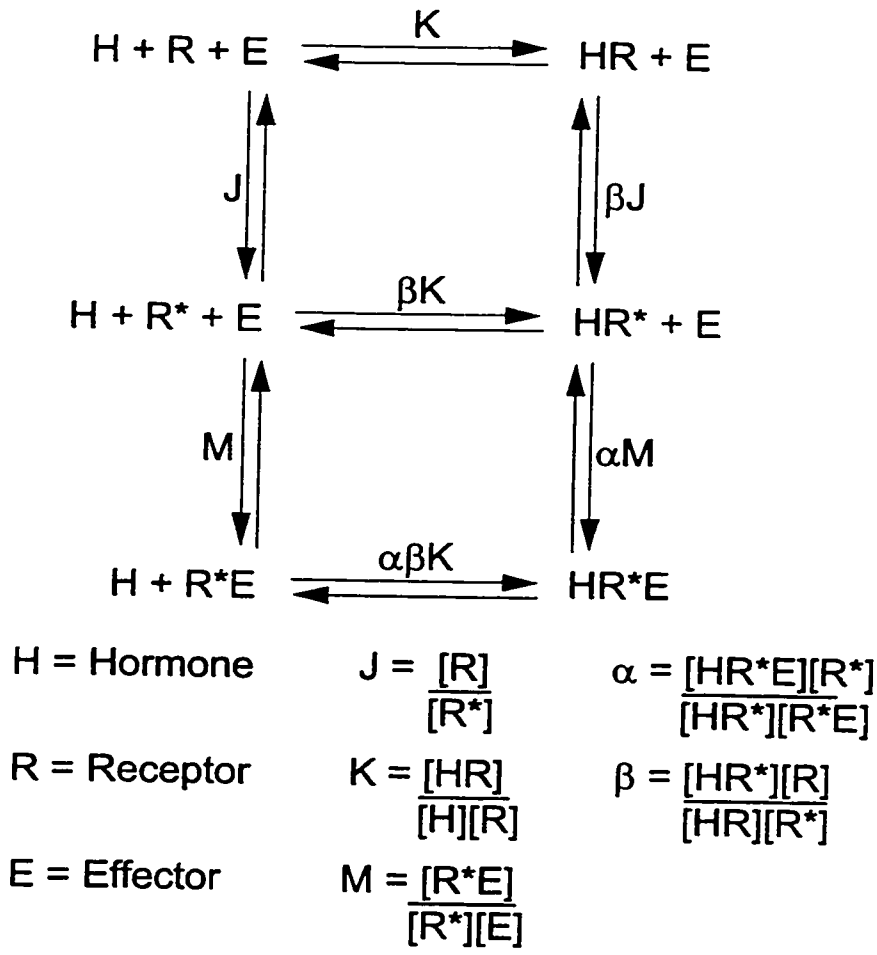


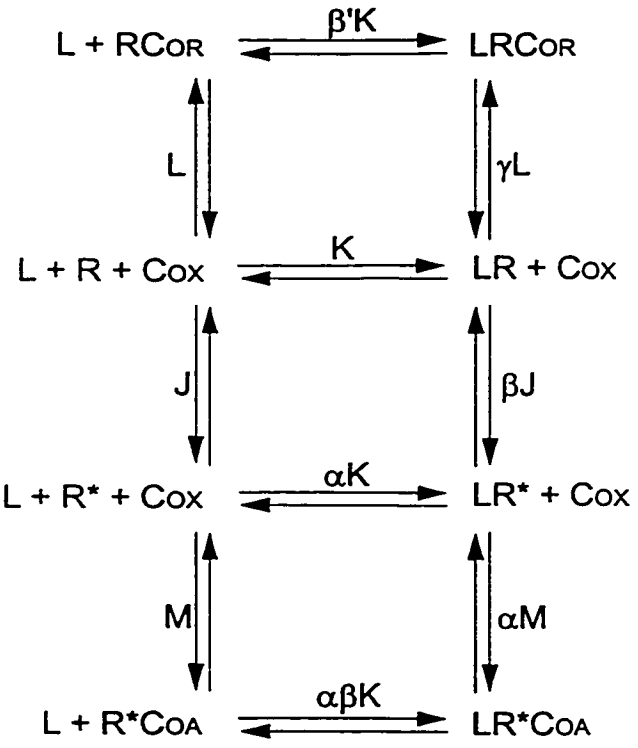
Figure 8.1: The allosteric ternary complex model of receptor function.
This model is composed of three components, hormone (H), receptor (R) and biological effector (E) (Samama et al., 1993). In this model, R undergoes an isomerization between the inactive and active states (R and R*) that is governed by the equilibrium constant J. The allosteric effect of hormone on this equilibrium is given by β. The effector of the biological activity (E) interacts only with the R* state and the effect of hormone on the association between R* and E is given by α. The role of ligand in this model is to influence the conversion between R and R* and to stabilize the complex of R* and the effector molecule. The effect of ligand on the isomerization of the receptor is governed by β, while the effect of ligand on the stability of the ternary complex is given by α. Finally, the efficacy of an agonist to effect a biological response depends on the combination of both α and β: αβ.

antagonist has a higher affinity for the R state, altering the equilibrium between the R and R* states to reduce basal activity.

8.2 MODIFIED ALLOSTERIC TERNARY COMPLEX MODEL

Figure 8.2 depicts an adaptation of the allosteric ternary complex model to accommodate the action of nuclear receptors that is composed of three components: ligand (L); nuclear receptor (R); and the biological effectors (coactivator (COA), corepressor (COR), coactivator or corepressor (COX)). In this model, R undergoes an isomerization between the inactive and active states (R and R*) that is governed by the equilibrium constant J. This R to R* transition represents the conformational change that involves the repositioning of Helix 12 of the ligand binding domain to form the coactivation complex (COA) binding interface. Conversely, the R form is recognized by the corepression complex (COR), an interaction that may involve Helix 1 of the ligand binding domain. Finally, it is assumed that the corepression complex (COR) interacts with only the R state while the coactivation complex (COA) is recruited only by the R* state.

The role of ligands in this model is to stabilize the R or R* state of the receptor as well as stabilize the ternary complex of ligand, nuclear receptor and biological effector. The allosteric effects of agonists and negative antagonists on the stabilization of either the R or R* states is governed by β and β' respectively. The stabilizing effect of agonist on the association between R* and COA is given by α while the stabilizing effect of negative antagonist on the association between R and COR is given by γ. The bipartite role of agonists in the stabilization of the R* state and the LR*Coa complex as governed by $\alpha\beta$ is evidenced in the fact that the concentrations of agonist required to achieve 50%



L = Ligand

R = Nuclear Receptor

CoA = Coactivator

CoR = Corepressor

Cox = Coactivator/Corepressor

$$J = \frac{[R]}{[R^*]} \qquad \alpha = \frac{[LR^*CoA][R^*]}{[LR^*][R^*CoA]}$$

$$K = \frac{[LR]}{[L][R]} \qquad \beta = \frac{[LR^*][R]}{[LR][R^*]}$$

$$L = \frac{[RCoR]}{[R][CoR]} \qquad \beta' = \frac{[LR][R^*]}{[LR^*][R]}$$

$$M = \frac{[R^*CoA]}{[R^*][CoA]} \qquad \gamma = \frac{[LRCoR][R]}{[LR][RCoR]}$$

Figure 8.2: Allosteric ternary complex model applied to nuclear receptors.
Modification of the allosteric ternary complex model of Samama *et al.*, 1993 to accommodate the actions of nuclear receptors. This model is composed of three components, ligand (L), nuclear receptor (R) and the biological effectors (coactivator (CoA), corepressor (CoR), coactivator/corepressor (Cox)). In this model, R undergoes an isomerization between the inactive and active states (R and R*) that is governed by the equilibrium constant J. The allosteric effects of agonists and negative antagonists on this equilibrium is governed by β and β' respectively. CoR interacts with only the R state while the CoA interacts only with the R* state. The effect of agonist on the association between R* and CoA is given by α while the effect of negative antagonist on the association between R and CoR is given by γ.

binding to a nuclear receptor are lower than the concentration of agonist required to solicit a half-maximal biological response (Katzenellenbogen et al., 1996).

Evidence from a number of studies supports the application of this model to the action of nuclear receptors. For instance, the isomerization of a nuclear receptor between two interconvertible forms has been found to occur with ER. In this case, the receptor isomerizes between two interconvertible states that have different affinities for estrogen, presumably R and R*(Dayani et al., 1987). Protease mapping of RAR and PR indicate the existence of different conformations of these receptors in the presence of agonists and antagonists (Keidel et al., 1994; Schulman et al., 1996). Interestingly, in the absence of ligand, the proteolytic degradation of both receptors resulted in a mixture of proteolytic fragments that corresponded to the agonist and antagonist bound forms of these receptors suggesting the presence of a mixed population of R and R* states. Finally, the existence of an equilibrium between two interconvertible repressive and activating states is used to explain the findings that the activity of TR in the absence of agonist is due to a balance between the alternating action of separate repressive and activating functions (Schulman et al., 1996). While the authors propose a model of nuclear receptor action similar to that depicted in Figure 8.2, this model does not depict all possible states reflecting a lack of appreciation for classical receptor pharmacology.

It is also predicted from this model that in the absence of ligand, nuclear receptors would demonstrate a partial activity. In fact, TR in the absence of a bound corepressor, exhibits partial activity which, according to this model is due to the isomerization between the R and R* states of TR and recruitment of COA (Lavinsky et al., 1998; Hörlein et al., 1995). However, the presence of a corepressor bound to the unliganded

TR results in lower activity (Hörlein et al., 1995). This is due to a stabilization of the R state and the active repression by the recruited corepression complex.

Since the R state of nuclear receptors recruits the biologically active corepression complex, the action of a negative antagonist serves not only to antagonize transcriptional activation, but also to effect an opposing biological response: i.e. repression of transcription. While an antagonist has no effect on the basal level of receptor activity, a negative antagonist would be governed by the same principles as an agonist. In fact, the stabilization of the R state would be governed by β' and the stabilization of the LRCOR complex would be given by γ. Interestingly, N-CoR binds more strongly to RAR in the presence of 'antagonist' supporting the predicted action of a negative antagonist (Hörlein et al., 1995).

The role of antagonists in nuclear receptor signalling is complicated by the fact that the inactivation of corepressors results in antagonists acting as partial agonists (Zhang et al., 1998; Lavinsky et al., 1998). The inactivation of corepressors in these cases was achieved by the addition of inactivating antibodies or the addition of a separate antagonist-bound nuclear receptor that would sequester corepressors (Zhang et al., 1998; Lavinsky et al., 1998). According to the allosteric ternary complex model, the removal of corepressors from the system would result in the uncovering of the basal activity due to the isomerization between the R and R* states. These studies are deficient in that they do not report the activity achieved by the removal of the corepressor in the absence of any agonist or antagonist. This additional data would reveal the normal basal activity due to the receptor isomerization. With the determination of this basal level of activity, the assessment of whether a ligand acts as a negative antagonist, antagonist or partial agonist

could be achieved. Therefore, the evaluation of the biological efficacy of a ligand must recognize both the isomerization of the nuclear receptor and the active recruitment of a repressive biological activity by the R state.

The allosteric ternary complex model was conceived to accommodate the existence of mutant receptors that are constitutively active such as the β-adrenergic receptor (Samama et al., 1993). The existence of a point mutant of RXR (RXRαF318A) that mimics agonist-bound RXRα by recruiting components of the coactivation complex in the absence of agonist and constitutively activating transcription, further supports this model being applied to nuclear receptors (Vivat et al., 1997).

Conversely, this model also predicts mutations that result in a constitutively repressive nuclear receptors. For example, a mutant of RAR that lacks the AF-2 domain acts as a constitutively active transcriptional repressor (Baniahmad et al., 1998). Similarly, mutants of RXR and TR in which the AF-2 domain has been disrupted also act as constitutive repressors (Schulman et al., 1996). In the case of the AF-2 deleted RXR mutant, it still retained the ability to heterodimerize with RAR to bind DNA (Schulman et al., 1996).

8.3 PPAR AND THE ALLOSTERIC TERNARY COMPLEX MODEL

The results of *in vitro* and *in vivo* experiments suggest that PPAR can interact with coactivators in the absence of agonist (Miyata et al., 1998; Zhu et al., 1996; Dowell et al., 1997a; Yoshida et al., 1997). According to the allosteric ternary complex model, PPAR exists in the R* state in these assays and according to the model of coactivator recruitment, the AF-2 domain is positioned to create the coactivator binding surface (Fig. 1.2). Since PPAR has been characterized as an agonist activated nuclear receptor, these

results present a challenge to the assumption that the mechanism of PPAR activation involves the same conformational changes as other nuclear receptors such as RAR, RXR and TR. However, PPAR exhibits many characteristics that are consistent with the typical mechanism of nuclear receptor activation including the fact that the structure of the ligand binding domain is similar to that of other nuclear receptors and the fact that the apo- and holo- forms of the ligand binding domain show a similar positioning of the AF-2 domain to that of the comparable forms of the ER (Nolte et al., 1998; Brzozowski et al., 1998). Furthermore, PPAR binds ligand and requires the AF-2 domain for activity (Dowell et al., 1997a). Thus, in order to reconcile the fact that PPAR predominantly exists in the R* state in solution with the fact that this nuclear receptor is agonist-activated requires an alteration of the isomerization constant of PPAR in the cellular environment (Fig. 8.2, J=[R]/[R*]).

The possibility exists that protein-DNA and/or protein-protein interactions may alter the isomerization constant (J) of PPAR. For example, the complexing of PPAR to RXR and the DNA binding site may result in an alteration in J and the requirement for agonist to stabilize R* state and trigger the recruitment of the coactivation complex and transcriptional activation. This scenario would be similar to the stabilization of the R state of TR in the TR/RXR complex bound to a DR1 binding site, a condition in which the DNA binding site acts as an allosteric inhibitor of TR activation (Hörlein et al., 1995; Lefstin and Yamamoto, 1998). Protein-protein interactions may also affect the isomerization constant (J) of PPAR. The prime candidate for this modulatory effect is RXRα since PPAR interacts constitutively with RXRα in solution and while bound to DNA. The allosteric modulation of PPAR activity by RXR is not unprecedented since

RXRα agonists are capable of inducing PPAR to rearrange its AF-2 domain and create the coactivator binding surface (Schulman et al., 1998).

In the absence of exogenous agonist, PPAR demonstrates partial transcriptional activation activity which is assumed to be due to the presence of endogenous ligands (Marcus et al., 1993). Alternatively, since the binding of corepressors to DNA bound PPAR cannot be demonstrated (Le Douarin et al., 1995; DiRenzo et al., 1997), the partial activity of PPAR in the absence of ligand could be due to isomerization between the R and R* states.

The application of the allosteric ternary complex model to PPAR has a number of other interesting implications. For instance, this model would predict the theoretical existence of a spectrum of PPAR ligands from negative antagonists to agonists. The importance of PPAR antagonists may now be clinically relevant in light of the role of PPARγ agonists in the progression of atherosclerotic lesions (Tontonoz et al., 1998; Nagy et al., 1998). Thus PPARγ antagonists may serve to prevent disease progression. In addition, a PPARγ null animal model does not currently exist and thus, a γ subtype antagonist would aid in elucidating the biological role of this nuclear receptor. Furthermore, if clinical agonists of one PPAR subtype activate another subtype with undesired consequences, the therapy could include an appropriate combination of subtype specific antagonists and agonists.

8.4 CONCLUSIONS

The action of nuclear receptors is governed by their activity as ligand activated receptors that utilize an effector molecule to elicit biological responses. For this reason, the allosteric ternary complex model was applied to nuclear receptors to provide a framework within which an evaluation of the mechanism of these receptors can be accomplished. While this model will not be able to predict the diverse and possibly numerous means of influencing the activity of nuclear receptors, it does provide a means to evaluate, understand and classify these regulatory mechanisms.

9. SYNOPSIS

The studies presented in this thesis have identified and characterized a number of aspects of the mechanism by which peroxisome proliferators activate the transcription of target genes. This includes the identification of PPARs as the factors that bound to the PPREs of the HD and AOx genes to mediate the activation of these target genes in response to peroxisome proliferators. Furthermore, it was shown that the binding of PPARs to PPREs required the requisite binding partner RXRα. Since PPARα interacted with RXRα in the absence of agonist and DNA binding, it is proposed that PPARα and RXRα form a heterodimeric complex that is then capable of binding to PPREs.

According to the model of nuclear receptor action, the complex of PPAR/RXR bound to the PPRE in the promoter of a target gene would recruit the coactivation complex in response to agonist. It was demonstrated that the 'putative' coactivator RIP140 interacted with PPARα. Paradoxically, RIP140 repressed PPARα mediated signalling, an effect inconsistent with the proposed role of RIP140 as a coactivator. Nevertheless, this added RIP140 to the repertoire of effector proteins with which PPARα interacts to regulate the transcription of target genes.

Furthermore, PPARα bound to RIP140 in the absence of agonist, an attribute inconsistent with the model of agonist dependent recruitment of the coactivation complex (Fig. 1.4). In order to resolve this discrepancy, the allosteric ternary complex model of β-adrenergic receptor (Samama et al., 1993) was applied to the action of nuclear

receptors. Interestingly, this provided an alternative explanation for the behavior of PPARα that was consistent with the current model of nuclear receptor activation. In essence, apo-PPARα in the absence of a modulatory factor, exists in the active conformation (i.e. can bind coactivators). However, when bound to DNA as a heterodimer with RXRα, agonist is required for creation of the coactivator binding surface and transcriptional activation. The interaction with RXRα or the PPRE may stabilize the inactive conformation of PPAR necessitating the binding of agonist for rearrangement into the active conformation and coactivator recruitment.

This research revealed not only aspects of the basic mechanism by which PPARs activate the transcription of target genes but also a number of strategies used modulate the activity of PPARα by different factors (Fig. 9.1). The discovery that COUP-TF1 and HNF-4 bound to the AOx and HD PPREs and modulated PPAR signalling revealed that other factors could compete with PPAR/RXR for binding to the PPREs. This had not only the effect of antagonizing PPAR mediated activation of the target gene but also enabled the competing factor to exert its own effects on transcription (e.g. active repression by COUP-TF1). The discovery that LXRα, a novel nuclear receptor that interacted with PPARα, antagonized PPARα mediated activation of transcription demonstrated that the formation of the complex of PPARα/RXRα is in equilibrium with other factors that form heterodimers with these proteins. Thus, the presence of other factors that are able to sequester either component will antagonize the formation of the complex of RXRα/PPARα and disrupt PPARα mediated activation of transcription (Fig. 9.1).

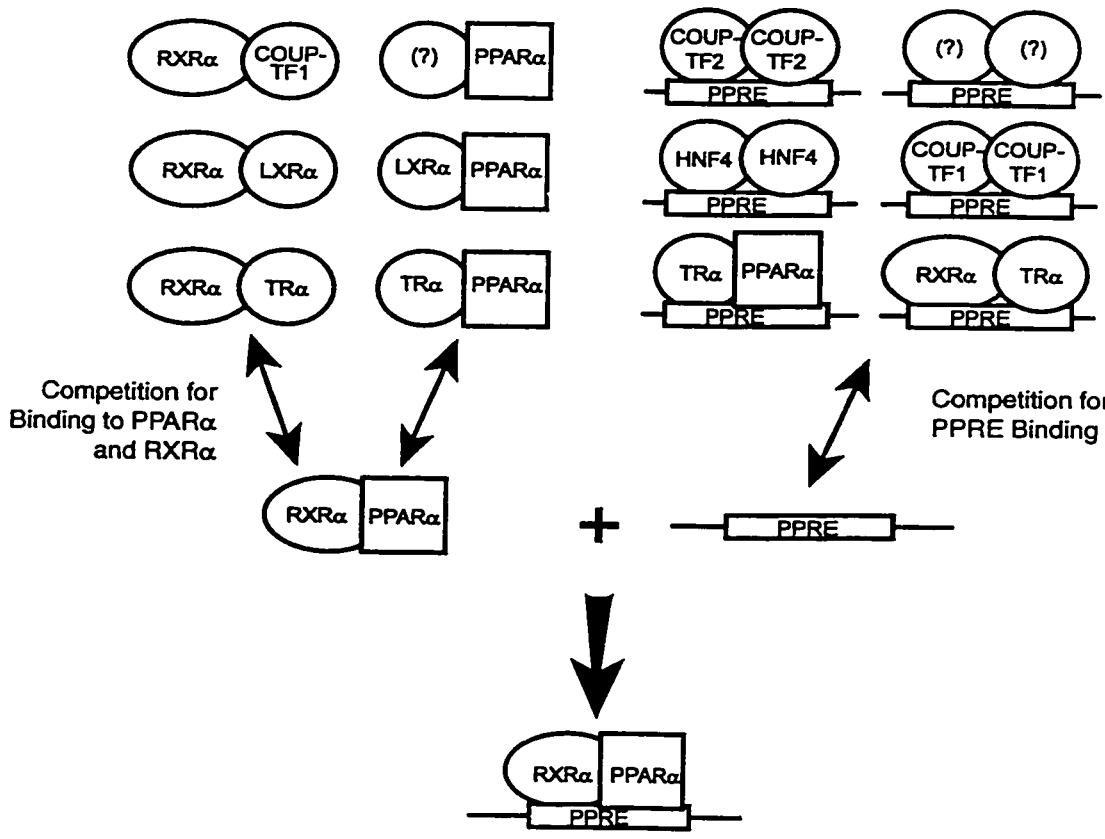


Figure 9.1: Two mechanisms by which factors modulate PPAR activity.
Two mechanisms by which factors can modulate the activity of PPARα include the sequestering of PPAR and RXR from forming a heterodimer and activating the transcription of target genes. Additionally, numerous factors bind to PPREs in addition to PPAR/RXR resulting in blocking of access to the PPRE. Furthermore, these factors exert their own intrinsic activity on the regulation of these target genes.

Future challenges to understanding PPARα mediated signalling include defining the mechanisms of specificity and insulation in PPAR mediated signalling pathways. Although, between the three PPAR subtypes, there is overlap in DNA binding specificity, agonist specificity and tissue expression patterns; each subtype appears to play a distinct role in the cell (Limberger et al., 1996; Lee et al., 1995). In order to reconcile this paradox, the determinants of specificity in subtype signalling must resolved. One example is the phosphorylation of the AF-1 domain of the γ subtype which inhibits not only the activity of PPARγ but also the biological manifestation of PPARγ activation: adipogenesis (Camp and Tarfuri, 1997; Adams et al., 1997). Another determinant of specificity is the DNA binding domain. Since we demonstrated that the binding of PPAR to a PPRE involves the formation of protein-DNA contacts that influence the ability of PPARs to activate transcription in response to agonist subtype (Marcus et al., 1993; Kassam et al., 1998; Juge-Aubry et al., 1997), the nature and identity of permissive and non-permissive contacts must be deciphered. In addition to the determinants of specificity, the means by which each signalling pathway is insulated from the others must be understood. For example, the ability of the PPRE to allosterically modulate the activity of bound PPARs in a subtype dependent manner may be a means of insulating the regulation of a target gene to one specific PPAR subtype (Marcus et al., 1993; Kassam et al., 1998; Juge-Aubry et al., 1997). Only through an appreciation of the full complement of the mechanisms of specificity and insulation involved in PPAR mediated signalling will one be able to resolve the relevant factors and regulators of each subtype signalling pathway.

Ultimately, the mapping of all protein-protein interactions, protein-DNA interactions and determinants of signal pathway specificity/insulation involved in PPAR mediated signalling will permit a direct prediction of the activity of a putative target gene from the DNA sequence of the gene's promoter. While this represents a Herculean task at present, a synthesis of current research disciplines and the evolving disciplines of genomics, proteomics and bioinformatics will make use of powerful new technologies such as mass spectrometry and gene arrays combined with the information in genome databases, to quickly bring this task into the realm of the possible.

10. APPENDIX A: PUBLICATIONS

The results presented in this thesis have been presented in whole or in part in the following publications:

Miyata, K.S., McCaw, S.E., Meertens, L.M, Patel, H.V., Rachubinski, R.A. and Capone, J.P. Receptor-interacting Protein 140 Interacts with and Inhibits Transactivation by the Peroxisome Proliferator-activated Receptor α. (1998) *Mol. Cell. Endocrinol.* **146,** 69-76

Meertens, L.M., **Miyata, K.S.,** Cechetto, J.D., Rachubinski, R.A. and Capone, J.P. A Mitochondrial Ketogenic Enzyme Regulates its Gene Expression by Association with the Nuclear Hormone Receptor PPARα. (1998) *EMBO J.* **17,** 6972-6978

Miyata, K.S., McCaw, S.E., Patel, H.V., Rachubinski, R.A. and Capone, J.P. The Orphan Nuclear Hormone Receptor LXRα Interacts with the Peroxisome Proliferator-Activated Receptor and Inhibits Peroxisome Proliferator Signalling. (1996) *J. Biol. Chem.* **271,** 9189-9192

Winrow, C.J., Kassam, A.K., **Miyata, K.S.,** Marcus, S.L., Hunter, J., and Capone, J.P. (1996) Interplay of the Peroxisome Proliferator-Activated Receptor and Thyroid Hormone Receptor Signalling Pathways in Regulating Peroxisome Proliferator-Responsive Genes. *Ann. New York Acad. Sci.* **804,** 214-230

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Marcus, S.L., **Miyata, K.S.,** Zhang, B., Subramani, S., Capone, J.P., and Rachubinski, R.A. (1993) 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. *Proc. Natl. Acad. Sci. USA* **90,** 5723-5727

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