FUNCTIONAL STUDIES OF THE NOVEL NUCLEAR HORMONE RECEPTOR LXRα

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By

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ABSTRACT

The regulation of gene expression at the transcriptional level is one of the paramount mechanisms for maintaining control of growth, development and metabolic homeostasis. The Liver X Receptor (LXRα) is a novel member of the nuclear hormone receptor superfamily of transcription factors, which was originally isolated in our laboratory. Subsequent studies have revealed that LXRα is an essential transcriptional regulator of cholesterol homeostasis and a number of potent LXRα activators, including the oxysterol 22(R)-hydroxycholesterol have also been identified. As other members of the superfamily, LXRα exerts its regulatory control of target genes directly by binding to LXRα-responsive enhancer elements (LXREs), located upstream of the target gene promoter. Our laboratory initially demonstrated that LXRα heterodimerizes with the Retinoid X Receptor (RXRα) and cooperatively binds to a synthetic LXRE (DR4-LXRE), which consists of direct repeats of the hexad core consensus sequence spaced by four nucleotides. To date, two naturally occurring LXREs have been identified, including the LXRE-ΔMTV element, located in the promoter region of the mouse mammary tumor virus long terminal repeat and the CYP7A-LXRE element, located in the proximal promoter region of the rat cholesterol 7α-hydroxylase gene.

In order to delineate the mechanism by which LXRα mediates the transcriptional regulation of target genes, a series of highly integrated characterization studies were initiated. Our initial interest was identifying the transactivation properties of LXRα.

Thus, a series of transient transfection studies were performed, which investigated the effect of various LXREs, ligands/activators and cell lines on LXRα-mediated transactivation. Ultimately, these studies revealed that the LXRα-mediated transcriptional response was highly varied and specifically dependent upon the response element, ligand and cell line employed. Thus, these investigations indicate the specificity and great diversity in the nuclear hormone receptor-mediated transcriptional regulation of target genes. Furthermore, these studies resulted in the establishment of a viable and efficient transient transfection assay for further LXRα *in vivo* investigations.

Nuclear hormone receptors, including LXRα, are comprised of several modular domains termed A/B, C, D and E. A number of recent studies have implicated the highly divergent A/B domain of variety of nuclear receptors, and their isoforms, as a participant in transactivation. Specifically, these nuclear receptors have been shown to posses, within their respective A/B domains, an autonomous ligand-independent transactivation function termed the AF-1 domain, which can either function independently or can synergize with the E domain of the same receptor. Thus, determination of whether or not the 97 amino acid A/B domain of LXRα participated in LXRα-mediated transactivation became a main focus in our investigation of LXRα. *In vitro* EMSA analysis revealed that deletion of up to 63 amino acids of the N-terminal region of the LXRα A/B domain did not effect either LXRα/ RXRα heterodimerization nor cooperative binding to LXREs. *In vivo* transient transfection assays further illustrated that the N-terminal 63 amino acids of the LXRα A/B domain were dispensable for LXRα/RXRα-mediated transactivation. Therefore, as determined by the limitations of these assays, the N-

terminal 63 amino acids of the LXRα A/B domain do not participate in neither transactivation nor heterodimerization and subsequent binding to LXREs.

Transcriptional regulation, mediated by members of the nuclear hormone receptor superfamily, has been shown to involve multiple auxiliary co-factors, which modulate receptor-mediated transactivation. These co-factors can either serve to repress (co-repressors) or activate (co-activators) transcription not only through blocking or facilitating interactions, respectively, between receptors and the basal transcription machinery but also through chromatin remodeling. Thus, the identification of LXRα-interacting co-factors and the subsequent investigation of their ability to modulate LXRα-mediated transactivation, were of particular interest. We demonstrated, *via* utilization of *in vitro* GST-binding assays, that LXRα interacts with RIP 140, SRC-1a and SMRT co-factors in a ligand-independent manner. Furthermore, these studies illustrate that the LXRα AF-2 core domain is necessary for efficient RIP 140 and SRC-1a binding. Surprisingly, this domain appears to impede, although not absolutely, the SMRT/LXRα interaction, which has also been observed for the Retinoic Acid Receptor (RAR)/SMRT interaction. Functional studies of LXRα, RXRα and RIP 140 indicate that RIP 140 antagonizes LXRα/RXRα-mediated transactivation, which suggests that RIP 140 may serve to attenuate the transcriptional response of nuclear receptors modulated by other, more potent co-activators, as previously suggested in Peroxisome Proliferator-activated receptor α (PPARα)/RIP 140 studies. As well, it is apparent that neither the RIP 140/LXRα interaction nor the RIP 140-mediated repression of LXRα activity is effected

upon deletion of the N-terminal 63 amino acids of the LXRα A/B domain. Interestingly, functional studies of LXRα, RXRα and the partial SRC-1a clone, which lacks the N-terminal PAS-bHLH domain, indicate that this SRC-1a clone antagonized LXRα/RXRα-mediated transactivation. While this result may simply demonstrate the necessity for a full length SRC-1a clone it may also indicate SRC-1 isoform-specific differences as previously illustrated in Estrogen Receptor (ER)/SRC-1 studies. Lastly, preliminary functional studies of LXRα, RXRα and SMRT indicate that SMRT has no significant effect on LXRα/RXR-mediated transactivation. These tentative results indicate that while LXRα and SMRT interaction in solution, SMRT may not be able to interact with LXRα when bound to DNA, and is thus unable to modulate LXRα-mediated transcriptional activation as previously demonstrated for the PPARγ and the orphan receptor Rev Erb.

Taken together, the investigations presented in this study of LXRα, further our understanding of not only the mechanism by which LXRα mediates its transcriptional response, but also how nuclear receptors achieve specificity and diversity in the activation of target gene expression.

DEDICATIONS

I would like to dedicate this body of work to my family. I thank my Mother and Father, Lynnda and Brian, my Brother David and my Michael for their encouragement, understanding and support. I would also like to thank my Aunt Janice Zwicker and my Grandparents, Leonard and Ruby Davis and Vivian McCaw for their continued belief in me. I am truly blessed to have been cared for and loved by them.

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aa	amino acid(s)
AD	activation domain
AF-1, -2	activation function-1, -2
Aox	acyl-CoA oxidase
AP-1	activating protein-1
ATP	adenosine triphosphate
BARE	bile acid response element
bHLH-PAS	basic-helix-loop-helix-PAS domain
BRE	IIB recognition element
BSA	bovine serum albumin
CBP	CREB-binding protein
9-*cis* RA	9-*cis* retinoic acid
CMV	cytomegalovirus
COUP-TFII	chicken ovalbumin upstream-promoter transcription factor II
CREB	3',5'-monophosphate-regulated enhancer binding protein
CsCl	cesium chloride
CYP7A	cholesterol 7α-hydroxylase
DBD	DNA binding domain
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNTPs	deoxyribonucleotide triphosphates
DR	direct repeat
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERE	estrogen receptor response element
ETOH	ethanol
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein
GST	glutathione S-transferase
HD	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
HDAC-1, -2	histone deacetylase 1, 2
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNF-3, -4	hepatocyte nuclear factor 3, 4
hr(s)	hour(s)
HRE	hormone response element
IPTG	iso-propyl-thiogalactoside
Kb	kilobase pairs
KDa	kilodaltons
LBD	ligand binding domain

LDL	low density lipoprotein
Leu	leucine
LXR	liver X receptor
LXRE	liver X receptor response element
MBP	maltose binding protien
MCS	multiple cloning site
min(s)	minute(s)
MOPS	3-(N-Morpholino)propanesulfonic acid
MTV	mouse mammary tumor virus
MVA	mevalonic acid
N-CoR	nuclear receptor co-repressor
NER	steroid hormone nuclear receptor
NP-40	Nonidet P-40
OD	optical density
ONPG	o-nitophenyl-β-D-galactopyranoside
OR-1	orphan receptor 1
PBS	phosphate buffered saline
p/CAF	p300/CBP-associated factor
p/CIP	p300/CBP/co-integrator-associated factor
PCR	polymerase chain reaction
PEG	polyethylene glycol
PKC	protein kinase C
PKA	protein kinase A
PMSF	phenylmethylsulfonylfluoride
PPAR	peroxisome proliferator-activated receptor
PPO	2,5-diphenyloxazole
PPRE	peroxisome proliferator response element
22(R)-OH-CH	22(R)-hydroxycholesterol
RAR	retinoid acid receptor
RIP 140	receptor-interacting protein 140
R.L.U.	relative light units
RNA	ribonucleic acid
RXR	retinoid X receptor
SCAP	SREBP cleavage activation protein
SDEV	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec(s)	second(s)
SF-1	steroidogenic factor-1
SMRT	silencing mdiator (co-represor) for retinoid andthyroid-hormone receptors
SRC-1	steroid receptor co-activator-1
SRE	sterol regulatory elements
SREBP-1, -2	sterol regulatory element binding protein-1, -2
SV40	simian virus 40

TBP	TATA binding protein
TE	Tris-EDTA
TFIIA,B,D,E,F,H	transcription factor IIA, IIB, IID, IIE, IIF and IIH
TIF2	transcriptional intermediary factor 2
TK	thymadine kinase
TR	thyroid-hormone receptor
Tris	Tris(Hydroymethyl)Aminoethane
Trp	tryptophan
UR	ubiquitous receptor
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER ONE

INTRODUCTION

1.0 An Overview of Transcription

The transcriptional regulation of gene expression is one of the paramount mechanisms employed by eukaryotes in order to control growth, development and metabolic homeostasis. Transcription of mRNA encoding genes is mediated by the enzyme RNA Polymerase II which associates with a host of auxiliary protein factors, collectively referred to as the General Transcription Factors (including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, where TF stands for transcription factor) (reviewed in Orphanides *et al.*, 1996). In brief, initiation of transcription occurs upon the recognition of the core promoter by TFIID (mediated by the TFIID subunit TBP (TATA binding protein) which binds to the TATA DNA element) and the resulting interaction is stabilized upon the binding of TFIIA. The TFIID-TFIIA-promoter complex is then recognized by TFIIB which binds TBP, the RNA Polymerase II/TFIIF complex and a recently identified core promoter element, the IIB recognition element (BRE), located upstream of the TATA element (Lagrange *et al.*, 1998). The pre-initiation complex is then completed upon binding of TFIIE and TFIIH which are involved in a number of processes including promoter melting, excision repair and helicase activity. The formation of the pre-initiation complex is further facilitated and stabilized by another group of transcription factors termed transactivators which bind to 'enhancer' elements located upstream of the core promoter. These transactivators presumably modulate

transcriptional activity *via* direct or indirect interactions with the basal transcription factors, such as TBP and TFIIB, thereby facilitating the formation of a stable pre-initiation complex and increasing the rate of gene expression (Leong *et al.*, 1998, Rochette-Egly *et al.*, 1997 and reviewed in Shibata *et al.*, 1997, Chang and Jaehning, 1997, Mangelsdorf and Evans, 1995, Bagchi *et al.*, 1992). Upon formation of a stable pre-initiation complex RNA Polymerase II is able to synthesize the RNA transcript.

1.2 LXRα as a Nuclear Receptor

The nuclear hormone receptors constitute a superfamily of intracellular ligand-modulated transcription factors (transactivators), which exert their regulatory control of target genes directly. By binding to hormone response elements (HREs) (enhancer elements), located upstream of the target gene core promoter, these receptors can presumably activate or repress transcription *via* interactions, or lack thereof, with the transcription machinery, as previously described. A novel member of this superfamily was originally isolated in our laboratory and was subsequently termed the Liver X Receptor (LXR) (refer to Figure 1) (Miyata *et al.*, 1996). Concurrently, Willy and colleagues (1995) reported the cloning of a cDNA encoding this receptor. To date, two isotypes of LXR have been isolated: LXRα (Willy *et al.*, 1995, Miyata et al., 1996) and its rat homolog RLD-1 (Apfel *et al.*, 1994) and LXRβ, also referred to as OR-1 (Teboul *et al.*, 1995), NER (Shinar *et al.*, 1994) and UR (and its rat homolog rUR) (Song *et al.*, 1994). Amino acid sequence comparisons between LXRα and the related receptors, as illustrated in Figure 2, indicate that the DNA binding and ligand binding domains of

```
1   MSLWLGAPVPDIPPDSAVELWKPGAQDASSQAQGGSSCIL    40

    REEARMPHSAGGTAGVGLEAAEPTALLTRAEPPSEPTEIR    80

    PQKRKKGPAPKMLGNELCSVCGDKASGFHYNVLSCEGCKG    120

    FFRRSVIKGAHYICHSGGHCPMDTYMRRKCQECRLRKCRQ    160

    AGMREECVLSEEQIRLKKLKRQEEEQAHATSFPPRASSPP    200

    QILPQLSPEQLGMIEKLVAAQQQCNRRSFSDRLRVTPWPM    240

    APDPHSREARQQRFAHFTELAIVSVQEIVDFAKQLPGFLQ    280

    LSREDQIALLKTSAIEVMLLETSRRYNPGSESITFLKDFS    320

    YNREDFAKAGLQVEFINPIFEFSRAMNELQLNDAEFALLI    360

    AISIFSADRPNVQDQLQVERLQHTYVEALHAYVSIHHPHD    400

    RLMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLS    440

    EIWDVHE 447
```

Figure 1: Predicted amino acid sequence of LXRα. The underlined region indicates the DNA binding domain and the core AF-2 domain is indicated by a dashed line. This amino acid sequence is identical to the sequence reported by Willy and colleagues (1995), with two exceptions; a phenylalanine in place of a leucine at position 192 and an alanine in place of an arginine at position 196 as indicated by the asterisks.

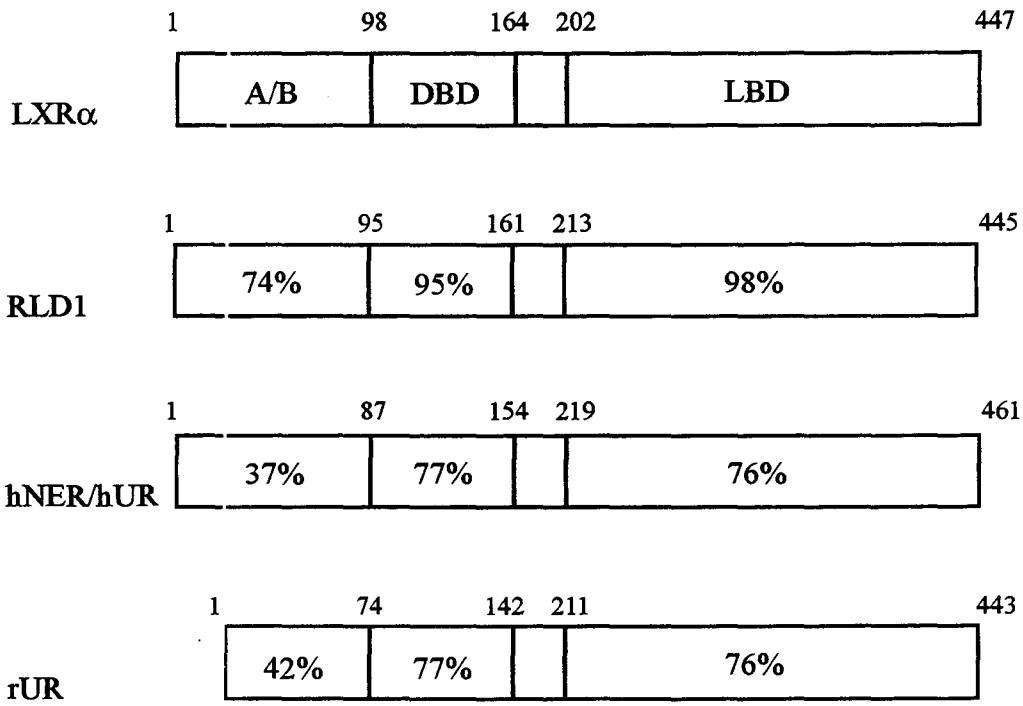


Figure 2: Amino acid sequence comparisons between LXRα and related receptors. The percent sequence identities among the amino terminal (A/B) domain, DNA binding domain (DBD) and the ligand binding domain (LBD) of the rat orphan receptor RLD1 (Apfel *et al.*, 1994), rat ubiquitous receptor rUR (Song *et al.*, 1994, Teboul *et al.*, 1995) and human hNER (Shinar *et al.*, 1994) are illustrated. Sequence comparisons were carried out using the Clustal Program within the DNAStar software package.

these receptors share significant sequence identity, while their respective N-terminal A/B domains are quite divergent.

LXRα, as its name implies, is abundantly expressed in the liver, kidney, heart and intestine and is also present, to a lesser extent, in the spleen, adrenals and skeletal muscle. Recent studies have implicated LXRα as an essential regulator of cholesterol homeostasis and may also have a wide ranging effect on hepatic lipid metabolism (Janowski *et al.*, 1996, Forman *et al.*, 1997, Lehmann *et al.*, 1997, Peet *et al.*, 1998). LXRβ, in contrast to LXRα, is ubiquitously expressed (liver, heart, kidney, testis, ovary, adrenal, uterus, prostate, vagina, lung, spleen, brown fat, retina, skin, skeletal muscle and brain). While the role of LXRβ has yet to be elucidated, one report suggests that in the fetal brain, LXRβ (OR-1) has a widespread role in modulating gene activity and that this modulation in postnatal and adult brains is limited to distinct neuronal populations (due to its wide-spread expression in the fetal brain compared to the localized expression in postnatal and adult brains) (Kainu *et al.*, 1996). Interestingly, a recent study has reported the occurrence of large amounts of aberrant LXRβ (NER) product (generated by alternative splicing) in cancer cell lines and primary cancer tissue (breast and colon) which is absent in normal tissues (Saito *et al.*, 1997). Whether or not this aberrant LXRβ product is involved in the development or progression of cancer in certain tissues, remains to be seen.

1.2.1 Nuclear Receptor Structure and Modular Domain Functions

Nuclear hormone receptors, including the Liver X receptor α (LXRα), are categorized as such according to familial homology between several modular domains

termed A/B, C, D, E and F (reviewed in Schoonjans *et al.*, 1996 and Shibata *et al.*, 1997, Mangelsdorf and Evans, 1995) (refer to Figure 3). While the structure of LXRα has yet to be elucidated *via* crystallographic techniques, structural information can be inferred from those nuclear receptors which have been crystallized (Bourget *et al.*, 1995, Renaud *et al.*, 1995, Nolte *et al.*, 1998, Darimont *et al.*, 1998 and reviewed in Mangelsdorf and Evans, 1995) due to this familial homology. The structure and function of each of the modular domains is outlined below.

1.2.1.1 The A/B domain

In general, the isoforms of a nuclear hormone receptor share a high degree of homology throughout their C, D and E regions but are divergent in their A/B domains. This fact, combined with the observation that some nuclear receptor isoforms are expressed in distinct tissue-specific patterns, raises the possibility that isoform-specific activity may be mediated by the A/B domain. In fact, a number of nuclear receptors have been shown to contain an autonomous ligand-independent transactivation function (AF-1 domain) (Nagpal *et al.*, 1993, Wilkinson and Towle, 1997, Werman *et al.*, 1997, Metzger *et al.*, 1995, Sjöberg and Vennström, 1995, and reviewed in Schoonjans *et al.*, 1996). Many of these studies have revealed that the activity of the AF-1 domain of these receptors may be promoter context, DNA response element and cell line-specific (Berry *et al.*, 1990, Nagpal *et al.*, 1992, Nagpal *et al.*, 1993, Metzger *et al.*, 1995, Sjöberg and Vennström, 1995, Ikonen *et al.*, 1997, Wilkinson and Towle, 1997). In some instances, the AF-1 domain has been shown to work synergistically with the AF-2 domain (located in domain E) of the same receptor (Pierrat et al., 1992, Metzger et al., 1995, Nagpal et al.,

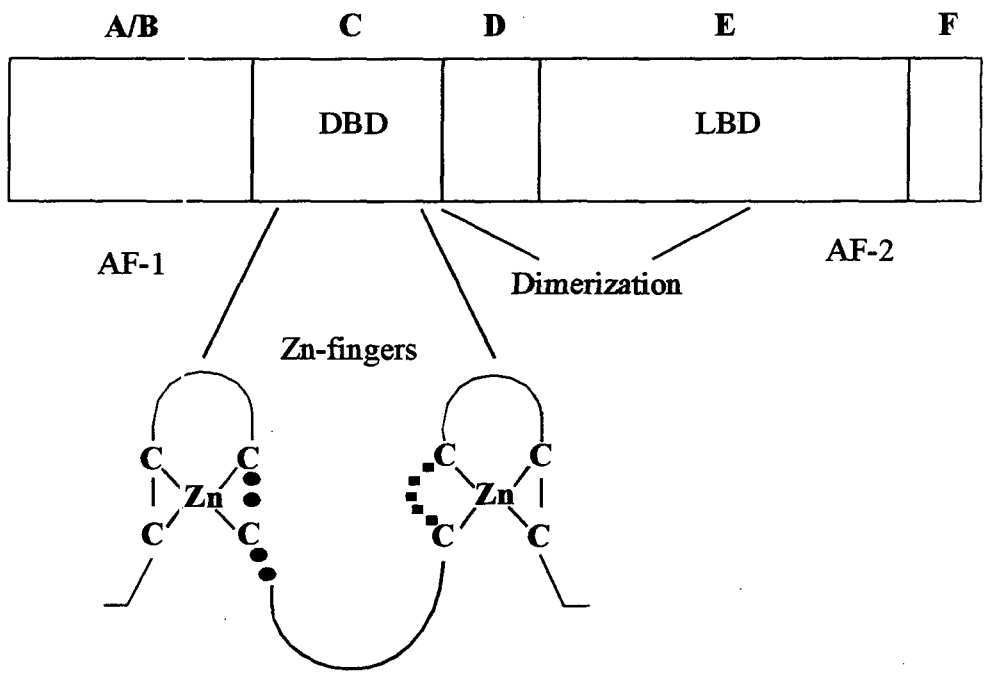


Figure 3: Schematic representation of the functional domains of the nuclear hormone receptors. Modified from Schoonjans et al., 1996 where the P box amino acids are represented by circles and the D box amino acids are represented by squares. The DNA binding domain (DBD) and the ligand binding domain (LBD) are indicated.

1993, Sjöberg and Vennström, 1995). Furthermore, recent studies report that AF-1/AF-2 domain synergism may be a direct consequence of a ligand-dependant interaction between the N and C-terminal regions which may be mediated by the binding of co-factors (such as CBP, SRC-1 and the related p160 proteins) to both the AF-1 and AF-2 domains (Kraus *et al.*, 1995, Ikonen *et al.*, 1997, Oñate *et al.*, 1998, Webb *et al.*, 1998).

While a number of reports have provided ample evidence that the AF-1 domain possess an autonomous activation function, it has been suggested that there exists many discrete regions within the AF-1 domains which possibly function independently and, in some cases, differently depending upon the cell type and ligand employed (Metzger *et al.*, 1995, McInerney *et al.*, 1996, Werman *et al.*, 1997). In fact, one study suggested the existence of an N-terminal repression moiety within the Peroxisome Proliferator Activated Receptor γ2 (PPARγ2) AF-1 activation domain (Werman *et al.*, 1997).

A further mode of regulation of the activities of the various receptors resides in the fact that a number of receptor AF-1 domains (as well as other domains) have been shown to be phosphorylated. In fact, phosphorylation of both the Estrogen Receptor (ER) (Kato *et al.*, 1995) and Retinoid Acid Receptor (RAR) (Rochette-Egly *et al.*, 1997) results in an increase in the transcriptional activity of the receptor. In contrast, phosphorylation of the PPARγ A/B domain reduces its activity (Adams *et al.*, 1997). Although no phosphorylation sites have been identified in the LXRα A/B domain (a putative recognition sequence for PKC is located in the hinge region of both LXRα and RLD-1), it may prove to play a role in LXRα transcriptional activity. Whether or not LXRα possesses an AF-1 domain in its A/B domain is the investigative focus of Section 3.2.

1.2.1.2 Domain C (The DNA Binding Domain)

Domain C is responsible for binding of the receptors to hormone response elements (located in the proximal promoters of target genes) as it constitutes a highly conserved DNA binding domain (DBD) which contains two cysteine zinc finger motifs. Specific contacts between the receptor and the DNA are mediated by the 6 amino acids of the P box, located at the carboxyl end of the first zinc finger. The P box sequence of LXRα (CEGCKG) is identical to that of TRs, RXR, RARs and PPARs. Protein-protein interactions are, in part, dependent upon the 5 amino acid sequence of the D box, located within the second zinc finger.

1.2.1.3 Domain D (The Hinge Region)

Domain D is a non-conserved hinge region, which allows bending and/or conformational alterations within the receptor. This region has also demonstrated the ability to interact with auxiliary transcription co-factors such as SMRT and N-CoR (Chen and Evans, 1995, Hörlein *et al.*, 1995, Kurokawa et al., 1995, and reviewed in Perlmann and Vennström, 1995). The hinge regions of LXRα and RLD-1 (but not LXRβ) have also been shown to contain a putative recognition sequence for PKC (RXXSXR; RSFSDR for both LXRα and RLD-1). A recent study reports that agents which trigger other signal transduction pathways significantly enhanced the activation of RLD-1 by 22(R)-hydroxycholesterol which could be abolished upon the addition of protein kinase inhibitors (Huang *et al.*, 1998). Thus, LXRα/RLD-1 mediated transactivation may be regulated by phosphorylation catalyzed by protein kinases.

1.2.1.4 Domain E (The AF-2 Core Domain)

Domain E is a complex and tightly integrated region comprised of a ligand binding domain (LBD), dimerization surfaces, nuclear localization signals, co-factor association surfaces and a ligand-dependent transactivation functional region (AF-2 core domain, also referred to as Helix 12) (Danielian *et al.*, 1992, and reviewed in Glass *et al.*, 1997, Mangelsdorf and Evans, 1995, Schoonjans *et al.*, 1996). Ligand-dependent regulation of target genes by nuclear hormone receptors is mediated by their respective E domains. Based upon the crystal structures of unliganded Retinoid X Receptor (RXRα) (Renaud et al., 1995) and both liganded and unliganded Retinoic Acid Receptor (RARγ) (Bourget et al., 1995), it is believed, upon binding of ligand, the E domain undergoes a conformational change which may cause the disassociation of bound co-repressors and the recruitment of co-activators. Specifically, the AF-2 core domain (amphipathic helix 12), which is highly conserved among nuclear receptors including LXRα (refer to Figure 20), undergoes a conformational shift from an extended conformation in the absence of ligand to a compacted conformation with the LBD (usually helices 3, 4 and 5) in the presence of ligand. Recent crystallographic studies of PPARγ (Nolte *et al.*, 1998) and TRβ (along with biochemical studies) (Darimont *et al.*, 1998), have demonstrated that this compact conformation of the liganded LBD constitutes a hydrophobic groove which can interact with the LXXLL motif-containing α-helix of co-activators. This observation is consistent with previous studies which report that loss or mutation of the AF-2 core domain (helix 12) results in the abolishment of ligand-dependent transcriptional activation, AF-1/AF-2 synergism and the loss of co-factor binding (although binding of

various co-factors has also been shown to be dependant upon other areas of the E domain) (Danielian *et al.*, 1992, Cavaillès *et al.*, 1995, Joyeux *et al.*, 1997, Jeyakumar *et al.*, 1997, Treuter *et al.*, 1998, Miyata *et al.*, 1998, and reviewed in Shibata *et al.*, 1997, and Glass *et al*, 1997).

1.2.1.5 Domain F

The F domain is generally a small and highly variable domain, located at the C-terminus, which has no known biological function (reviewed in Schoonjans *et al.*, 1996). It does not appear that LXRα possesses this domain.

1.2.2 DNA Binding of LXRα and LXRα Response Elements

Members of the nuclear hormone receptor superfamily are intracellular ligand-activated transcription factors, which exert their regulatory control of target genes directly by binding to hormone response elements (HREs) located in the promoter of target genes. The nonsteroid members of the nuclear receptor superfamily, such as LXR and RXR, recognize HREs which consist of the hexad core consensus sequence AGGTCA arranged into different configurations and spaced by a variant number of nucleotides (generally 0-5 nucleotides). These HREs can be arranged into configurations referred to as direct repeats (DRs), inverted repeats (IRs) or everted repeats (ERs). These differences in HRE orientation, spacing and sequence (of the core, spacer and flanking sequences) directly determine such specificity as the mode of receptor binding (monomers, homodimers and heterodimers), orientation of the receptors on the HRE, affinity of the receptors for the HRE, and responsiveness to ligand for one or both of the

nuclear receptor partners (reviewed in Mangelsdorf and Evans, 1995, Glass, 1996, Schoonjans *et al.*, 1996).

The Liver X Receptor (LXRα and LXRβ) has demonstrated the ability to hetero-dimerize with the Retinoid X Receptor (RXRα) and bind cooperatively to direct repeats of the hexad core consensus sequence spaced by four nucleotides (DR4s; DR4-LXRE) (Willy *et al.*, 1995, Miyata *et al.*, 1996, Apfel *et al.*, 1994, Teboul *et al.*, 1995, Song *et al.*, 1994). To date, two other LXR response elements (LXREs) have been reported; the LXRE-ΔMTV (Willy *et al.*, 1995) and the CYP7A-LXRE (Lehmann *et al.*, 1997), both of which are classified as DR4s (refer to Table 1).

The discovery of a functional, high affinity binding site for LXRα, located in the promoter sequence of ΔMTV (LXRE-ΔMTV), was reported in initial LXRα studies (Willy *et al.*, 1995). This promoter was derived form the mouse mammary tumor virus LTR (long terminal repeat) (Ringold, 1979) in which the glucocorticoid response elements have been deleted (Hollenberg and Evans, 1988). A subsequent report has demonstrated that the LXRα/RXRα heterodimer binds to the LXRE-ΔMTV with RXR occupying the 5' half site and LXRα occupying the 3' half site (Willy and Mangelsdorf, 1997). This polarity has also been observed for the Vitamin D Receptor (VDR), Thyroid Hormone Receptor (TR) and the Retinoic Acid Receptor (RAR) heterodimers with RXR on DR3, DR4 and DR5 DNA response elements respectively (reviewed in Glass, 1996, Mangelsdorf and Evans, 1995). Furthermore, the same study reports that subtle changes in the nucleotide sequence of the LXRE-ΔMTV half sites, spacer and flanking regions can have a significant effect on the ability of the heterodimer to be activated by ligand

while having no significant effect on DNA binding. Alternatively, a number of reports which study the DR4-LXRE have demonstrated that mutations of the spacer sequence can have a significant effect on DNA binding (Apfel *et al.*, 1994). This observation may account for the different results reported in Miyata *et al.*, 1996 and Lehmann *et al.*, 1997, where the formation of a weak homodimeric complex of LXRα was seen on a DR4-LXRE in the latter study which was not observed in the former study (where the spacer sequence between the two DR4 motifs was different).

The most recent LXRE, the CYP7A-LXRE identified for LXRα and LXRβ, was located in the proximal promoter region of the rat cholesterol 7α-hydroxylase gene (rCYP7A) (Lehmann *et al.*, 1997). This gene encodes the enzyme responsible for the initial and rate-limiting step in the conversion of cholesterol to bile acids (Li *et al.*, 1990, and reviewed in Myant and Mitropoulos, 1977).

Table 1: Functional Liver X Receptor Response Elements (LXREs)

LXRE	Sequence	Promoter	Location in promoter
DR4-LXRE	TTCTGACCTCCTGTGACCTGG	Synthetically prepared	
LXRE-ΔMTV	CTTGCGGTTCCCAGGGTTTAAATAAGTTCATCTA	5'regulatory region of the mouse mammary tumor virus LTR (ΔMTV)	-123 to –94
CYP7A-LXRE	CCTTTGGTCACTCAAGTTCAAGTG	Rat cholesterol 7α–hydroxylase gene	-72 to -57

1.2.3 LXR Activators

LXRα and LXRβ have been termed an orphan nuclear hormone receptors as no ligands have been shown to bind to the LBDs of the receptors directly. However, a

number of reports have demonstrated that a variety of oysterols are able to activate both LXRα and LXRβ, the most potent of which include 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol , (20R, 22R)-dihydroxycholesterol, 24(S)-hydroxycholesterol and 24(S),25-epoxycholesterol (Janowski *et al.*, 1996, Lehmann *et al.*, 1997, Forman *et al.*, 1997, Lala *et al.*, 1997). Oxysterols are oxygenated derivatives of cholesterol which are produced as downstream intermediates in the steroid and bile acid synthesis pathways, and are therefore involved in the cholesterol homeostasis/mevalonic acid (MVA) biosynthetic pathways (reviewed in Kandutsch *et al.*, 1978, Luu and Moog, 1991, Goldstein and Brown, 1990, Parish *et al.*, 1995). These observations are consistent with a recent report which demonstrated that the constitutive transcriptional activity observed for LXRα (RLD-1)/RXRα complexes (Apfel *et al.*, 1994, Willy *et al.*, 1995), which was shown to require the LBDs of both receptors, was dependant upon MVA biosynthesis occurring in the eukaryotic cells employed (Forman *et al.*, 1997). LXRα activity can be repressed upon the addition of inhibitors of the MVA biosynthetic pathway (Mevastatin and Lovastatin) which can, in turn, be relieved upon the addition of specific products of the pathway including MVA itself and the aforementioned potent oxysterols. Interestingly, one metabolite of the MVA pathway, geranylgeraniol, was shown to actually inhibit the LXRα-mediated constitutive activity.

The LXRα/RXRα heterodimer has been classified as a member of a subset of receptors termed the permissive heterodimers (which also includes RXR/PPAR) due to its unique ability to be activated by LXR activators, RXR ligand (9-*cis* retinoic acid) or both together, resulting in a more than additive effect (Janowski *et al.*, 1996, Willy and

Mangelsdorf, 1997) (reviewed in Glass, 1996, Mangelsdorf and Evans 1995, Chambon, 1996). Surprisingly, studies have demonstrated that retinoid activation of the LXRα/RXRα heterodimer requires only the activation domain of LXRα (AF-2 domain) (Willy and Mangelsdorf, 1997). This unique form of communication between heterodimer partners, where the activation potential of one receptor can be mediated by ligand binding of its partner, has been termed the phantom ligand effect (Schulman *et al.*, 1997).

A recent study reports that the activation of LXRα (RLD-1) by 22(R)-hydroxy-cholesterol could be enhanced upon the addition of agents known to stimulate the PKC and/or the PKA signaling pathways (Huang *et al.*, 1998). This enhancement was, in turn, abolished upon addition of protein kinase inhibitors. Thus, this reports suggests that other signal transduction pathways may regulate transactivation by oxysterol-activated LXRα (RLD-1) *via* phosphorylation catalyzed by protein kinases.

1.3 LXRα as the Key Regulator of Cholesterol Homeostasis

1.3.1 The MVA Biosynthetic Pathway and Cholesterol Homeostasis

Cholesterol plays a critical role in not only the structural formation of all interior and exterior cell membranes but also as a precursor of essential substances including bile acids and steroid hormones. The mevalonate (MVA) pathway synthesizes the precursors involved in cholesterol biosynthesis (refer to Figure 4). This pathway is also responsible for the production of substances including ubiquinone, dolichol and farnesyl residues, all of which are involved in a number of critical cellular functions. A balance, however, must be maintained between mevalonate synthesis and cholesterol homeostasis as excess

cholesterol accumulation can result in diseases such as atherosclerosis and hyper-cholesterolemia. This balance is achieved through two mechanisms: transcriptional and post-transcriptional feed-back regulation of *de novo* cholesterol biosynthesis and the receptor-mediated endocytosis of cholesterol and the transcriptional feed-forward regulation of genes responsible for the conversion of cholesterol stores to bile acids and steroid hormones (reviewed in Brown and Goldstein, 1986, Goldstein and Brown, 1990, Brown and Goldstein, 1997).

Regulation of cholesterol biosynthesis occurs at the level of transcription for both the cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase genes as well as the low density lipoprotein receptor (LDL) gene *via* a sterol-mediated feed-back mechanism which involves the membrane bound transcription factor termed sterol regulatory element binding protein 2 (SREBP-2) (reviewed in Brown and Goldstein, 1997). When cellular sterol levels are low, the SREBP cleavage activation protein (SCAP) activates (either directly or indirectly) a two step proteolytic cascade, which ultimately results in the release of the NH_2-terminal domain of the SREBP from the membrane. Upon release, this SREBP fragment then enters the nucleus and binds to DNA elements, termed sterol regulatory elements (SREs), located in the promoter regions of HMG-CoA synthase, HMG-CoA reductase and LDL receptor genes. Once bound, these transcription factors up-regulates gene expression through either direct or indirect communications with the basal transcription machinery (Oliner *et al.*, 1996). However, when cellular sterol levels are high, cleavage of SREBP-2 is inhibited resulting in the repression of transcription of these genes.

Cholesterol homeostasis is also believed to be regulated by a number of mechanisms, which achieve post-transcriptional control of HMG-CoA reductase. These mechanisms include translational control of HMG-CoA reductase (possibly through differential splicing mediated by an unknown MVA metabolite), degradation of the protein (sterol mediated) and inactivation of the enzyme (phosphorylation by an AMP-dependent kinase) (Goldstein and Brown, 1990).

The feed-forward mechanism, by which cholesterol homeostasis is further achieved, is thought to occur through a reduction of cholesterol stores by conversion to bile acids or steroid hormones. Steroid hormone synthesis has been demonstrated to be governed by the orphan nuclear receptor, steroidogenic factor-1 (SF-1) (Lala *et al.*, 1997, Parker and Schimmer, 1997). Bile acid synthesis occurs as a result of the up-regulation in the expression of the cholesterol 7α-hydroxylase (CYP7A) gene which encodes the initial and rate limiting enzyme responsible for the conversion of cholesterol to bile acids (Li *et al.*, 1990 and reviewed in Myant and Mitropoulos, 1977). Transcriptional regulation of the CYP7A gene has proven to be quite complex and mediated by both hormones (up-regulation) and bile acids (down-regulation). Nonetheless, a number of studies have begun to unravel the complexity by demonstrating the existence of multiple and overlapping nuclear hormone receptor response elements (HREs), located in the proximal promoter region of the CYP7A gene. A number of transcription factors including COUP-TFII (chicken ovalbumin upstream promoter-transcription factor II), HNF-4 and HNF-3 (hepatocyte nuclear factors), RXR and RAR, have been shown to bind to these HREs and stimulate CYP7A promoter activity (Crestani *et al.*, 1995, Crestani *et al.*, 1996,

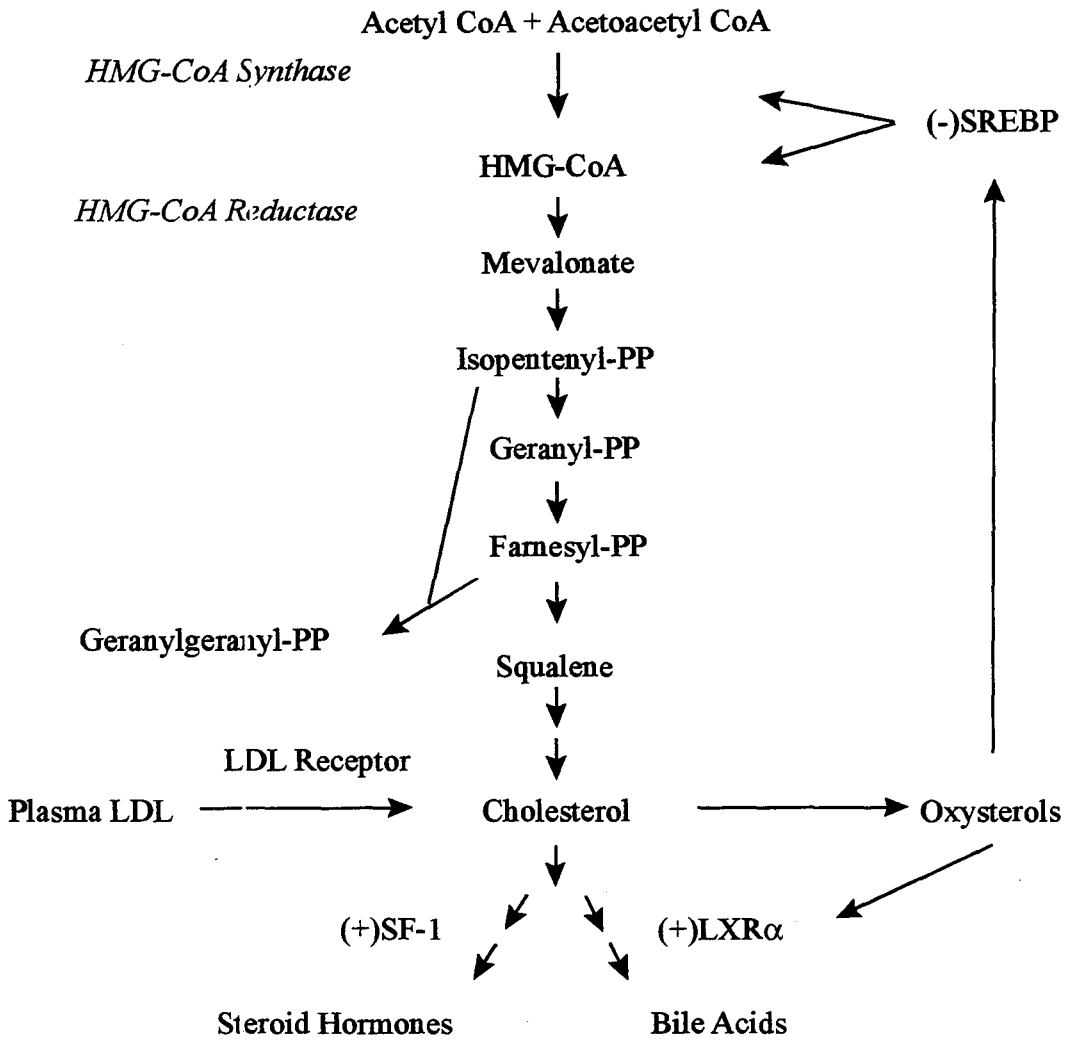


Figure 4: Proposed model for the regulation of cholesterol homeostasis. Adapted from Goldstein and Brown, 1990, Forman *et al.*, 1997 and Peet *et al.*, 1998. Cholesterol and mevalonate homeostasis is achieved through two mechanisms: feed-back repression of biosynthesis through sterol-mediated inhibition of the transcription factor SREBP and feed-forward induction of sterol-activated transcription factors LXRα and SF-1 resulting in the conversion of cholesterol stores to bile acids or steroid hormones.

Stroup *et al.*, 1997, Crestani *et al.*, 1998). A recent report identified a high affinity binding site for LXRα (CYP7A-LXRE) in the CYP7A gene (Lehmann *et al.*, 1997), which overlaps the COUP-TFII binding site (Stroup *et al.*, 1997). Furthermore, two bile acid response elements (BARE I and II) have been identified, which overlap the aforementioned HREs, and presumably aid in the binding of hydrophobic bile acid activated-receptors which mediate the feedback transcriptional repression of the CYP7A gene (Crestani *et al.*, 1994, Chiang and Stroup, 1994, Crestani *et al.*, 1998). Thus, it becomes evident that the regulation of CYP7A gene expression is a result of the competition between various transcription factors for these overlapping response elements and the resulting cross-talk of different signal transduction pathways.

1.3.2 The Role of LXRα in Cholesterol Homeostasis

The observations that LXRα is both positively and negatively regulated by products of the MVA biosynthetic/cholesterol homeostasis pathways, coupled with the fact that an LXRE was located in the proximal promoter region of the CYP7A gene, indicate that LXRα is a key sensor/transcriptional regulator of these pathways (refer to Figure 4). Indeed, a recent report which investigated the effect of an Lxrα gene-knockout in mice, demonstrated that LXRα(-/-) mice exhibit impaired cholesterol and bile acid metabolism ability (Peet *et al.*, 1998). This study specifically reports that LXRα(-/-) mice lose the ability to regulate dietary cholesterol leading to an accumulation of hepatic cholesteryl esters in the liver which results in hepatic failure. Furthermore, LXRα(-/-) mice not only failed to induce the transcription of the gene encoding CYP7A but also exhibited aberrant regulation of several other crucial genes involved in sterol and fatty acid synthesis

including HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase (FPP), squalene synthase, SREBP-2, SREBP-1, stearoyl CoA desaturase-1 and fatty acid synthase, which implies LXRα has a wide ranging effect on hepatic lipid metabolism. Thus, evidence from this report implicates LXRα as an essential regulator of cholesterol homeostasis as no other mechanisms, including LXRβ, appear to be able to compensate for its loss.

1.4 Transcription Co-factors: Co-activators and Co-repressors

Transcriptional regulation, mediated by members of the nuclear hormone receptor superfamily, has been shown to involve interactions with multiple auxiliary co-factors which function either to repress or activate transcription through interactions (or lack thereof) to the basal transcription machinery and/or participate in chromatin remodeling (reviewed in Shibata *et al.*, 1997, Glass *et al.*, 1997, Pazin and Kadonaga, 1997). Co-factors, which have demonstrated an ability to act as transcriptional activators, are termed co-activators, while those, which appear to inhibit nuclear receptor-mediated activation, are termed co-repressors.

Co-repressors, such as the silencing mediator of RARs and TRs (SMRT) (Chen and Evans, 1995) and the nuclear receptor co-repressor (N-CoR) (Hörlein *et al.*, 1995), presumably exert their repressive effects by binding to unliganded receptors and thereby block interactions with other co-factors (co-activators and basal transcription factors) required for initiation of transcription of the target gene (Chen *et al.*, 1996, Kurokawa *et al.*, 1996, Lavinsky *et al.*, 1998 and reviewed in Perlmann and Vennström, 1995 and Shibata *et al.*, 1997). Recent studies have reported that multiple mechanisms exist, which

impart specificity of nuclear hormone receptor interactions with various co-repressors. These mechanisms include the nature of the ligand, the levels of available SMRT and N-CoR, DNA response element binding and subsequent heterodimer partner polarity, and regulation of the nuclear hormone receptors by multiple signal transduction pathways (protein kinase-dependent signaling cascades which can inhibit co-repressor recruitment, as observed for ER, PR and RAR, or stimulate co-repressor recruitment, as observed for PPARγ) (Kurokawa *et al.*, 1995, Lavinsky *et al.*, 1998). Furthermore, it has been reported that both SMRT and N-CoR-mediated transcriptional repression is in part due to an interaction with both mSin3A/B and Histone Deacetylase (HDAC1 for SMRT and mRPD3 (HDAC2) for N-CoR), resulting in the formation of a multisubunit repressor complex (Nagy *et al.*, 1997, Heinzel *et al.*, 1997 and reviewed in Pazin and Kadonaga, 1997). Thus, this repressor complex is believed to induce chromatin remodeling, through core histone deacetylation, thereby blocking transcription factors access to the DNA template and resulting in transcriptional repression. However, once ligand binding is achieved, a conformational change is thought to occur which causes the co-repressor complex to dissociate. This displacement of co-repressors subsequently permits the recruitment of co-activators to the nuclear hormone receptor complex.

A number of co-activator proteins, which bind to various nuclear hormone receptor complexes, have been reported to date (reviewed in Janknecht and Hunter, 1996, Shibata *et al.*, 1997, Glass *et al.*, 1997). These include the steroid receptor co-activator-1 (SRC-1) (Oñate *et al.*, 1995, Kamei *et al.*, 1996, Zhu *et al.*, 1996, Jayakumar *et al.*, 1997, Kalkhoven *et al.*, 1998, Lee *et al.*, 1998), the CREB-binding protein and its homolog

p300 (CBP/p300) (Chrivia *et al.*, 1993, Eckner *et al.*, 1994, Chakravarti *et al.*, 1996, Dowell *et al.*, 1997), the p300/CBP/co-integrator associated protein (p/CIP) (Torchia *et al.*, 1997), the p300/CBP-associated factor (P/CAF) (Yang *et al*, 1996, Korzus *et al.*, 1998), the glucocorticoid receptor-interacting protein (GRIP1) and the transcriptional intermediary factor (TIF2) (Voegel *et al.*, 1996, Hong *et al.*, 1996, Walfish *et al.*, 1997), and the receptor-interacting protein 140 (RIP 140) (Cavaillès *et al.*, 1995). In addition to their ability to bind nuclear hormone receptors, several of these co-activators have demonstrated an ability to bind each other, as in the case of SRC-1 (and related p160 proteins) with CBP/p300 (Kamei *et al.*, 1996, Yao *et al.*, 1996, Korzus *et al.*, 1998), CBP/p300 with p/CIP (Torchia *et al.*, 1997), and CBP/p300 with P/CAF (Yang *et al.*, 1996). Furthermore, several of these co-activators, like the nuclear hormone receptors to which they bind, have been shown to interact with basal transcription factors, such as TBP and TFIIB, as well as with RNA Polymerase II itself (Leong *et al.*, 1998, Rochette-Egly *et al.*, 1997, Takeshita *et al.*, 1996 and reviewed in Mangelsdorf and Evans, 1995, Shibata *et al.*, 1997, Chang and Jaehning, 1997). A number of these co-activators, including CBP/p300 and P/CAF, have also exhibited intrinsic histone acetylase activity, which is believed to induce chromatin remodeling, through core histone acetylation, thereby permitting transcription factors access to the DNA template and resulting in transcriptional activation (Korzus *et al.*, 1998 and reviewed in Janknecht and Hunter, 1996, Pazin and Kadonaga, 1997, Shibata *et al.*, 1997, Glass *et al.*, 1997). Thus, in light of these findings, many have suggested the existence of multisubunit co-activator

complexes, formed in response to ligand, which facilitates the assembly of a stable pre-initiation complex, resulting in efficient transcriptional activation of target genes.

Co-activator binding to nuclear hormone receptors is believed to occur *via* a ligand-induced conformational change, which shifts the AF-2 core domain (helix 12) from an extended conformation in the absence of ligand to a compacted conformation with the LBD (usually helices 3, 4 and 5) in the presence of ligand, presumably resulting in the dissociation of the bound co-repressor complex (Renaud *et al.*, 1995, Bourget *et al.*, 1995). This compact conformation of the liganded LBD constitutes a hydrophobic groove, as demonstrated by crystallographic studies (Nolte *et al.*, 1998, Darimont *et al.*, 1998), which can interact with the nuclear hormone receptor LXXLL binding motif possessed by co-activators (Heery *et al.*, 1997, Torchia *et al.*, 1997). Furthermore, recent studies report that ligand-dependent binding of co-activators, such as CBP, SRC-1 and the related p160 proteins, to both the A/B (AF-1) and AF-2 (LBD) domains of the same receptor, may mediate AF-1/AF-2 synergism (Kraus *et al.*, 1995, Ikonen *et al.*, 1997, Oñate *et al.*, 1998, Webb *et al.*, 1998). These observations are consistent with previous studies which report that loss or mutation of the AF-2 core domain (helix 12) and/or other helices (namely 3, 4 and 5) of the LBD results in the abolishment of ligand-dependent transcriptional activation, AF-1/AF-2 synergism and the loss of co-factor binding (Danielian *et al.*, 1992, Cavaillès *et al.*, 1995, Henttu *et al.*, 1997, White *et al.*, 1997, Collingwood *et al.*, 1997, Joyeux *et al.*, 1997, Jeyakumar *et al.*, 1997, Treuter *et al.*, 1998, Miyata *et al.*, 1998, and reviewed in Shibata *et al.*, 1997, and Glass *et al*, 1997).

However, it should be noted at this point that while ligand binding to nuclear hormone receptors appears to be a prerequisite for co-activator binding, a number of studies have demonstrated efficient binding of co-activators to unliganded receptors, the significance of which remains to be seen. Some of these interactions include RIP 140 and LXRα (Miyata *et al.*, 1998), RIP 140 and PPARα (Miyata *et al.*, 1998, Treuter *et al.*, 1998), SRC-1 with PPARγ (Zhu et al., 1996) and p300 with PPARα (Dowell *et al.*, 1997).

Co-activator binding to nuclear receptors, as previously described, is dependent upon the α-helical LXXLL motif (s) present in co-activators. Introduction of mutations within these motifs results in either a reduction or abolishment of co-activator/receptor interaction and a concordant loss in transcriptional transactivation (Heery *et al.*, 1997, Torchia *et al.*, 1997) Recently, a number of studies have demonstrated that co-activator binding to nuclear receptors occurs with respect to a two-fold receptor-specific code of interaction, mediated by the multiple LXXLL motifs located within each co-activator (McInerney *et al.*, 1998, Darimont *et al.*, 1998). The first level of specificity resides in the ability of LXXLL adjacent residues to modulate specific interactions of various co-activators with the LBDs of different and specific receptors (McInerney *et al.*, 1998, Darimont *et al.*, 1998). The second level of specificity involves the number of LXXLL motifs, located within a co-activator, which are differentially utilized by the various nuclear receptors (McInerney *et al.*, 1998). Specifically, this study reports that while only a single LXXLL motif of SRC-1 is sufficient for ER-mediated activation, receptors, binding as heterodimers with RXR (i.e. TR, RAR and PPAR) or PR homodimers, required different combinations of two, appropriately spaced LXXLL motifs. These

observations are consistent with previous studies, which demonstrate the requirement of two consecutive SRC-1 LXXLL motifs for interactions with both subunits of a PPARγ homodimer (Nolte *et al.*, 1998) and a requirement for two SRC-1 LXXLL motifs for cooperative binding to both DNA-bound RAR/RXR and PPARγ/RXR heterodimers (Westin *et al.*, 1998)

To date, only one co-factor has been shown to bind to LXRα (RIP 140; Miyata *et al.*, 1998 and Section 3.3 of this study); however, recent evidence, presented in this report, suggests that LXRα is also capable of interacting with the co-activator SRC-1and the co-repressor SMRT (Section 3.3.8).

1.4.1 An Overview of the Co-factor RIP 140

RIP 140 is a recently identified protein that has previously been shown to interact with a number of nuclear hormone receptors including ER, TR, RAR and RXR in a ligand-dependent manner (Cavaillès *et al.*, 1995, L'Horset *et al.*, 1996, and reviewed in Shibata *et al.*, 1997, Glass *et al.*, 1997). RIP 140 has been described as a member of the transcriptional co-activating protein family as it posses intrinsic activation potential when tethered directly to a promoter (Cavaillès *et al.*, 1995). Interactions between RIP 140 and nuclear hormone receptors occur *via* LXXLL signature motifs, nine of which are spread throughout RIP 140 (Heery *et al.*, 1997). Furthermore, two distinct nuclear hormone receptor binding sites have been characterized, site1 (aa 27-241) and site2 (aa 753-981), within RIP 140 (L'Horset *et al.*, 1996). The same report also demonstrates that these sites possess similar properties but not identical, as interaction with specific nuclear hormone receptors can be mediated primarily by one site over another. To date, all

receptor/RIP140 interactions have been shown to be dependent upon an intact LBD and AF-2 core domain (helix 12) (Cavaillès *et al.*, 1995, L'Horset *et al.*, 1996, Joyeux *et al.*, 1997, Collingwood *et al.*, 1997, Henttu *et al.*, 1997, White *et al.*, 1997, Treuter *et al.*, 1998, Miyata *et al.*, 1998).

Two recent studies have reported that RIP140 also interacts with PPAR; however, Miyata and colleagues (1998) identifies this interaction as ligand-independent in solution, whereas Treuter and colleagues (1998) observes a ligand-enhanced interaction in solution (although RIP 140 interactions with PPARα/RXRα bound to DNA proved to be ligand-independent). In contrast to a role for RIP 140 as a co-activator, the PPAR ligand–independent interaction with RIP140 leads to a repression in transcriptional activity of PPARα/RXRα in both mammalian cells (Miyata *et al.*, 1998, Treuter *et al.*, 1998) and yeast cells (Treuter *et al.*, 1998). Similarly, transient transfection studies of RIP 140 and the ER receptor (Cavaillès *et al.*, 1995), as well as studies performed in yeast (Joyeux *et al.*, 1997), demonstrate that only minimal activation is achieved upon co-transfection/co-transformation, which is subsequently repressed as RIP 140 concentrations, in transfections, are increased. RAR-mediated activation was also shown to be repressed upon co-transfection with RIP 140 (Chakravarti *et al.*, 1996). Furthermore, RIP 140 fails to interact not only with CBP (Kamei *et al.*, 1996) but also with basal transcription factors such as TBP and TFIIB (Cavaillès *et al.*, 1995), indicating an inability to function as a co-activator/bridging molecule with the transcription initiation complex. As well, a recent study has demonstrated the inability of RIP 140 to functionally substitute for SRC-1 (Torchia *et al.*, 1997). Thus, despite the similarities between RIP140 and other

co-activators such as the SRC-1 family, a true 'co-activator' role for RIP140 has come under question. Our laboratory, and others, have suggested that RIP140, under certain experimental conditions, acts to attenuate the transcriptional response of nuclear hormone receptors by preventing functional interactions with other, more potent co-activators.

1.4.2 An Overview of the Co-activator SRC-1

The steroid receptor co-activator 1 (SRC-1) was originally identified as a human progesterone receptor (hPR) interacting protein, *via* a yeast dihybrid screen of a human β lymphocyte cDNA expression library (Oñate *et al.*, 1995). Subsequent studies have shown this initial SRC-1a was a partial clone and the full-length SRC-1a (F-SRC-1a), in both human (Kalkoven *et al.*, 1998) and mouse (Kamei *et al.*, 1996, Yao *et al.*, 1996), was subsequently isolated and shown to possess an N-terminal extension, which exhibited a high degree of homology to a PAS-A-basic-helix-loop-helix (bHLH) domain, beyond the original SRC-1a N-terminus. Furthermore, four other murine SRC-1 clones have been isolated including SRC-1b (an N-terminal variant of F-SRC-1a), SRC-1c, d and e (C-terminal variants of the F-SRC-1a), and one human C-terminal variant SRC-1e (Kamei *et al.*, 1996, Kalkoven *et al*, 1998).

As its name implies, SRC-1 has been identified as a member of the transcriptional co-activating protein family and has been shown to interact with and activate a number of receptors including PR, ER, RAR, RXR, TR, GR and PPARγ in a ligand-dependent or ligand–enhancing manner (reviewed in Shibata *et al.*, 1997, Glass *et al.*, 1997). These interactions have been shown to be dependent upon the LXXLL signature motif, four of which are located in SRC-1a (and three in SRC-1e) (Heery *et al.*, 1997, Kalkoven *et al.*,

1998). In turn, the AF-2 domain (helix 12) of various receptors has been shown to be required for efficient SRC-1 binding and subsequent activation but also makes contacts with SRC-1*via* its A/B domains and other areas of the LBD (Takeshita *et al.*, 1996, Jeyakmur *et al.*, 1997, Oñate *et al.*, 1998 and reviewed in Shibata *et al.*, 1997). Taken together these observations indicate that SRC-1 may mediate AF-1/AF-2 synergism, by facilitating a functional interaction between the A/B and AF-2 domains of the same receptor (Ikonen *et al.*, 1997, Webb *et al.*, 1998, Oñate *et al.*, 1998).

Further indication that SRC-1 functions as a co-activator for the nuclear hormone receptors resides in four main pieces of evidence; first, a dominant-negative form of SRC-1a serves to block nuclear receptor function (Oñate *et al.*, 1995). Second, microinjection antibodies against SRC-1 also block nuclear receptor function (Torchia *et al.*, 1997). Third, SRC-1 has been shown to interact with CBP/p300 which, in turn, has been shown to interact with p/CAF (both of which exhibit histone acetylase activity) suggesting the formation of multimeric complexes with nuclear receptors, which facilitates transcriptional activation of target genes. Fourth, SRC-1 interacts *in vitro* with the basal transcription factors TBP and TFIIB (Takeshita *et al.*, 1996) (although Kalkoven *et al.*, 1998 report that SRC-1a and e were unable to bind these factors). Interestingly, Kalkoven *et al.*, 1998 have also demonstrated that SRC-1 isoforms differ in their ability to potentiate ER-mediated activation, where SRC-1e appears to be the more potent co-activator and SRC-1a can result in a repression of ER-mediated transcription depending upon the promoter context. Lastly, SRC-1 has also been shown to bind to the transcription factor AP-1 subunits c-Jun and c-Fos and can potentiate AP-1-mediated

transactivation (like CBP/p300), indicating that SRC-1/CBP/p300 may mediate transrepression between AP-1 and nuclear receptors *in vivo* (Lee *et al.*, 1998).

1.4.3 An Overview of the Co-repressor SMRT

The silencing mediator (co-repressor) for retinoid and thyroid hormone receptors (SMRT) was initially identified as an RXR (although weakly) interacting protein *via* a yeast dihybrid screen of a human lymphocyte cDNA library (Chen and Evans, 1995). As its name implies, SMRT acts as a co-repressor of a GAL-4 dependent reporter when fused to the GAL-4 DNA binding domain (Chen and Evans, 1995). SMRT has been shown to interact with RAR, TR and marginally to RXR in a ligand-independent manner where addition of ligand reduces these interactions (although in the case of RXR the effect observed was slight) (Chen and Evans, 1995). Furthermore, SMRT interacts with the RXR/RAR heterodimer in mammalian cells in the absence of ligand but fails to interact in its presence (Chen *et al.*, 1996). Subsequently, SMRT was shown to mediate the transcriptional silencing of RAR and TR (but not RXR where no effect was observed under the experimental conditions employed) (Chen and Evans, 1995). SMRT is further implicated as a nuclear receptor co-repressor based on three other main points of evidence; first, the receptor-interacting domain of SMRT antagonizes the repressive effect of both RAR and TR and, therefore, acts as a dominant-negative form of SMRT (Chen *et al.*, 1996). Second, SMRT is highly related to another identified nuclear receptor co-repressor, N-CoR (Chen *et al.*, 1996, Lavinsky *et al.*, 1998 and reviewed in Perlmann and Vennström, 1995, Shibata *et al.*, 1997). Third, SMRT has also been shown to interact with both mSin3A and the histone deacetylase 1 (HDAC1) and has therefore

been implicated in transcriptional repression as deacetylation of core histones (thought to prevent access of transcription factors to the DNA template) (Nagy *et al.*, 1997 and reviewed in Pazin and Kadonaga, 1997).

A recent report has demonstrated that multiple mechanisms exist, which impart specificity of nuclear hormone receptor interactions with various co-repressors such as SMRT and N-CoR which include the nature of the ligand, the levels of available SMRT and N-CoR, DNA response element binding and subsequent heterodimer partner polarity, and regulation of the nuclear hormone receptors by multiple signal transduction pathways (protein kinase-dependent signaling cascades which can inhibit co-repressor recruitment, as observed for ER, PR and RAR, or stimulate co-repressor recruitment, as observed for PPARγ) (Kurokawa *et al.*, 1995, Lavinsky *et al.*, 1998).

1.5 Previously Completed Work

The versatility of the yeast dihybrid system provides the user with an *in vivo* assay for detecting protein-protein interactions in *Saccharomyces cerevisiae* as well as an effective method of genetic screening for novel proteins. We employed this system as a genetic screen for proteins, which interact with RXRα. In screening a Hela cell cDNA library, fused to the GAL 4 activation domain (GAD) with hRXRα, bound to the GAL 4 DNA binding domain (GBD), we were able to isolate SM1 (Miyata *et al.*, 1996), a truncated form of a novel nuclear receptor later cloned and termed LXRα (Willy *et al.*, 1995, Miyata *et al.*, 1996). Concurrently, Kenji Miyata, using mPPARα (fused to the GBD) and the same GAD-cDNA library, also isolated an LXRα clone (Miyata *et al.*,

1996). Subsequently, the full length LXRα clone was constructed as outlined in Section 2.1.7.3 (Miyata *et al*, 1996) (refer to Figure 1).

Initial characterization studies of LXRα, performed by Kenji Miyata and Hansa Patel, revealed that the 1.9kb LXRα mRNA is expressed in highest abundance in the liver and heart but is also detected in the kidney, intestine, spleen, lung, pancreas, skeletal muscle and testis (Miyata *et al.*, 1996 as confirmed by Willy *et al.*, 1995). As well, *in vitro* studies using maltose binding protein (MBP)-RXRα fusion proteins in protein binding assays showed that LXRα interacts with RXRα in the absence of a DNA target site (Miyata *et al.*, 1996).

As part of the LXRα characterization studies we investigated the DNA binding specificity of LXRα and RXRα using electrophoretic mobility shift analysis (EMSAs). For these experiments we synthesized a series of oligonucleotides which contained AGGTCA half sites, present as direct repeats, spaced by zero to five nucleotides (DR0 to DR5). Using *in vitro* translated LXRα and RXRα we were able to show that LXRα heterodimerized with RXRα and bound preferentially to the DR4 response element and weakly to the DR3 and DR5 response elements (Miyata *et al.*, 1996). Furthermore, LXRα and RXRα were also shown to bind to the LXRE-ΔMTV, as previously determined by Willy and colleagues (1995).

Interestingly, SM1 and RXRα heterodimer formation on the DNA response elements DR4 and LXRE-ΔMTV was greatly diminished in comparison to LXRα/RXRα/DR4 and LXRα/RXRα/LXRE-ΔMTV complex formation (demonstrated in Section 3.2.1). The

SM1 clone is missing the first 61 amino acids of the N-terminus (A/B domain) and also possess an N-terminal extension sequence different than the native LXRα sequence. Furthermore, as demonstrated in Section 3.2.1, EMSA analysis revealed the existence of a second band shift (shift B) whose mobility was slightly slower than that of the LXRα/RXRα/LXRE complex mobility. Interestingly, this second species was absent in EMSA experiments with SM1/RXRα/LXRE. These observations subsequently led to investigations of the A/B domain of LXRα, presented in Section 3.2.

Initial transient transfection assays with LXRα and RXRα demonstrated that LXRα/RXRα can constitutively activate transcription of a reporter gene that contained the DR4 LXRE but not the DR1 response element (Miyata *et al.*, 1996). The addition of 9-*cis* retinoic acid, a known RXR ligand, only potenitated this activation slightly (also seen in Section 3.1.1) (Miyata *et al.*, 1996, Willy *et al.*, 1995).

LXRα has also been shown to interact with PPARα as determined by both a yeast two-hybrid assay and protein binding assays with MBP-PPARα fusions; however, an LXRα/PPARα response element has yet to be identified (Miyata *et al.*, 1995). EMSA analysis demonstrated that LXRα inhibits binding of RXRα/PPARα heterodimers to both rat fatty acyl-CoA oxidase (Aox) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) PPREs (Miyata *et al.*, 1996). Similarly, PPARα inhibits binding of LXRα/RXRα heterodimers to both DR4 and DR5 LXREs (Miyata *et al.*, 1996). Furthermore, transient transfection assays indicated that LXRα can antagonize RXRα/PPARα-mediated transcriptional activation (Miyata *et al.*, 1996). These

observations, taken together, implicate LXRα as a negative regulator of PPARα-mediated transcriptional activation.

Thus, taken together, these preliminary studies of LXRα contributed to our understanding of the role of LXRα in not only novel and distinct retinoid response pathways, but also in modulating PPAR-signaling pathways in the cell, thereby indicating a convergence of distinct receptor signaling pathways. Ultimately, these initial investigations opened the door for further functional analysis of LXRα.

1.6 Project Overview

Upon commencement of this project, little was known about LXRα apart from those initial investigations outlined in Section 1.5. Thus, in order to determine the mechanism by which LXRα functions as a transcriptional regulator of target genes, a number of integrated characterization studies were designed, which focused on three main areas of LXRα investigation; first, examination of the transactivation properties of LXRα, under various conditions *in vivo*, second, delineation of the role of the LXRα A/B domain in transactivation *via* LXRα A/B domain deletion studies and third, the identification of potential co-factors which may functionally interact with LXRα, thereby aiding in the LXRα-mediated transcriptional response.

Investigations of the transactivation properties of LXRα, subsequent to those initially described in Section 1.5, were facilitated by the identification of both the LXRE-ΔMTV response element and LXRα activators, which includes 22(R)-hydroxy-cholesterol. These studies were performed in order to ascertain if LXRα-mediated

transcriptional activity exhibits DNA response element (LXRE), activator/ligand or cell line specific effects as reported in many other nuclear receptor transactivation studies. In doing so, a viable and efficient transient transfection assay was developed for subsequent LXRα investigations *in vivo*. Thus, these studies contribute to the delineation of the mechanisms and conditions that exist within the cell, which modulate LXRα-mediated transactivation.

Studies of the highly divergent A/B domain of various nuclear receptors have implicated this domain as a participant in transactivation and thus, have provided great insight into how these receptors regulate/modulate transcription of target genes. Specifically, a number of these investigations have revealed the existence of an autonomous ligand-independent activation domain (AF-1 domain), which can either function independently or synergize with the AF-2 domain of the same receptor, thought to be mediated by the binding of various co-activators. Thus, investigation of the LXRα A/B domain was of significant importance and ultimately utilized a series of LXRα N-terminal A/B domain deletion constructs. These studies focused on the role, if any, the N-terminal 63 amino acids of the LXRα A/B domain played in RXRα heterodimerization and subsequent binding to LXREs, co-factor (RIP 140) binding and transactivation. The results from these investigations may further serve to indicate the presence or absence of a functional AF-1 domain within this region of LXRα.

The identification of LXRα co-factors is of particular interest since transcriptional regulation, mediated by other members of the nuclear hormone receptor superfamily, has been shown to involve multiple auxiliary co-factors, which function either to repress or

activate transcription not only through interactions (or lack thereof) between the basal transcription machinery and nuclear receptors but also through chromatin remodeling. Through a fortuitous sequence of events, the previously identified co-factor RIP 140 was isolated, as a partial clone, from a human liver cDNA library *via* the two-hybrid yeast system utilizing PPARα as bait. This finding ultimately facilitated LXRα and RIP 140 interaction and functional studies. Subsequent interaction and functional investigations between LXRα and both the co-repressor, SMRT, and the co-activator, SRC-1, were also performed. These co-factor/LXRα investigations further our understanding of the mechanism by which nuclear receptors achieve specificity and diversity in the activation of target gene expression.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

The chemicals and reagents employed in this research, and the corresponding companies from which they were obtained, are outlined below:

agarose (electrophoresis grade)	Life Technologies
agarose (NuSieve-GTG)	FMC Bioproducts
agar	Becton Dickinson and Company
amino acids	Sigma Chemical Company
ampicillin	Sigma Chemical Company
Bacto-peptone	Difco Laboratories
BioRad protein assay	BioRad Laboratories
bovine serum albumin	Pharmacia Biotech
cesium chloride (molecular biology grade)	Life Technologies
charcoal, Dextran coated	Sigma Chemical Company
chloramphenicol	Sigma Chemical Company
coenzyme A	Sigma Chemical Company
deoxynucleotide triphosphates (dNTPs)	Pharmacia Biotech
dithiothreitol (DTT)	Sigma Chemical Company

dimethylsulphoxide (DMSO)	Caledon Laboratories
Glutathione Sepharose 4B	Pharmacia Biotech
22(R)-Hydroxycholesterol (22 (R)-OH-CH)	Research Plus
isopropylthio-β-D-galactoside (IPTG)	Life Technologies
kanomycinsulfate	Boehringer Mannheim
L-glutamine	Life Technologies
luciferin	Biosynth
MEM sodium pyruvate solution (100mM)	Life Technologies
molecular weight standards	
50 bp DNA ladder	Life Technologies
1 Kb DNA ladder	Life Technologies
1Kb Plus DNA ladder	Life Technologies
low range SDS-PAGE standards	BioRad Laboratories
high range SDS-PAGE standards	BioRad Laboratories
Nonidet P-40 (NP-40)	BDH Chemicals
penicillin (5,000/ml)/ streptomycin (5,000µg/ml)	Life Technologies
phenylmethylsulphonylflouride (PMSF)	Boehringer Mannheim
Protease inhibitor cocktail tablets	Boehringer Mannheim
polydIdC	Pharmacia Biotech
PPO (2,5-diphenyloxazole) scintanalyzed	Fischer Scientific
5X reporter lysis buffer	Promega
9-*cis* retinoic acid (9-*cis* RA)	Sigma Chemical Company Biomol Research Laboratories

Salmon sperm DNA	Sigma Chemical Company
Sephadex G-50	Pharmacia Biotech
serum, calf	Life Technologies
serum, fetal bovine	Sigma Chemical Company
sodium dodecyl sulfate (SDS)	Sigma Chemical Company
Triton X-100	BDH Chemicals
Wy-14,643	ChemSyn Laboratories
X-GAL (5-bromo-4-chloro-3-indoyl-β-D-galactoside)	Life Technologies
yeast extract	Becton Dickinson Company
yeast nitrogen base (without amino acids)	Difco Laboratories

2.1.2 Enzymes

The enzymes that were employed in this research , and the corresponding companies from which they were obtained, are outlined below. All enzymatic reactions were performed as recommended by the manufacturer.

calf intestinal alkaline phosphatase	New England Biolabs
DNA polymerase I (Klenow)	New England Biolabs
lysozyme	Sigma Chemical Company
restriction endonucleases	New England Biolabs
RNAse A	Pharmacia Biotech
SP6 RNA polymerase	Promega
T3 RNA polymerase	Promega
T7 RNA polymerase	Promega

T7 DNA polymerase	Pharmacia Biotech
T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase	New England Biolabs
Vent DNA polymerase	New England Biolabs

2.1.3 Radiochemicals

L-[^{35}S]-methionine (1151 Ci/mmol; 10μCi/μl)	NEN Life Science Products
[α-^{32}P]dATP (3,000 Ci/mmol; 10μCi/μl)	Amersham Canada Ltd. NEN Life Science Products
[^{14}C]-labeled molecular weight markers	
high range molecular weight markers	Life Technologies
broad range molecular weight markers	Amersham Canada Ltd.

2.1.4 Yeast Strains and Growth Conditions

The PCY2 yeast strain, employed in this study, is of the genotype: MATα Δ*gal*4 Δ*gal*80 URA3::GAL1-*lacZ* *lys*2-801^{amber} *his*3-Δ200 *trp*-Δ63 *leu*2 *ade*2-101^{ochre}, and was a generous gift from Dr. P. Chevery, The Johns Hopkins University School of Medicine, Baltimore, MD (Chevery and Nathans, 1992). The Y190 yeast strain, employed in this study, is of the genotype: MATa, *leu*2-3,112, *ura*3-52, *trp*1-901, *his*3-Δ200, *ade*2-101, *gal*4Δ*gal*80Δ URA3 GAL-*lacZ*, LYS GAL-HIS3, *cyh*^{r}, (Harper *et al.*, 1993). Yeast were grown at 30°C in either YPD media (rich media containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose) or in synthetic complete (SC) media (0.67% bacto-yeast nitrogen base (without amino acids), 2% bacto-agar, 2% glucose and 0.2% drop-out mix (contains all amino acids except those being selected for)).

2.1.5 Bacterial Strains and Growth Conditions

The DH5α bacterial strain, employed in this study, is of the genotype: F^-, ϕ80Δ*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *hsd*R17 (r_k^-, m_k^-) phoA supE44 λ^- *thi*-1 *gyr*A96 *rel*A1. Bacteria was routinely grown at 37°C in 2YT media (1.6% bacto-tryptone, 1% bacto-yeast and 0.5% NaCl) which was supplemented with either 100 μg/ml ampicillin or 30 μg/ml kanamycin under selective conditions.

2.1.6 Mammalian Cell Lines

The BSC40 cell line are African Green Monkey Kidney cells derived from the BSC-1 cell line adapted to 40°C (Brockman and Nathans, 1974) and were obtained from the Massachusetts Institute of Technology (MIT).

The COS-1 cell line are African Green Monkey Kidney cells, established from CV-1 simian cells transformed by an origin-defective mutant of SV40 which codes for wild-type T antigen (Gluzman, 1981), and were obtained from the American Type Culture Collection (ATCC).

The Hep G2 cell line are human liver hepatoblastoma cells (Aden *et al.*, 1979, Aden *et al.*, 1980) and were obtained from the American Type Culture Collection (ATCC).

2.1.7 Oligonucleotides

The oligonucleotides outlined in Table 2 were employed for PCR amplification, construction of vectors, DNA sequencing or radioactive probe preparations for EMSA analysis. All oligonucleotides were prepared by the Central Facility of the Institute for Molecular Biology (MOBIX), McMaster University or Dalton Chemical Laboratories, North York.

Table 2: Oligonucleotides

Oligo-Nucleotide	Sequence	Purpose
AB 1118	5'TAATACGACTGCACTATAGGG3'	T7 promoter primer
AB5597	5'GATTTAGGTGACACTATAG3'	SP6 promoter primer
AB4589	5'GATCCCAGGTCACAGGAGGTCAGAA3'	Dr4 (A)
AB4590	5'GATCTTCTGACCTCCTGTGACCTGG3'	Dr4 (B)
AB6291	5'GCGCGGATCCGGTACCATGTCCTTGTGGCTGGGGGC3'	LXR (for) PCR
AB6292	5'GCGCGGATCCGATATCTCATTCGTGCACATCCCAGATC3'	LXR (rev) PCR
AB6554	5'GCGCGGTACCATGGGCGCACAGGATGCAAGCAG3'	RIPΔ1 (for) PCR
AB6555	5'GCGCGGTACCATGGCCAGGATGCCCCACTCTGC3'	RIPΔ2 (for) PCR
AB6556	5'GCGCGGTACCATGACAGCCCTGCTCACCAGGGC3'	RIPΔ3 (for) PCR
AB6557	5'GGGGTTAGGCCTTGTCCCCACACACACTGCATG3'	RIP deletions (rev) PCR
AB6011	5'TGTATCTTATGGTACTGTAACTG3'	PGL2 sequencing primer
AB6746	5'GATCCTTGCGGTTCCCAGGGTTTAAATAAGTTCATCTA3'	LXRE-ΔMTV (A)
AB6747	5'GATCTAGATGAACTTATTTAAACCCTGGGAACCGCAAC3'	LXRE-ΔMTV (B)
AB9324	5'GGGCTGGCAAGCCACGTTTGGTG3'	GST sequencing primer (5')
Dalton 1	5'GCAGACCGGCCCAACGTGCAG3'	LXR sequencing primer at NdeI site
AB14336	5'GCGCGGATCCGCTCACGGTGGGAGCTTTTTGTCCTGCAG3'	LXR (rev) primer for AF-2 domain deletion
AB13685	5'GGAGTGAGAGTATCACCTTCCTCAAGG	LXR internal sequencing primer at the EcoRI site

2.1.8 Plasmids

2.1.8.1 Commercially Available Vectors

pSG5: Obtained from Stratagene, pSG5 is an ampicillin resistant eukaryotic expression vector containing an SV40 early promoter and an SV40 poly-A signal and a T7 promoter upstream of the multiple cloning site (MCS).

pRc/CMV: Obtained from Invitrogen, pRc/CMV is an ampicillin resistant eukaryotic expression vector containing enhancer-promoter sequences from the immediate-early

gene of the human cytomegalovirus (CMV) and a poly-A signal from the bovine growth hormone (BGH) gene. It also possesses both a T7 and an SP6 promoter flanking either side of the MCS.

pGL2: Obtained from Promega, pGL2 is an enhancerless SV40 promoter/luciferase expression vector which is ampicillin resistant.

pGEX-2T (GST): Obtained form Pharmacia Biotech, pGEX-2T is an ampicillin resistant bacterial expression vector which contains a tac promoter, a lac operon and a thrombin recognition (cleavage) site upstream of the GST gene. The MCS of the vector is adjacent to the GST domain.

pGEX-2TK (GST): Obtained form Pharmacia Biotech, pGEX-2TK is identical to pGEX-2T except that this vector contains a protein kinase site between the GST domain and the MCS and downstream of the thrombin recognition site.

pGBT9: Obtained form Clontech Laboratories, pGBT9 is an ampicillin resistant DNA-binding domain hybrid cloning vector (GAL4 DNA-binding domain (aa 1-147)) which can be employed to generate target protein fusions with the GAL4 DNA binding domain. pGBT9 also carries a TRP1 selectable marker which allows yeast auxotrophs to grow on limiting synthetic media.

pGAD 424: Obtained form Clontech Laboratories, pGAD 424 is an ampicillin resistant activation domain hybrid cloning vector (GAL4 activation domain (aa 768-881)) which can be employed to generate protein fusions with the GAL4 activation domain. pGAD 424 also carries a LEU2 selectable marker which allows yeast auxotrophs to grow on limiting synthetic media.

2.1.8.2 Plasmids Constructed By Others

pSG5-RXRα: contains full length human RXRα, cloned into the EcoRI site of pSG5 (Marcus *et al.*, 1993).

pSPUTK: a generous gift from Dr. Andrews (Falcon and Andrews, 1991), pSPUTK is a transcription/translation vector whose MCS site is flanked by SP6 and T7 promoters.

pSPUTK-SM1: contains an amino terminal truncated clone of LXRα (aa 62-447), obtained from Jamie Ingles, which also possesses an extension piece (employed for cloning purposes) at the amino terminus ($^{5'}$M,G,C,R,N,S,A,R,A,T$^{3'}$) which is different from that of the native LXRα sequence.

pSPUTK-RIP1: contains an amino terminal truncated clone of LXRα (aa 6-447), obtained from Hansa Patel.

pRc/CMV-RIP1: contains an amino terminal truncated clone of LXRα (aa 6-447), obtained form Hansa Patel.

pRc/CMV-LXRα: contains full length human LXRα, obtained from Hansa Patel (Miyata *et al.*, 1996).

pGBT9-LXRα: contains full length human LXRα, obtained from Hansa Patel, and cloned into the BamHI site of pGBT9.

pGEX-2TK-mPPARα (GST-PPARα): contains full length mPPARα cloned into the BamHI site of pGEX-2TK, obtained from Lisa Meertens.

TK-LXRE3-LUC: was a generous gift from Dr. Mangelsdorf (Willy *et al.*, 1995) and contains three copies of the LXRE-ΔMTV DNA response element cloned into the HindIII site of a TK-LUC vector in a tandem repeat configuration (→→→).

pGAD-RIP 140: contains aa 164-869 of RIP 140, obtained from Kenji Miyata, and was isolated from a Matchmaker human liver cDNA library (cloned into pGAD10 (Clontech)) in a yeast two-hybrid analysis using mPPARα as bait (Miyata *et al.*, 1998).

pEF-BOS: was a generous gift from Dr. Parker (Cavaillès *et al.*, 1995), and is an ampicillin resistant eukaryotic expression vector and contains an elongation factor 1α (EF-1α) promoter region and a poly-A signal from human granulocyte colony-stimulating factor (G-CSF) cDNA (Mizushima and Nagate, 1990).

pEF-BOS-RIP 140: was a generous gift from Dr. Parker (Cavaillès *et al.*, 1995) and contains full length RIP 140.

pBluescript II SK(-)-RIP 140: was a generous gift from Dr. Parker (Cavaillès *et al.*, 1995) and contains full length RIP 140.

pGEX-2TK-(N-term)-RIP 140 (GST-RIP 140 (N)): contains aa 1-479 of RIP 140 cloned downstream and in frame of the GST gene (Miyata *et al.*, 1998).

pGEX-2TK-(C-term)-RIP 140 (GST-RIP 140 (C)): contains aa 656-1158 of RIP 140 cloned downstream and in frame of the GST gene (Miyata *et al.*, 1998).

pBK-CMV-SRC-1: was a generous gift from Dr. O'Malley (Oñate *et al.*, 1995) and contains a truncated form of SRC-1 (aa 378-1441) (Shibata *et al.*, 1997) cloned into the eukaryotic expression vector pBK-CMV (Stratagene) which is kanamycin resistant and possesses both a T3 and a T7 promoter flanking either side of the MCS site.

pCMX-SMRT: was a generous gift from Dr. Evans (Chen and Evans, 1995) and contains full length human SMRT cDNA but does not contain the aa 1330-1375

alternative spliced insert. The pCMX vector is an ampicillin resistant eukaryotic expression vector with a CMV promoter and also contains a T7 promoter.

2.1.8.3 Plasmids Constructed For Project:

pGAD-SM1: encodes aa 61-447 of LXRα and was isolated form a Hela cell cDNA library constructed in the GAD vector pGADGH (Clontech) in a yeast two-hybrid analysis using RXRα as bait (Miyata *et al.*, 1996).

pSPUTK-LXRα:

a) Cloning full length LXRα cDNA was performed in collaboration with the Rachubinski laboratory (Miyata *et al.*, 1996). Using pGAD-SM1 as a probe, a 1.7kbp cDNA was isolated from a λgt11 human liver cDNA library (Stratagene) whose sequence was shown to contain the entire 447 amino acid long open reading frame of LXRα (Miyata *et al.*, 1996). This cDNA by convention was designated LXRα and is essentially identical at the amino acid level to the published sequence of LXRα (Willy *et al.*, 1995). The only differences noted are a phenylalanine in place of a leucine at position 192 and an alanine in place of an arginine at position 196.

b) The entire open reading frame of LXRα was amplified from the human liver cDNA library plasmid by the polymerase chain reaction (PCR) with oligonucleotides AB6291 and AB6292 an was cloned into the BglII sites of pSPUTK. This construct was then digested with ApaI and BglII and replaced with the ApaI/BglII fragment from pSPUTK-RIP1 in order to obtain the full length clone with minimal PCR product. The final pSPUTK-LXRα was sequenced.

pSPUTK-RIPΔ1: is an amino terminal truncated clone of LXRα (aa 24-447) and thus lacks the initial 23 amino acids. This plasmid was constructed *via* the PCR technique which employed the oligonucleotides AB6554 and AB6557 in order to amplify bp 70-316 of LXRα from pSPUTK-LXRα. This PCR product was then cleaved with KpnI and StuI and was used to replace the KpnI/StuI fragment removed from pSPUTK-LXRα. The PCR product portion of this clone was sequenced.

pSPUTK-RIPΔ2: is an amino terminal truncated clone of LXRα (aa 44-447) and thus lacks the initial 43 amino acids. This plasmid was constructed *via* the PCR technique which employed the oligonucleotides AB6555 and AB6557 in order to amplify bp 130-316 of LXRα from pSPUTK-LXRα. This PCR product was then cleaved with KpnI and StuI and was used to replace the KpnI/StuI fragment removed from pSPUTK-LXRα. The PCR product portion of this clone was sequenced.

pSPUTK-RIPΔ3: is an amino terminal truncated clone of LXRα (aa 64-447) and thus lacks the initial 63 amino acids. This plasmid was constructed *via* the PCR technique which employed the oligonucleotides AB6556 and AB6557 in order to amplify bp 190-316 of LXRα from pSPUTK-LXRα. This PCR product was then cleaved with KpnI and StuI and was used to replace the KpnI/StuI fragment removed from pSPUTK-LXRα. The PCR product portion of this clone was sequenced.

pRc/CMV-RIPΔ2: is an amino terminal truncated clone of LXRα (aa 44-447) and thus lacks the initial 43 amino acids. This plasmid was constructed by digesting pRc/CMV-RIP1 with HindIII/EcoRI and isolated a 1.4 kb fragment (C-terminus of LXRα and part

of the pRc/CMV) and the large remaining fragment (fragment 1). Subsequently, pSPUTK-RIPΔ2 was digested with HindIII and EcoRI and the 1kb piece was isolated. The fragment 1 (from pRc/CMV-RIP1) was ligated to the 1kb HindIII/EcoRI piece isolated from pSPUTK-RIPΔ2. The resulting clone was then digested with EcoRI and the 1.4kb piece from pRc/CMV was cloned into the resulting site.

pRc/CMV-RIPΔ3: is an amino terminal truncated clone of LXRα (aa 64-447) and thus lacks the initial 63 amino acids. This plasmid was constructed by digesting pRc/CMV-RIP1 with HindIII/EcoRI and isolated a 1.4 kb fragment (C-terminus of LXRα and part of the pRc/CMV) and the large remaining fragment (fragment 1). Subsequently, pSPUTK-RIPΔ2 was digested with HindIII and EcoRI and the 1kb piece was isolated. The fragment 1 (from pRc/CMV-RIP1) was ligated to the 1kb HindIII/EcoRI piece isolated from pSPUTK-RIPΔ2. The resulting clone was then digested with EcoRI and the 1.4kb piece from pRc/CMV was cloned into the resulting site.

pSG5-LXRα: contains full length LXRα and was constructed *via* the PCR technique with oligonucleotides AB6291 and AB6292 from pSPUTK-LXRα. This PCR product was digested with BamHI and inserted into the BamHI site in pSG5. The resulting clone was then digested with KpnI and EcoRV and replaced with the KpnI/EcoRV LXRα fragment from pSPUTK-LXRα so that no PCR product remained. LXRα, in this vector, is in a backward orientation (and can not be transcribed/translated from the T7 promoter) and was therefore employed solely as a cloning intermediate.

pGEX-2T-LXRα (GST-LXRα): contains full length LXRα fused, in frame, to the GST gene. This plasmid was constructed by digesting pSG5-LXRα with BamHI and inserting the LXRα BamHI fragment into the BamHI site of pGEX-2T.

pGEX-2T-LXRα-ΔAF-2 (GST-LXRαΔAF-2): contains a carboxyl terminal truncated clone of LXRα (aa 1-437) and thus lacks the reported AF-2 domain of LXRα (Willy et al., 1995). This plasmid was constructed *via* the PCR technique with oligonucleotides AB6291 and AB14336 from pSPUTK-LXRα. The PCR product was digested with BamHI and cloned into the BamHI site of pGEX-2T. The resulting clone was digested with KpnI and NdeI and replaced with the KpnI/NdeI fragment isolated from pGEX-2T-LXRα in order to obtain a clone with minimal PCR product. The remaining PCR product of this clone was sequenced (NdeI site to the end).

pGEX-2T-RXRα (GST-RXRα): contains full length human RXRα fused, in frame, to the GST gene. This plasmid was constructed by digesting pGEX-2T with EcoRI and was subsequently blunted using the Klenow fragment of DNA polymerase. RXRα was isolated from pGEM7Zf(+)-RXRα using BglII and the resulting ends were also blunted with Klenow. The RXRα blunt fragment was then cloned into the blunted pGEX-2T vector.

pDR4(X2)luc: contains two copies of the synthetic DR4 direct repeat element, in an everted configuration, in the enhancerless SV40 promoter/luciferase expression vector pGL2. The plasmid was constructed by digesting the double-stranded oligonucleotides

(AB4589 and AB4590) with BamHI and subsequently cloned the fragment into the BglII site of pGL2 (Miyata *et al.*, 1996) (←→).

2.2 Methods

2.2.1 Purification of Plasmid DNA

2.2.1.1 Small Scale Plasmid DNA Purification

The method for small scale (miniprep) preparation of plasmid DNA employed in this study is a lysis by boiling method (Sambrook *et al.*, 1989). Plasmid DNA was extracted from 1.5 ml of pelleted saturated bacterial culture, grown overnight at 37°C in 5 ml of 2YT supplemented with either ampicillin (final concentration 100 μg/ml) or kanamycin (final concentration 30 μg/ml). The resulting pellet was resuspended in 350 μl of STET buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8.0), 1 mM EDTA pH 8.0) and 5% Triton X-100) and 25 μl of fresh lysozyme (10 mg/ml in 10 mM Tris-HCl pH 8.0). The sample was subsequently incubated in a boiling water bath for 40 secs followed by a 10 min centrifugation in order to pellet the cell debris. The resulting supernatant was collected in a fresh tube and 200 μl of NH_4Ac and 700 μl of isopropanol was added. The sample was then frozen in liquid nitrogen for 5 mins and centrifuged at 14,000 rpm at 4°C for 20 mins. The resulting DNA pellet was washed with 70% ethanol and the DNA was ultimately resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) containing RNAse A (100 μg/ml).

2.2.1.2 Large Scale Plasmid DNA Purification (Qiagen method)

The method for large scale (maxiprep) preparation of plasmid DNA, using Qiagen prepared DNA purification columns (composed of a modified anionic silica-gel resin that

binds dsDNA and excludes such contaminants as RNA and cell debris), is based on the alkaline lysis procedure. Each plasmid was also subjected to chloramphenicol amplification. Thus, for each plasmid preparation, a 50 ml flask of 2YT media (containing either ampicillin (100 μg/ml) or kanamycin (30 μg/ml)) was inoculated with a single colony and was grown overnight in a shaking incubator at 37°C. After 16 hrs, 25 ml of the overnight culture was added to 500 ml of 2YT media (containing either ampicillin (100 μg/ml) or kanamycin (30 μg/ml) and was grown to an OD600 of 0.6. Chloramphenicol was subsequently added to the culture at a final concentration of 34 mg/ml and the culture was grown for another 16 hrs in a shaking incubator at 37°C. This overnight culture was then harvested the next day and was applied to the Qiagen column. Preparation of the cellular extract, application to the column, subsequent washes, elution and DNA precipitation were performed as recommended by the manufacturer. The resulting DNA pellet was resuspended in sterile TE (pH 8.0).

2.2.1.3 Large Scale Plasmid DNA Purification (CsCl method)

The method for large scale (maxiprep) preparation of plasmid DNA, using a CsCl gradient method, is based on the alkaline lysis procedure. Cultures were grown as previously described in Section 2.2.1.2 with chloramphenicol amplification. The resulting culture was ultimately harvested the by centrifugation at 5,000 rpm for 15 mins at 4°C and the resulting pellet was resuspended in 21 ml of cold Solution I (50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 μg/ml RNAse A, 50 mM glucose) and incubated at room temperature for 10 mins. Subsequently, 44 ml of Solution II (200 mM NaOH, 1% SDS) was added and the mixture was gently mixed and incubated on ice for

10 mins. Lastly, 22 ml of cold Solution III (3.0M KAc (pH 5.5)) was added and the mixture was incubated on ice for 10 more mins. The cell debris was pelleted at 4°C at 16,000 rpm for 20 mins. The supernatant was filtered through sterile gauze into a sterile graduated cylinder and 0.6X the volume of isopropanol was added. Once transferred to a fresh bottle, the mixture was incubated at 4°C for 1 hr and subsequently spun at 4°C for 30 mins at 16,000rpm. The resulting pellet was washed with cold 70% ethanol and was resuspended in 9.5ml of sterile, deionized water. To this solution, 10 g of CsCl (molecular biology grade) was added and mixed well which was followed by an addition of 1ml of ethidium bromide (10 mg/ml). In order to clear the solution, the mixture was spun at 5, 000 rpm at room temperature for 5 mins. The supernatant was then transferred to a 13 ml ultracentrifuge tube, balanced with CsCl (1 g/ml) and heat sealed. The tubes were then loaded into the ultracentrifuge rotor and spun at 55K for 16 hrs at 20°C. After the completion of the spin, the lower dark red band (supercoiled DNA) was removed with a sterile hypodermic needle and put in a sterile disposable 50 ml Falcon tube. An equal volume of water saturated butanol was then added, mixed and the upper pink layer was discarded in order to remove all traces of the ethidium bromide. This step was repeated until the solution is completely clear. The resulting solution was then precipitated with 6X TE (pH 8.0) and 3X volume of ethanol and incubated at -20°C for 30 mins. The DNA was then pelleted at 13,000 rpm at 4°C for 30 mins. The resulting DNA pellet was resuspended in TE (pH 8.0) and the DNA was quantitated by fluorometry.

2.2.2 DNA Quantification By Fluorometry

Plasmid DNA quantitation was determined by fluorometry using a method outlined by Hoefer and Hoefer Mini-Fluorometer (TKO 100). All DNA quantitations were measured relative to the Calf thymus DNA standard (1 μg/μl). Plasmid DNA was diluted into 2 ml of 1X TNE buffer (1 mg/ml Hoescht 33258 dye, 0.2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4)).

2.2.3 Transformation of Plasmid DNA into Bacterial Cells

2.2.3.1 Preparation of Chemically Competent Bacterial Cells

DH5α cells were made competent *via* a modified RbCl method outlined by Ausubel *et al.*, 1997. A single colony, obtained from a freshly streaked plate of DH5α bacterial cells, was used to inoculate 50 ml of 2YT media (no addition of antibiotic) and was grown for 16 hrs in a 37°C shaking incubator. This primary culture of noncompetent cells was then used to inoculate 250 ml of 2YT and was grown at 37°C to an OD600 of approximately 0.45. The cells were then chilled on ice for 10 mins and spun at 4°C for 20 mins at 3,000 rpm. The pellet was gently resuspended in 20 ml of ice cold RF1 solution (100 mM RbCl, 50 mM $MnCl_2 \cdot 4H_2O$, 30 mM KOAc, 10 mM $CaCl_2 \cdot 2H_2O$, 15% glycerol; adjusted to pH 5.8 with acetic acid; filter sterilized) and incubated on ice for 1hr. The cells were then pelleted by centrifugation at 4°C for 20 mins at 3,000 rpm and then resuspended in 8 ml of ice cold RF2 solution (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2 \cdot 2H_2O$, 15% glycerol; adjusted to pH 6.8 with NaOH; filter sterilized). The cells were aliquoted into pre-chilled sterile eppendorf tubes and were stored at -70°C.

2.2.3.2 Transformation of Bacterial Cells By Heat Shock

Approximately 50 µl of competent DH5α cells were incubated on ice with 5-10 ng of plasmid DNA for 30-40 mins. The resulting mixture was then heat shocked for 90 secs in a 42°C water bath. Subsequently, the cells were placed on ice and were incubated for an additional 5 mins. The cells were incubated at 37°C in 1ml of 2YT for 1 hr and then plated on a 2YT agar plate (with antibiotics) and incubated at 37°C overnight.

2.2.4 Sequencing

DNA sequencing was performed using a ^{T7}Sequencing^{TM} Kit obtained from Pharmacia Biotech. The template DNA was denatured by mixing 16 µl of plasmid DNA (5 µg) with 1 µl of 4 M NaOH and 1 µl of 4 mM EDTA (pH 8.0) followed by incubation at room temperature for 5 mins. Then 2 µl of 2 M NH_4Ac and 60 µl of ice cold ethanol was added and the solution was flash frozen in liquid nitrogen. The solution was spun at 4°C for 30mins at 14,000rpm and the resulting pellet was washed with 70% ethanol and resuspended in 20 µl of sterile water. Annealing of the primer to the denatured template was achieved by incubating 10 µl of the DNA (2µg) with 2 µl of primer (2 ρmol) and 2 µl of annealing buffer. The resulting mixture was then incubated at 60°C for 10 mins and then incubated at room temperature for an additional 10 mins. Following this step, 3 µl of labeling mix, 1 µl of [α-^{32}P]dATP and 2 µl of diluted T7 DNA polymerase, was added to the mixture and incubated for 5 mins at room temperature. The termination reaction was achieved by adding 4.5 µl of the mixture to four pre-warmed eppendorf tubes each containing 2.5 µl of one of the four nucleotide mixes and incubating at 37°C for 5 mins.

Following this reaction, 5 µl of the stop solution (97.5% deionized formamide, 10 mM EDTA (pH 7.5), 0.3% bromophenol blue and xylene cyanol FF) was added to each tube. Each of these sample tubes were then heated at 80°C for 2 mins and then were loaded onto an 8% acrylamide/7.0 M urea sequencing gel pre-warmed to 50°C. The gel was run at a constant 80 watts for approximately 2.5-3 hrs per run, dried and exposed to Kodak X-ray film.

2.2.5 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was employed in several cloning strategies (as described in Section 2.1.7.3) and was performed as outlined by Ausubel *et al.*, 1997. The PCR reaction was comprised of 10 µl of 10X Thermophilic Buffer, containing 2mM $MgSO_4$ (New England Biolabs), 0-6 µl of 100 mM $MgSO_4$, resulting in a final concentration of 2 to 8 mM (New England Biolabs), 10 µl of each 4 mM dNTP, resulting in a final concentration of 1 mM each, 50-100 ng of DNA (x µl), 1 µl of each primer (1 µM/100 ρmol), 1 µl of Vent^{TM} DNA Polymerase (2,000 U/ml) and topped up with water to give a total reaction volume of 100 µl. The initial PCR reaction cycle was performed in the absence of the Vent DNA Polymerase and was added just prior to the initial extension step so as to optimize the initial annealing of the primer to the template DNA. Thus, the first cycle began with a 5 mins denaturing step at 95°C, a 1-2 mins annealing step at 55°C-70°C depending upon the primers employed (where the annealing temperature was calculated with the formula Tm= 81.5+16.6(log 0.0334)+41x(GC content/total AGCT content)-(500/total AGCT content)), and a 1-2 mins extension step at

72°C (the extension time varies with the length of the PCR product where 1 Kb/min is the general rule). The subsequent 29 cycles were performed for 1min at 95°C, 1-2 mins at the annealing temperature and 1-2 mins at 72°C. Upon completion of the PCR reaction, the samples were kept at 4°C and were subsequently analyzed *via* a 1% agarose or 4% NuSieve gel.

2.2.6 *In Vitro* Transcription and Translation

In vitro transcription and translation reactions were carried out using either the Promega uncoupled system (T7, SP6 or T3) or the Promega T3 TNT® coupled system. The Promega uncoupled system begins with the transcription reaction which was comprised of 5 µg of plasmid DNA (1 µg/µl) (which contains a T7, SP6 or T3 promoter), 10 µl of 5X Transcription buffer, 5 µl of 100 mM DTT, 1.5 µl Rnasin ribonuclease inhibitor (40 U/µl), 2.5 µl of 10 mM ATP, CTP and UTP, 2.5 µl of 1 mM GTP, 5 µl of 5 mM m^7G (5')ppp(5')G, 3 µl of T7, SP6 or T3 RNA polymerase (15 U/µl) and 10.5 µl of sterile nuclease free water. This reaction mixture was subsequently incubated for 2 hrs in a 37°C water bath. This reaction was then subjected to two PCI extractions (25:24:1 ratio of phenol:chloroform:isoamyl alcohol) followed by three chloroform reactions in order to extract the RNA. The RNA was pelleted *via* addition of 600 µl of ethanol, flash freezing in liquid nitrogen, and centrifugation at 14,000 rpm for 30 mins at 4°C. The resulting RNA pellet was washed repeatedly with 70% ethanol and was resuspended in 25 µl of sterile water and 1 µl of Rnasin ribonuclease inhibitor. Lastly, the RNA solution was stored in 2 µl aliquots and stored at -70°C.

The Promega uncoupled system translation reactions were performed with the previously prepared RNA (2 µl/50 µl translation reaction). The translation reaction was comprised of 2 µl of RNA, 35 µl of rabbit reticulocyte lysate (nuclease treated), 1 µl Rnasin ribonuclease inhibitor (40U/µl), 1 µl of 1 mM amino acid mixture (either in the absence or presence of methionine), 4 µl of L-[^{35}S]-methionine (10 mCi/ml)(for radioactive translates only) and 7 µl of sterile water. This mixture was then incubated for 1 hr in 30°C water bath and ultimately stored at -70°C. Radioactive translates were tested on a 10% SDS-PAGE gel which was dried and exposed to Kodak X-ray film.

The Promega T3 TNT® coupled system permits the transcription and translation reactions to occur in the same reaction mixture. The coupled reaction mixture was comprised of 25 µl of TNT® rabbit reticulocyte lysate, 2 µl of TNT® Reaction buffer, 1 µl of TNT® T3 RNA polymerase, 1 µl of amino acid mixture (minus methionine for radioactive reactions), 2 µl of L-[^{35}S]-methionine (10 mCi/ml)(for radioactive reactions only), 1 µl Rnasin ribonuclease inhibitor (40 U/µl), 1-2 µg of plasmid DNA (1 µg/µl) and topped up with sterile, nuclease free water to a final volume of 50 µl. The mixture was then incubated for 1 5-2 hrs in a 30°C water bath and ultimately stored at -70°C. The radioactive translates were tested on a 10% SDS-PAGE gel which was dried and exposed to Kodak X-ray film.

2.2.7 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed using a modified technique outlined by Fried and Crothers, 1981. The EMSA technique was employed in

this study in order to determine the binding of proteins to various DNA binding sequences and occurs as a two step process; first the DNA radioactive probe is prepared and the second step is the preparation and running of the EMSA (bandshift) reaction on a 4% nondenaturing acrylamide gel. The preparation of the radioactive DNA probe begins with the annealing of the two oligonucleotides in order to obtain dsDNA. This was achieved by heating the two oligos at 75°C for 10 mins and then slowly cooled (approximately 1°C/min) to room temperature. The annealed DNA was then run and purified from a 4% NuSieve gel (containing 0.5 μg/ml of ethidium bromide) using NA45 paper. The DNA was run onto the NA45 paper and was eluted by heating the paper for 30 mins at 65°C in DEAE elution buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl). The paper was subsequently removed and the solution was spun at 14,000 rpm for 5 mins in order to remove any agarose gel debris. The resulting supernatant was then isolated and mixed with 10 μl of $MgCl_2$ and 1 ml of ethanol and centrifuged at 14,000 rpm at 4°C for 30 mins. The DNA pellet was washed with 70% ethanol and resuspended in sterile water (resulting in approximately 20 pmol/μl). The DNA probe was then radioactively labeled by mixing 1 μl of the previously prepared DNA solution, 2.5 μl of 10X Klenow buffer, 10 μl [α-^{32}P]dATP, 1 μl of 5 mM dCTP, dTTP and dGTP, 8.5 μl of sterile water and 1 μl of Klenow for 20 mins at room temperature. The reaction was terminated by the addition of 0.5 μl of 0.5 M EDTA and 75 μl was added to the mixture. The probe was then purified by passing the DNA

solution over two Sephadex G-50 columns and 1 µl of the resulting probe was measured using a scintillation counter.

The second step of the EMSA assay involves the preparation of the EMSA reaction followed by gel analysis. The EMSA binding reaction is composed of *in vitro* translated non-radioactive proteins, 1 µl of polydIdC (4 µg/µl), 1 µl of bovine serum albumin (BSA; 4 µg/µl), 1 µl of salmon sperm DNA (4 µg/µl), 5 µl of Buffer C (20 mM HEPES-KOH, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 25% glycerol). This reaction was allowed to incubate at room temperature for 5 mins and at which time 20 pmol of the previously prepared DNA probe was added, with x µl of sterile water for a total reaction volume of 15 µl. This final reaction mixture was incubated for 30 mins in a 30 °C water bath. Subsequent to the incubation period, 1 µl of EMSA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in sterile water) was added and mixed well. The EMSA reaction was then loaded on a 4% non-denaturing gel and run at a constant 200 volts at 4°C for 2-3hrs. The EMSA gel was dried and exposed to Kodak X-ray film.

2.2.8 Overexpression and Preparation of GST Fusion Proteins

The GST fusion proteins were overexpressed and the cell extracts were prepared for subsequent use in solid phase capture assays (Section 2.2.9) using a modified method outlined by Kaelin *et al.*, 1991. For each of the GST fusion proteins a single colony, isolated from a freshly streaked plate of DH5α bacterial cells harboring the GST proteins, was used to inoculate 50 ml of 2YT in the presence of ampicillin (100 µg/ml). This culture was grown to saturation overnight in a 37°C incubator. The following day, the 50

ml overnight culture was used to inoculate 500 ml of 2YT (2% glucose, ampicillin (100 μg/ml)) which was grown for 3-5 hrs at 30 °C until an OD600 of 0.7-0.8 was achieved. At this point, IPTG (0.1 mM final concentration) was added and the culture was grown for an additional 1.5-3 hrs at 30°C. In order to test the induction of the protein, samples of the culture were collected throughout the induction period, pelleted, resuspended in 50 μl of 2X SDS-PAGE loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled and finally loaded and run on a 10% SDS-PAGE gel. The proteins were visualized using staining solution (2.5 μg Coomassie Brilliant Blue R-250, 450 ml methanol, 100 ml acetic acid, 450 ml sterile water) which was soaked with the gel for 20-30 mins followed by an incubation period with destaining solution (5% methanol, 5% acetic acid) overnight. The gel was then dried and analyzed.

After the incubation period was complete, the 500 ml culture was chilled on ice for 15 mins and was then spun at 4°C at 6,000 rpm for 20 mins. The resulting pellet was washed twice with PBS buffer (260 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4) and repelleted. The cell extracts were then prepared by resuspended the pellet in 30 ml of cold NETN buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, .5% NP-40 and one tablet of Protease inhibitor cocktail (Boehringer Mannheim). The solution was then divided into 5 ml aliquots and each aliquot were lysed using a probe sonicator for 8, 30 sec bursts with a 30 sec incubation on ice between intervals. The aliquots were then pooled and underwent centrifugation at 10,000 rpm at 4°C for 10 mins. The resulting cell extract supernatant was then divided into 1 or 5 ml aliquots and stored at -70°C until employed in solid phase capture assays.

2.2.9 GST-binding Assay

GST-binding assays were performed using a modified protocol outlined by Kaelin *et al.*, 1991, Cavaillès *et al.*, 1995 and L'Horset *et al.*, 1996. The Glutathione Sepharose 4 B beads were prepared as outlined by Pharmacia Biotech into a 50% slurry. The GST fusion protein whole-cell extract was added to the beads (1-2 ml of extract/10 µl of 50 % slurry) so as to ensure saturation of the beads and the mixture was incubated for 1-2 hrs at 4°C with end-over-end mixing. Once the incubation was complete, the beads were washed twice with NETN buffer and twice with PBS. Subsequent to the wash steps, the beads (with attached GST fusion protein) were incubated with 2-20 µl of *in vitro* translated L-[^{35}S]-methionine labeled protein, 200 µl of IPAB buffer (150 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, 0.1% Triton X-100, 0.2% NP-40; adjusted to pH 7.9 with KOH; BSA (0.02 mg/ml final concentration);10 µl of 7X Protease inhibitor cocktail) and topped up to 250 µl with IPAB buffer (in the absence of BSA and Protease inhibitor cocktail). This mixture was incubated at 4°C for 1-2 hrs in the presence of vehicle, drug or in the absence of both. After the incubation period was complete, the beads were washed five times with IPAB buffer. The beads were pelleted at 2,000 rpm and all of the supernatant was removed. Subsequently, 50 µl of 2X SDS-PAGE loading buffer was added to the beads and boiled for 5 mins. Approximately 10-15 µl of the binding assay reaction was loaded onto a 10 % SDS-PAGE gel and run at 200 volts at room temperature.

Fluorography was performed on all [^{35}S]-methionine labeled protein gels and thus, after the SDS-PAGE gels of the solid phase capture assays were run, they were incubated

for 30 min this destaining solution. After fixing with destaining solution, the gels were incubated for 2, 1 hr intervals with fresh dimethylsulphoxide (DMSO). The gels were incubated for 2-3 hrs in DMSO containing 22.2% PPO (2,5-diphenyloxazole) and were then re-hydrated in sterile, deionized water for 30 mins. Once dried the gels were exposed to Kodak X-ray film. Quantification of the amount of protein that was retained by the GST-fusion proteins employed was performed with the use of a Molecular Dynamics Phosphoimager.

2.2.10 Transformation of Yeast (Lithium Acetate/PEG method)

Transformation of yeast was performed as outlined by Elble, 1992. A yeast culture was grown overnight at 30 °C to saturation as outlined in Section 2.1.4. Approximately 0.5 ml of the saturated yeast culture underwent centrifugation at 8,000 rpm for 1 min. The resulting pellet was mixed with 100 μg of sonicated salmon testes DNA (Sigma Chemical Laboratories), 1 μg of each transforming DNA plasmid and 0.5 ml of Plate solution (40% polyethylene glycol (PEG) 3350, 0.1 M LiAc, 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA) and incubated overnight at room temperature. Generally, the following day, the cells have settled to the bottom of the tube. Approximately 50 μl of these settled cells are removed from the solution, plated on the appropriate selective agar plate and incubated at 30°C overnight.

2.2.11 Qualitative Agarose Overlay Assay for Detection of β-galactosidase Activity

The agarose overlay assay was performed using a modified protocol outlined in Bohen and Yamamoto, 1993. The assay solution is comprised of a 100 ml solution of 0.5% agarose in 0.5 M $NaPO_4$ (pH 7.0) mixed with 1 ml of 10% SDS and 2.5 ml of 2%

X-Gal solution. The solution (approximately 10 ml) was then poured over the plates of the yeast transformants and incubated at room temperature until the agarose solution had solidified. The plates were then incubated at 37°C and closely observed for any colour changes (appearance of a blue colour).

2.2.12 Quantitative β-galactosidase Liquid Assay

The β-galactosidase liquid assay was performed as outlined by Ausubel *et al.*, 1997. Yeast cultures were grown to saturation overnight at 30°C under selective conditions. The following morning, the overnight yeast cultures were used to inoculate 10 ml of fresh selective yeast media and the cultures were grown until the cells reached mid- or late-log phase and the OD_{600} of each sample was measured. These cells were pelleted and resuspended in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.0 mM Mg_2SO_4, 50 mM β-mercaptoethanol; adjusted to pH 7.0). The cell mixture was then permeabilized with 5 cycles of freezing with liquid nitrogen and thawing in a 30°C water bath. Once the cells have been permeabilized, ONPG (0.2ml of 4 mg/ml) was added to each sample (and to a blank sample containing only the buffer Z with no cells) and the mixture was then well mixed and incubated at 30°C. Once a medium-yellow colour had developed the reaction was stopped with the addition of 0.5 ml of 1 M Na_2CO_3 and was mixed thoroughly. The cell debris was pelleted and the supernatant was isolated for OD measurements at 420, 550 and 600 nm. The units were subsequently calculated using the equation: $U= (1000 \times [(OD_{420})-(1.75 \times OD_{550})])/(\text{time} \times \text{volume} \times OD_{600})$; where time is the length of time in minutes, volume is the volume of culture used in the assay, OD_{600} measures the cell density at the beginning of the assay, OD_{420} measures the absorbance

by *o*-nitrophenol and light scattering by the remaining cell debris and OD_{550} measures the light scattering by the cell debris. All yeast samples were analyzed a minimum of twice in triplicate.

2.2.13 Transient Transfections and Luciferase Assays

2.2.13.1 Transient Transfection Assays

Transient transfections were performed using a modified version of the calcium phosphate co-precipitation protocol outlined by Graham and Van Der Eb, 1973. For this method, 20 μg of total DNA (target plasmid DNA and filler DNA (salmon sperm DNA at 1 mg/ml)), 0.25 M $CaCl_2$ (final concentration; filter sterilized) and sterile, deionized water was combined in a total volume of 500μl and well mixed. This mixture was then added dropwise to an equal volume of 2X HBSS buffer (0.28 M NaCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4 \cdot 7H_2O$; adjusted to pH 7.12 with NaOH; filter sterilized) while gently vortexing. The mixture was then allowed to incubate at room temperature for 20 min. Subsequent to the incubation period, 250 μl of the precipitate solution was added to each well of a 6 well plate, previously seeded the day before with approximately 2.5 x 10^5 cells/well. The precipitate was incubated with the cells at 37°C for 16 hrs. The following day, the cells were washed with twice with PBS, fresh media was added and the cells were allowed to incubate for 24 hrs. After this final incubation period, the cells are washed twice with PBS and harvested with the addition of 300 μl/well of 5X reporter lysis buffer. The cells are then scraped from the plate with a cell lifter and collected in chilled eppendorf tubes. Each sample was then vortexed at high speed for 10 secs and the cell debris was pelleted *via* centrifugation at 4°C for 1 min at 14,000 rpm. The

supernatant samples (lysates) were then collected in chilled eppendorf tubes and were kept on ice until employed in luciferase and protein assays.

2.2.13.2 Luciferase and Protein Assays

The luciferase assay was performed using a modified protocol as outlined by Promega. This method required the use of a Lumat LB 9507 luminometer which performs the actual mixing of the lysate (10-15 µl) to 100 µl of assay buffer (470 µM luciferin, 270 µM coenzyme A, 530 µM ATP, 33.3 mM DTT, 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA) and subsequently measures the relative light units (R.L.U.). All lysate samples were then analyzed for protein content *via* the Bradford Assay from BioRad. For this protocol, 200 µl of Coomassie Brilliant blue G-250 Dye Reagent (BioRad) was mixed with protein samples diluted to 800 µl with water. After a five min incubation period, the absorbance of the samples were measured at 595 nm. The R.L.U. values of the lysate samples were then adjusted to protein content accordingly. Transient transfection graphs were constructed by combining a minimum of two independent experiments performed in duplicate. The standard deviation was calculated for the combined experiments using the formula:

$SD = ((^{m}\Sigma_{s=1}{}^{n}\Sigma_{I=1}(y_{is}-M)^2)/(n_y-1))^{1/2}$, Where s is the series number, i is the point number in series s, m is the number of series for point y in graph, n is the number of data points in each series, y_{is} is the data value of series s and the ith point, n_y is the total number of data values in all series and M is the arithmetic mean.

CHAPTER THREE

RESULTS

3.1 Transient Transfection Studies of LXRα

3.1.1 Transactivation Properties of LXRα and LXRα Response Element Investigations

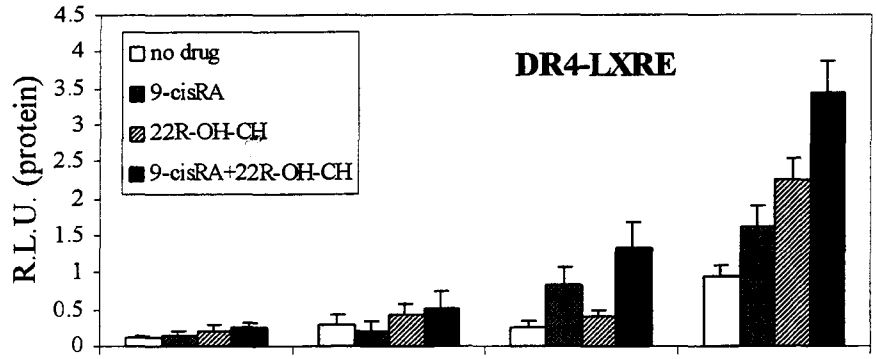
The transactivation properties exhibited by nuclear receptors have been shown to differ depending on the various DNA response element employed in these studies, indicating the specificity with which nuclear receptors regulate the transcription of target genes (Nagpal *et al.*, 1992, Kurokawa *et al.*, 1995, Sjöberg and Vennström, 1995, Mangelsdorf and Evans, 1995, Willy and Mangelsdorf, 1997, Kalkoven *et al.*, 1998). Thus, LXRα response element investigations, which employed the DR4-LXRE and LXRE-ΔMTV elements, were of particular interest. While these DNA elements share some similarities, as both are DR4s, it is the differences, which include spacer sequences, flanking sequences and the hexad sequences (identical in the case of DR4-LXRE and degenerate in the LXRE-ΔMTV element) that may influence the transactivation properties of LXRα (Apfel *et al.*, 1994, Willy and Mangelsdorf, 1997). These investigations were further facilitated by the identification of various LXRα activators including 22(R)-hydroxycholesterol. Therefore, initial transient transfections were performed in the BSC40 cell line, in the presence of the pDR4(X2)luc enhancerless SV40 promoter/luciferase reporter plasmid, which contained two copies of the DR4-LXRE in an everted configuration, alone or in combination with mammalian expression vectors containing LXRα and RXRα. The effects of exogenously added 9-*cis* RA (1μM), a

known RXRα ligand, and the potent LXRα activator 22(R)-OH-CH (7μM), alone or together, were also examined (refer to Figure 5A). These experiments also resulted in the establishment of a viable and efficient transient transfection assay for further LXRα investigations *in vivo*.

In analyzing the data it is important to note that many mammalian cell lines, including the BSC40, HepG2 and COS-1 cell lines, harbor endogenous nuclear receptors which can interact with LXRα, RXRα, and the DR4 response element. This is clearly demonstrated upon the addition of the drugs 9-*cis* RA and 22(R)-OH-CH, alone or in combination, which results in a slight enhancing effect on reporter activity. Nonetheless, co-transfection of LXRα and RXRα resulted in a 10-fold non-ligand dependent induction. This constitutive activity may be attributable to the presence of a functional AF-1 domain within the A/B domain of LXRα. Alternatively, such activity may be due to the presence of endogenous LXRα activators/ligands as reported for other cell lines (Forman *et al.*, 1997). These concepts are investigated in Section 3.2 and further examined in the discussion.

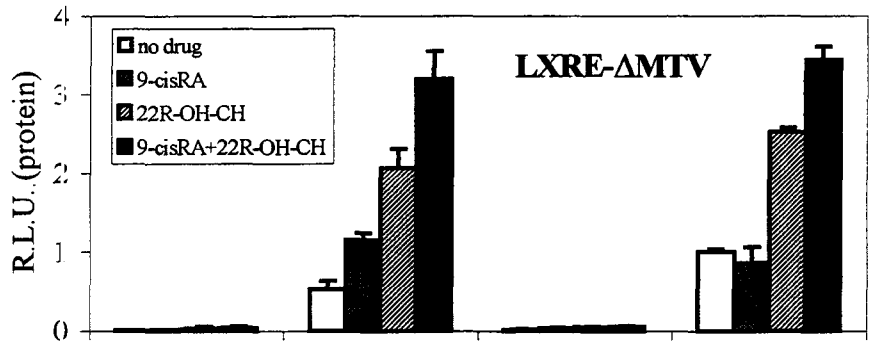
In summary, the LXRα/RXRα heterodimer displays a 10-fold non-ligand dependent induction, a 1.5-fold induction with the addition of 9-*cis* RA, an almost 2.5-fold induction with the addition of 22(R)-OH-CH and a 3.5-fold induction with the addition of both 9-*cis* RA and 22(R)-OH-CH. Thus, while an additive effect for 9-*cis* RA and 22(R)-OH-CH was observed for the DR4 response element the synergism reported for the TK-LXRE3-Luc reporter construct (Janowski *et al.*, 1996) was not observed in this DR4/BSC40 transient transfection system.

(A) BSC40 cell line



LXRα	-	+	-	+
RXRα	-	-	+	+

(B) BSC40 cell line



LXRα	-	+	-	+
RXRα	-	-	+	+

Figure 5: Transient transfection assays demonstrating the transactivation properties of LXRα and the variant LXRE construct dependent responses in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with either (A) pDR4(X2) luc (1.25 μg) or (B) TK-LXRE3-Luc (1.25 μg) luciferase reporter plasmids alone or in combination with expression vectors for LXRα or RXRα (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and/or the RXRα ligand 9-*cis* RA (final concentration 1 μM) were added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of (A) five or (B) three separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

While studies with the DR4-LXRE element were underway, Willy and colleagues (1995) identified the LXRE-ΔMTV element and was thus incorporated in these investigations. These transient transfection assays were performed in BSC40 cells in the presence of the TK-LXRE3-Luc thymidine kinase promoter/luciferase reporter plasmid, which contained three copies of the LXRE-ΔMTV response element in a tandem repeat configuration, alone or in combination with mammalian expression vectors containing LXRα and RXRα. As with the DR4/BSC40 system, the effects of exogenously added 9-*cis* RA (1μM) and 22(R)-OH-CH (7μM), alone or in combination, were investigated (Figure 5B).

A direct comparison between the DR4-LXRE and LXRE-ΔMTV elements is not possible as they are under the control of two different promoters, the number of DNA response element copies is varied and the configurations of the LXREs within the two clones is also different. Nonetheless, the results of these transfection assays, as presented in Figure 5B, clearly demonstrate the effectual differences between the DR4 and the LXRE-ΔMTV response elements *in vivo*. While endogenous nuclear receptors are present, they do not appear functional with the LXRE-ΔMTV element (Willy *et al.*, 1995). Thus, the effect on the LXRE-ΔMTV element alone and in the presence of exogenous RXRα with the addition of 9-*cis* RA and/or 22(R)-OH-CH result in an insignificant increase (presumably due to endogenous LXRα and RXRα). The most dramatic difference is the effect observed for exogenously transfected LXRα, attributable to the presence of endogenous RXRα, which may heterodimerize with the exogenous

LXRα and preferentially bind to the LXRE-ΔMTV response element over the DR4 *in vivo*, although no difference in binding affinity has been observed in *in vitro* studies (as demonstrated in Figures 9A and 9B). This activity is not significantly increased with the addition of exogenous RXRα with one exception; in the absence of drug where the co-transfection of LXRα and RXRα results in the doubling of the activation potential. The observation that high levels of LXRα-mediated activity can be achieved, in the absence of exogenously added RXRα has previously been reported (Willy *et al.*, 1995, Willy and Mangelsdorf, 1997, Janowski *et al.*, 1996).

Ultimately, the activation levels mediated by LXRα/endogenous RXRα and LXRα/exogenous RXRα heterodimers on the LXRE-ΔMTV are quite similar to those found for DR4; 1.5-fold induction with 9-*cis* RA, 2-2.5-fold induction with the addition of 22(R)-OH-CH and 3-3.5-fold induction with the addition of both drugs. The lack of synergism, upon the addition of the 9-*cis* RA and 22(R)-OH-CH together is consistent with results reported for the DR4 response element in the BSC40 cell line.

3.1.2 Transactivation Properties of LXRα in Various Cell Lines

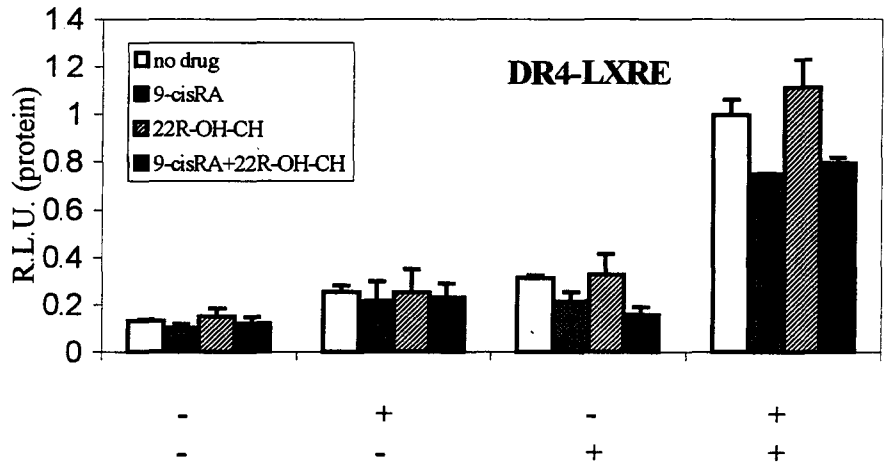
A number of reports, which studied the transactivation properties of various nuclear receptors, have illustrated that the activity of these receptors may exhibit cell line specificity (Berry *et al.*, 1990, Metzger *et al.*, 1995, McInerney *et al.*, 1996 , Sjöberg and Vennström, 1995). This specificity may be attributed to differences between the cell lines including the presence of endogenous receptors, co-factors and ligands as well as the presence of various signal transduction pathways, which may modify these receptors *via* phosphorylation or dephosphorylation, thereby modulating the activity of these

transcription factors. Thus the study of the transactivation properties of LXRα in various cell lines was achieved *via* transient transfection assays performed, as described in Section 3.1.1, with either the pDR4(X2)luc or TK-LXRE3-Luc reporter constructs, in both the HepG2 and COS-1 cell lines. The effects of exogenously added 9-*cis* RA (1μM) and 22(R)-OH-CH (7μM), alone or in combination, were examined.

Transient transfections, performed in the HepG2 cell line, with the pDR4(X2)luc reporter plasmid (Figure 6A), clearly demonstrate LXRα/RXRα-mediated constitutive activity, in the absence of drug, as observed for the BSC40 cell line. However, in contrast to the observations reported for the DR4/BSC40 transfection system, addition of 22(R)-OH-CH had no significant effect on activation potentials. Surprisingly, a slight inhibitory effect on LXRα/RXRα-mediated activity was observed upon addition of either 9-*cis* RA alone, or in combination with 22(R)-OH-CH. Similar results were achieved for transient transfections with the DR4 reporter construct in the COS-1 cell line (Figure 7A).

Subsequently, transfection assays were performed with the LXRE-ΔMTV reporter construct in the HepG2 cell line. As demonstrated in Figure 6B, LXRα/RXRα-mediated constitutive activity was observed as reported for the BSC40 cell line. Similarly, the addition of exogenous RXRα resulted in an increase reporter gene activity both in the absence of drug and the presence of 22(R)-OH-CH, although no such increase was observed in the presence of 9-*cis* RA and 9-*cis* RA/22(R)-OH-CH. However, the addition of 22(R)-OH-CH had no effect on activation levels, as observed for the DR4/HepG2 and DR4/COS-1 studies. Interestingly, in contrast to the results reported for the DR4 element in both COS-1 and HepG2 cells, the LXRE-ΔMTV element is 9-*cis* RA

(A) HepG2 cell line



(B) HepG2 cell line

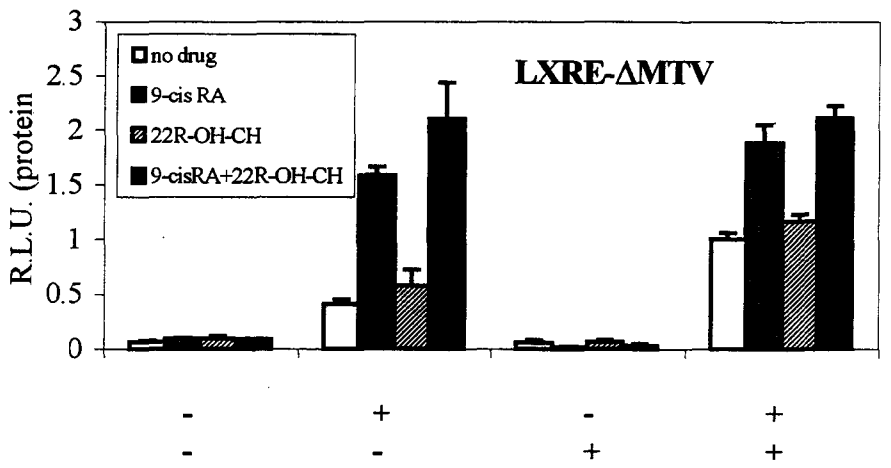
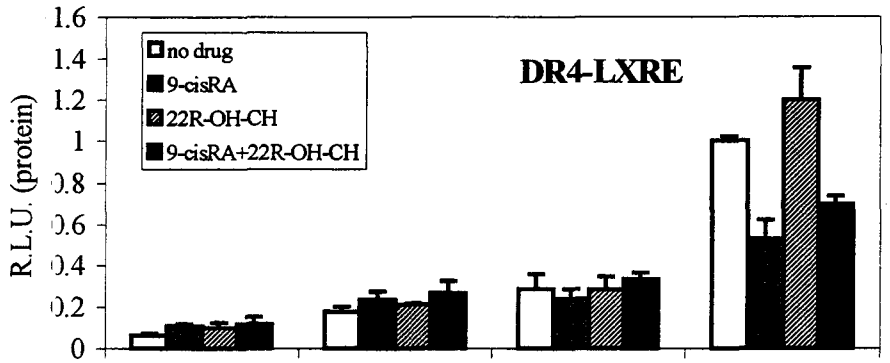


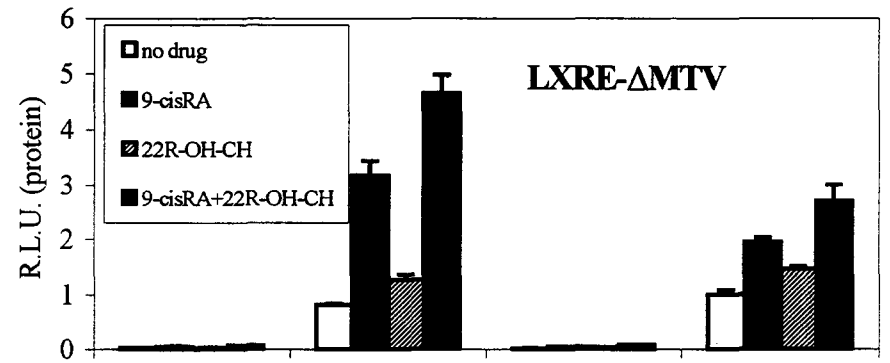
Figure 6: Transient transfection assays demonstrating the transactivation properties of LXRα and the variant LXRE construct dependent responses in the HepG2 cell line. HepG2 cells were transfected, utilizing the calcium phosphate method, with either (A) pDR4(X2) luc (1.25 μg) or (B) TK-LXRE3-Luc (1.25 μg) luciferase reporter plasmids alone or in combination with expression vectors for LXRα or RXRα (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and/or the RXRα ligand 9-*cis* RA (final concentration 1 μM) were added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

(A) COS-1 cell line



LXRα	-	+	-	+
RXRα	-	-	+	+

(B) COS-1 cell line



LXRα	-	+	-	+
RXRα	-	-	+	+

Figure 7: Transient transfection assays demonstrating the transactivation properties of LXRα and the variant LXRE construct dependent responses in the COS-1 cell line. COS-1 cells were transfected, utilizing the calcium phosphate method, with either (A) pDR4(X2) luc (1.25 μg) or (B) TK-LXRE3-Luc (1.25 μg) luciferase reporter plasmids alone or in combination with expression vectors for LXRα or RXRα (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and/or the RXRα ligand 9-*cis* RA (final concentration 1 μM) were added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

responsive. Neither a synergistic or additive effect was observed upon the addition of both 9-*cis* RA and 22(R)-OH-CH.

Some similarities exist between transfections performed in the HepG2 cells and the COS-1 cells for the LXRE-ΔMTV element (Figure 7B). These similarities include the observations that the addition of 22(R)-OH-CH has no effect on reporter gene activity, the LXRE-ΔMTV element is also 9-*cis* RA responsive, and no synergistic or additive effect was apparent upon the addition of both drugs. However, in contrast to those observations reported for the LXRE-ΔMTV, in both the BSC40 cell line (in the absence of drug) and in the HepG2 cell line (in the absence and presence of 22(R)-OH-CH), the presence of exogenously added RXRα had no significant effect (either in the absence or presence of 22(R)-OH-CH). More surprising is the observation that co-transfection of RXRα and LXRα resulted in a significant decrease in reporter gene activity. This effect is in contrast to that described for both the BSC40 and HepG2 cell lines and may be a cell-specific occurrence.

Thus, findings presented here indicate how transactivation properties of LXRα may be DNA response element, promoter context and cell line specific as has been reported in many other nuclear receptors studies (Berry *et al.*, 1990, Metzger *et al.*, 1995, Sjöberg and Vennström, 1995, McInerney *et al.*, 1996, Ikonen *et al.*, 1997). Further discussion of the LXRα/RXRα-mediated constitutive activity and the apparent DNA element and cell-specific differences observed in the absence and presence of exogenous ligand is presented in the discussion. Ultimately, in completing these groundwork studies, the *in*

vivo investigations of the LXRα A/B domain and LXRα/co-factor functional studies were made possible.

3.2 LXRα A/B Domain Deletion Studies

Nuclear hormone receptors, including LXRα, consist of several modular domains termed A/B, C, D, E and F (refer to Figure 3). While the C,D and E regions of nuclear receptors and their isoforms share a high degree of homology, their A/B domains are quite divergent, indicating that the A/B domain may be important in mediating nuclear receptor/isoform-specific transactivation. Indeed, a number of studies have revealed the existence of an autonomous ligand-independent activation domain (AF-1 domain) located within the A/B domain of a variety of nuclear receptors (Nagpal et al., 1993, Wilkinson and Towle, 1997, Werman et al., 1997, Metzger et al., 1995, Sjöberg and Vennström, 1995). Subsequent studies have demonstrated that the AF-1 domain can either function independently or can synergize with the AF-2 domain (LBD) of the same receptor through a functional interaction between these two domains, which is thought to be modulated by the binding of various co-activators (Pierrat et al., 1992, Nagpal et al., 1993, Metzger et al., 1995, Sjöberg and Vennström, 1995, Kraus et al., 1995, Ikonen et al., 1997, Oñate et al., 1998, Webb et al., 1998). Thus, investigation of the A/B domain of LXRα, which utilized a series of LXRα N-terminal A/B domain deletion constructs, became a main focus in the investigation of this novel receptor. These studies investigated the role, if any, the LXRα A/B domain played in heterodimerization and subsequent LXRE binding, transactivation, and co-factor binding (the latter aspect being presented in Sections 3.3.5 and 3.3.6).

3.2.1 Electrophoretic Mobility Shift Assays (EMSAs)

Initial interest in the A/B domain of LXRα was a result of EMSA studies which demonstrated SM1 (an LXRα clone missing the N-terminal 61 amino acids of the A/B domain, which also possessed an N-terminal extension piece different than that of the native LXRα sequence) heterodimeric formation with RXRα on the DR4-LXRE and LXRE-ΔMTV elements was greatly diminished in comparison to LXRα/RXRα/DR4 or LXRE-ΔMTV complex formation (re-demonstrated in Figures 9A and 9B). This observation led to the construction of the LXRα N-terminal deletion constructs (refer to Figure 8): RIP1 (lacking the first 5 aa), RIPΔ1(lacking the first 23 aa), RIPΔ2 (lacking the first 43aa) and RIPΔ3 (lacking the first 63aa, much like SM1 but without the N-terminal extension piece).

Once constructed, the LXRα A/B deletions were employed in EMSA experiments using both DR4-LXRE and LXRE-ΔMTV response elements as radioactively labeled probe (refer to Figures 9A and 9B respectively). Consequently, it was shown that RIP1/RXR, RIPΔ1/RXR, RIPΔ2/RXR and RIPΔ3/RXR complexes all bound to both elements with similar intensities as compared to that observed for the LXRα/RXRα heterodimer. In contrast, the SM1/RXR heterodimer binding intensity was considerably weaker. These results therefore suggest that deletion of up to 63 amino acids at the N-terminus of LXRα does not affect either LXRα/RXRα heterodimer nor subsequent complex formation on the LXRE response elements. Furthermore, alteration of the N-

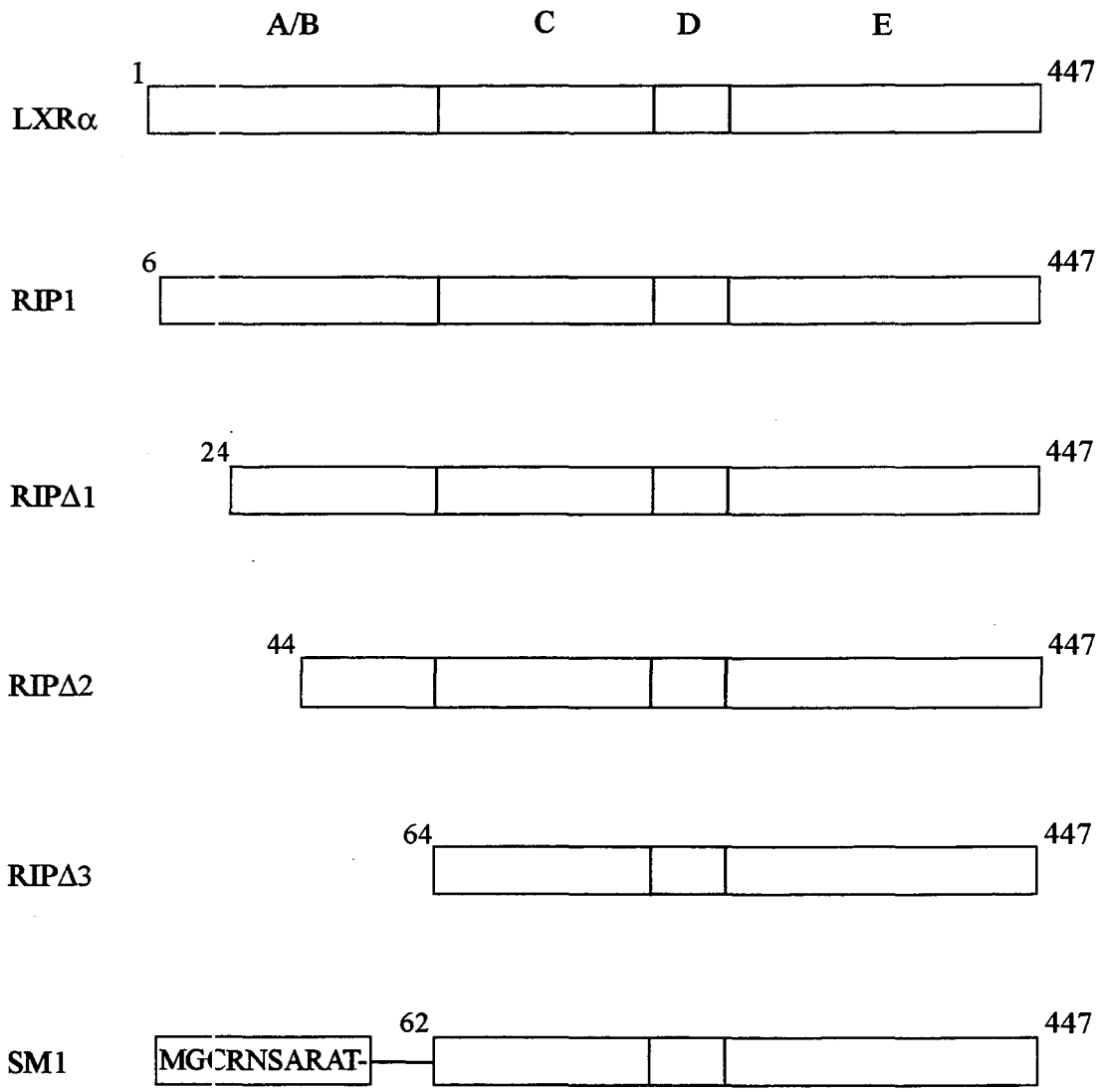
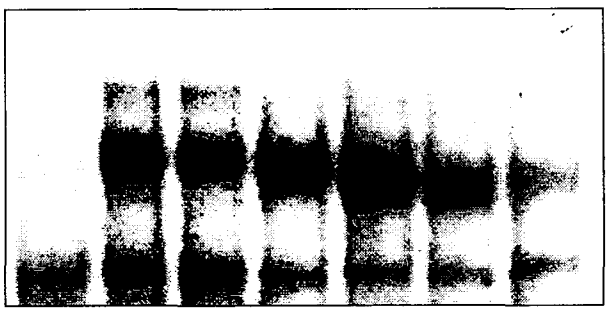
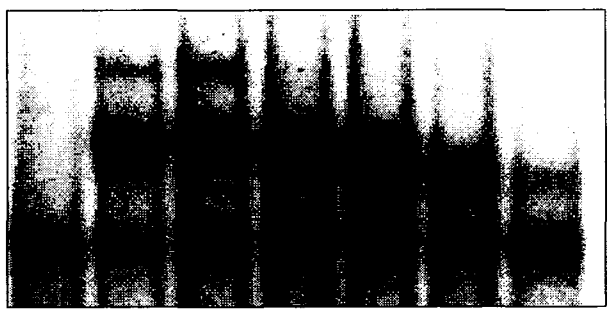


Figure 8: Schematic representation of the LXRα A/B domain deletion constructs. These constructs were ultimately employed in EMSA, transient transfection assay and GST-binding assay studies. The DNA binding domain (DBD) and the ligand binding domain (LBD) are indicated. The sequence of the N-terminal extension piece of SM1 is illustrated.

(A) DR4-LXRE



(B) LXRE-ΔMTV



Retic	+	–	–	–	–	–	–
RXRα	+	+	+	+	+	+	+
LXRα	–	+	–	–	–	–	–
RIP1	–	–	+	–	–	–	–
RIPΔ1	–	–	–	+	–	–	–
RIPΔ2	–	–	–	–	+	–	–
RIPΔ3	–	–	–	–	–	+	–
SM1	–	–	–	–	–	–	+

Figure 9: EMSAs which study the effect of LXRα A/B domain deletions on LXRα/RXRα binding on theDR4-LXRE and LXRE-ΔMTV elements.(A) *In vitro* translated, non-radioactive proteins LXRα, RIP1, RIPΔ1, RIPΔ2, RIPΔ3 or SM1 (2μl each) were individually incubated with RXRα (2μl) and the [α-^{32}P]dATP labeled DR4-LXRE probe or (B) the [α-^{32}P]dATP labeled LXRE-ΔMTV probe. Subsquent to the incubation period, the samples were run on a 4% non-denaturing acrylamide gel.

terminal sequence of LXRα from amino acid positions 53 to 61 greatly diminishes the DNA binding ability of the LXRα/RXRα heterodimer.

Further analysis of the EMSA Figures 9A and 9B, reveals the existence of a second band shift, termed Shift B, whose mobility is slightly slower that that of the LXRα/ RXRα/LXRE complex mobility. This second shift is lost upon deletion of the first 23 amino acids of LXRα but remains upon deletion of the first 5aa within the A/B domain. Titration EMSAs have indicated that secondary complex formation becomes greater as LXRα and RXRα translates are titrated while maintaining a constant amount of rabbit reticulocyte lysate (refer to Figure 10). Furthermore, the formation of the secondary complex appears to be dependent upon the presence of both LXRα and RXRα as no complex is observed under conditions where rabbit reticulocyte lysate is solely employed. Exactly what Shift B is comprised of is not known, however, the possibility exists that this shift is a result of an LXRα/RXRα ternary interaction with another nuclear receptor or co-factor. Therefore, while the biological significance of the complex formation is questionable, these results clearly indicate that formation is specific and is dependent upon not only the presence of LXRα and RXRα but also the presence of an intact LXRα A/B domain.

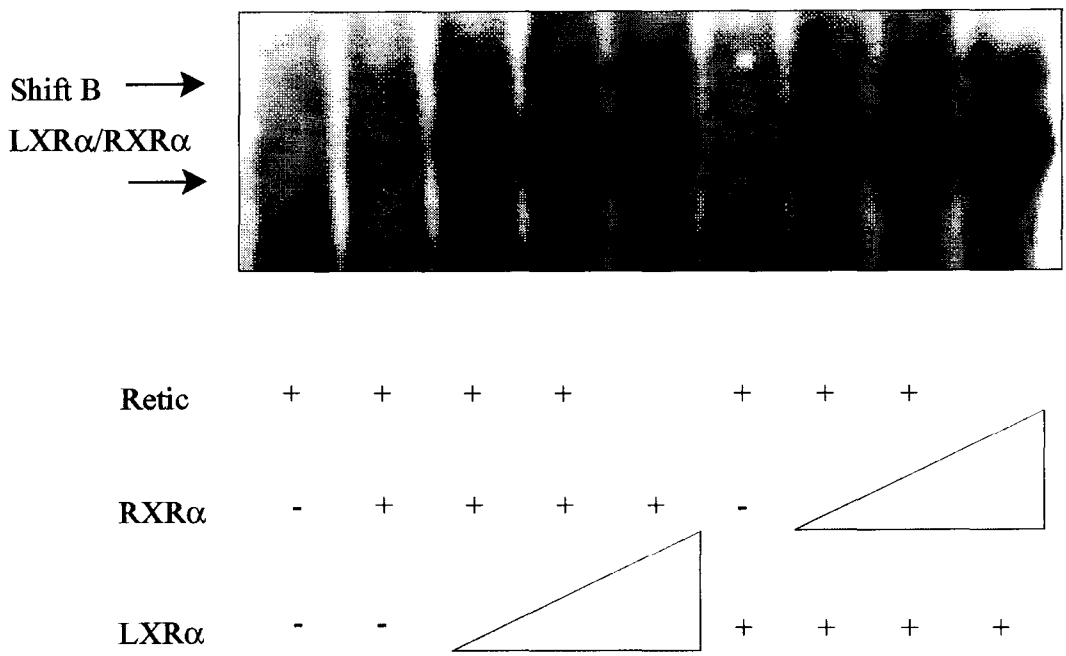


Figure 10: EMSA titration experiment demonstrating the correlation between an increase in Shift B formation and increasing amounts of either LXRα or RXRα protein. Titration studies of either LXRα or RXRα (0, 2, 3, or 4 µl) against constant amounts of rabbit reticulocyte lysate (Promega) was performed with [α-^{32}P]dATP labeled DR4-LXRE DNA probe. Subsequent to the incubation period, the samples were run on a 4% non-denaturing gel.

3.2.2 Transfection Studies of LXRα A/B Domain Deletions With the DR4-LXRE Response Element in the BSC40 Cell Line

While the LXRα A/B deletion EMSA studies did not show any significant effect on heterodimeric complex formation with RXRα, on either the DR4-LXRE or the LXRE-ΔMTV elements, the possibility remained that these deletions may exhibit differences in transient transfection experiments. Thus, in order to establish if a role for the LXRα A/B domain could be detected, transient transfection studies with the LXRα A/B domain deletions, in parallel with full length LXRα, were performed. These transfection assays were performed, in BSC40 cells, in the presence of the pDR4(X2)luc luciferase reporter plasmid (1.25μg) alone or in combination with LXRα, the LXRα A/B domain deletion clones or RXRα (0.5μg each). The effects of exogenously added 9-*cis* RA (1μM) and/or 22(R)-OH-CH (7μM) were examined. As illustrated in Figure 11, LXRα-mediated transactivation of the pDR4(X2)luc reporter construct, in the absence or presence of 9-*cis* RA and/or 22(R)-OH-CH, is comparable to that reported in Section 3.1.1 and Figure 6A for the BSC40 cell line. Interestingly, as the LXRα A/B domain was deleted, no significant difference, between LXRα A/B domain deletions and LXRα-mediated transactivation potentials, was observed either for LXRα/LXRα deletions transfected alone or co-transfected with RXRα. This result was achieved regardless of the conditions employed including the absence of drug, the presence of 9-*cis* RA, 22(R)-OH-CH or both. This study clearly demonstrates that LXRα A/B domain deletions of up to 63 amino acids have no effect on LXRα-mediated transactivation. Therefore, these

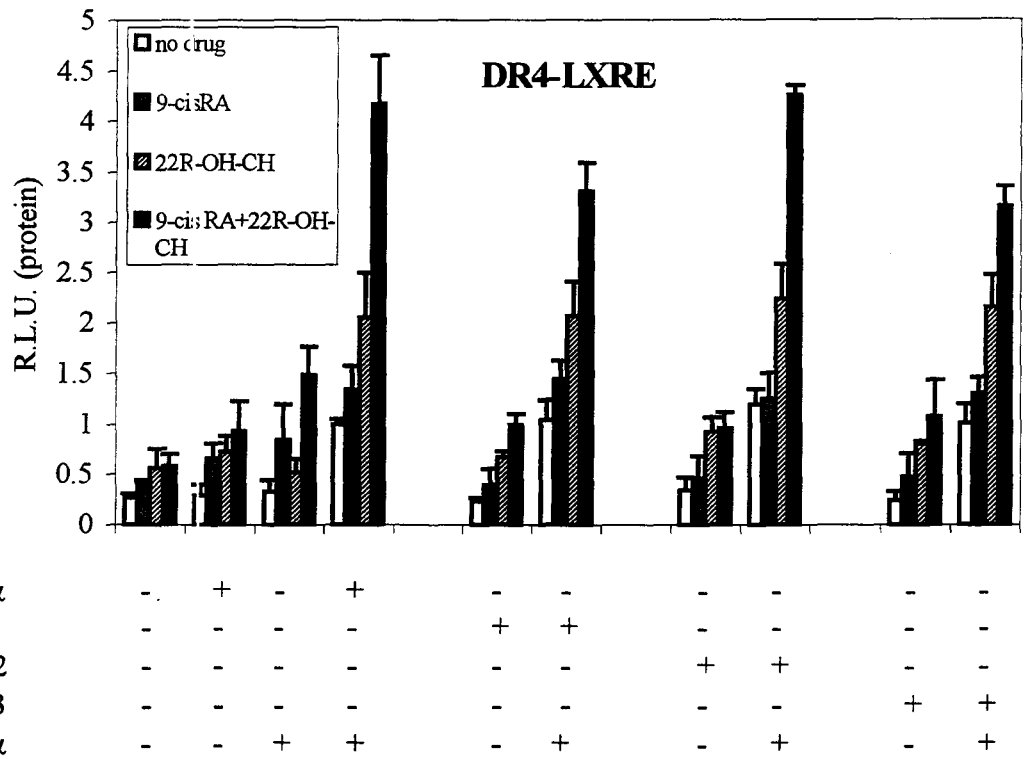


Figure 11: Transient transfection assays, with the pDR4(X2)luc reporter plasmid, demonstrating that no significant difference is observed between the transcriptional activity mediated by LXRα and that mediated by any of the LXRα A/B domain deletions, in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with the pDR4(X2) luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for LXRα, LXRα A/B domain deletions (RIP1, RIPΔ2 and RIPΔ3) or RXRα (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and/or the RXRα ligand 9-*cis* RA (final concentration 1 μM) were added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of five separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

transfection assays failed to detect a functional role for the N-terminal 63 amino acids of the LXRα A/B domain and may be indicative of a lack of a functional AF-1 domain in this region.

3.2.3 Transfection Studies of LXRα A/B Domain Deletions With the LXRE-ΔMTV Response Element in the BSC40 Cell Line

The possibility that a functional A/B domain response may be dependent on the DNA response element construct employed was subsequently addressed with transient transfections studies which utilized the LXRE-ΔMTV response element. Thus, transfection assays were performed, in BSC40 cells, in the presence of the TK-LXRE3-Luc luciferase reporter plasmid (1.25μg) alone or in combination with LXRα, the LXRα A/B domain deletion clones or RXRα (0.5μg each). The effect of exogenously added 22(R)-OH-CH (7μM) was investigated. As demonstrated in Figure 12, LXRα-mediated activation of the TK-LXRE3-Luc reporter plasmid, in the absence and presence of 22(R)-OH-CH, is comparable to previously reported activity (Section 3.1.1 and Figure 5B). Ultimately, this data further demonstrates that no significant difference between the transactivation mediated by full length LXRα and that mediated by any of the LXRα deletions were observed for all conditions employed. The only difference noted was the slight reduction in activation levels for the LXRα deletion RIP1, when transfected alone, the significance of which, if any, remains to be seen.

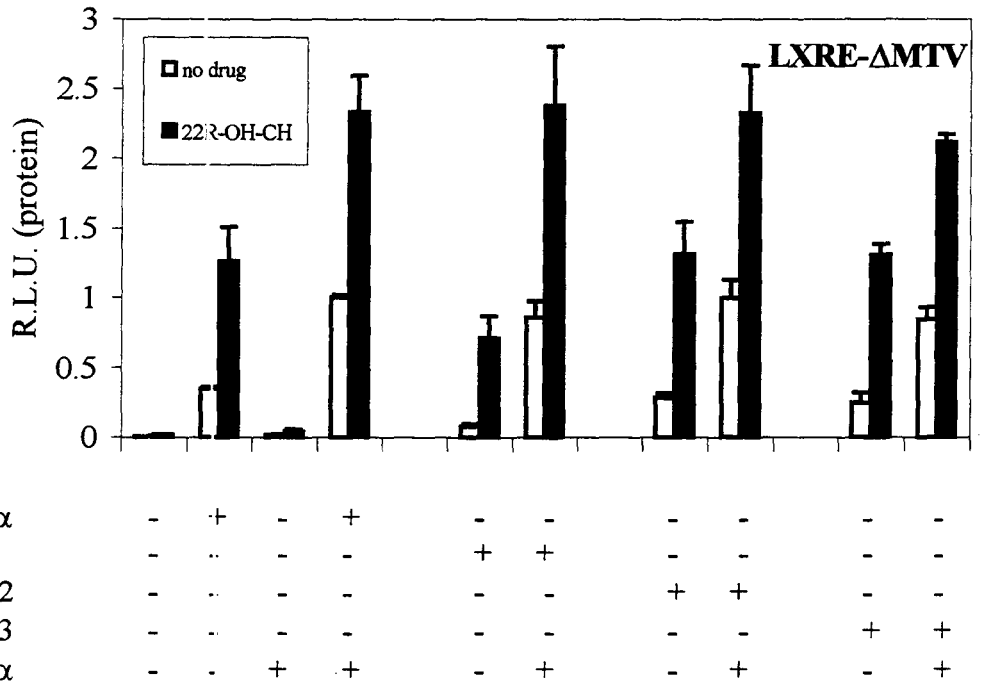


Figure 12: Transient transfection assays, with the TK-LXRE3-Luc reporter plasmid, demonstrating that no significant difference is observed between the transcriptional activity mediated by LXRα and that mediated by any of the LXRα A/B domain deletions, in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with the TK-LXRE3-Luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for LXRα, LXRα A/B domain deletions (RIP1, RIPΔ2 and RIPΔ3) or RXRα (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) was added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

3.2.4 Transfection Studies of LXRα A/B Domain Deletions With the DR4-LXRE Response Element in the COS-1 Cell Line

The investigation into whether or not a functional LXRα A/B domain response was dependent upon the cell line employed was achieved *via* transient transfection studies with the DR4-LXRE response element in the COS-1 cell line (refer to Figure 13). These assays were performed, in COS-1 cells, in the presence of the pDR4(X2)luc luciferase reporter plasmid (1.25μg) alone or in combination with LXRα, the LXRα A/B domain deletion clones or LXRα (0.5μg each). The effects of exogenously added 9-*cis* RA (1μM) and/or 22(R)-OH-CH (7μM) were also examined. Once again, these studies indicate that no significant difference in LXRα-mediated transactivation was detected as the LXRα A/B domain is deleted (up to 63 amino acids), under any of the conditions tested.

Thus, taken together, the LXRα A/B domain deletion (up to 63 amino acids) transient transfection studies, presented in Sections 3.2.2, 3.2.3 and 3.2.4, failed to delineate a functional role for the LXRα A/B domain within this region, regardless of DNA response element and cell line employed. These results may be attributable to a number of possibilities, which are offered and examined in the discussion.

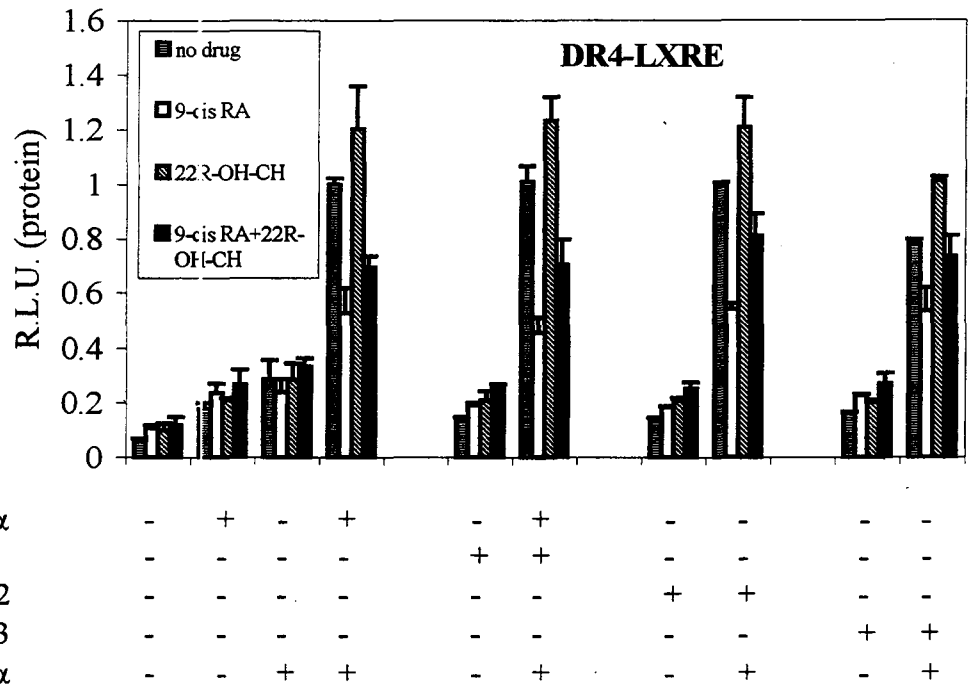


Figure 13: Transient transfection assays, with the pDR4(X2)luc reporter plasmid, demonstrating that no significant difference is observed between the transcriptional activity mediated by LXRα and that mediated by any of the LXRα A/B domain deletions, in the COS-1 cell line. COS-1 cells were transfected, utilizing the calcium phosphate method, with the TK-pDR4(X2)luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for LXRα, LXRα A/B domain deletions (RIP1, RIPΔ2 and RIPΔ3) or RXRα (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and/or the RXRα ligand 9-*cis* RA (final concentration 1μM) were added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

3.3 Interaction and Functional Studies of LXRα and the Transcription Co-factors RIP 140, SRC-1a and SMRT

Transcriptional regulation, mediated by nuclear hormone receptors, has been shown to involve multiple auxiliary co-factors (reviewed in Glass *et al.*, 1997 and Shibata *et al.*, 1997, Pazin and Kadonaga, 1997). These co-factors function to either activate (co-activators) or repress (co-repressors) through interactions, or lack thereof, respectively, with components of the basal transcription machinery and/or participate in chromatin remodeling. Thus, the identification of LXRα-interacting co-factors, and the investigation of their subsequent function in LXRα-mediated transactivation, was of particular interest. These studies were facilitated by the discovery that PPARα bound to a previously identified co-factor termed RIP 140 (Miyata *et al.*, 1998, Treuter *et al.*, 1998). Since LXRα and PPARα, as members of the nuclear receptor superfamily, are similar in several respects, including their ability to heterodimerize with RXR and their involvement in lipid metabolism as well as their ability to interact with each other, it stood to reason that LXRα may also interact with RIP 140. Subsequent studies with LXRα and the co-activator SRC-1a and the co-repressor SMRT were also undertaken as these two co-factors have been shown to modulate the transactivation mediated by a wide variety of nuclear receptors (Kurwokawa *et al.*, 1995, Chen and Evans, 1995, Oñate *et al.*,1995, Kamei *et al.*, 1996, Yao *et al.*, 1996, Zhu *et al.*, 1996, Jayakumar *et al.*, 1997, Kalkoven *et al.*, 1998, Lavinsky *et al.*, 1998, Lee *et al.*, 1998).

3.3.1 LXRα Interaction Studies with RIP 140 *In Vivo*

Initial studies with RIP140 were dependent upon the partial clone isolated from the human liver cDNA library with PPARα (RIP 140-GAD aa 164-869) (refer to Figure 14) via a yeast dihybrid screen. Therefore, in order to determine if LXRα and RIP 140 interacted *in vivo*, the yeast dihybrid system was utilized with full length LXRα fused to the GAL4 DNA binding domain (DBD) and the isolated RIP 140 clone (RIP140-GAD fused to the GAL4 activation domain). These plasmids were transformed into the PCY2 yeast strain (the PPARα/RIP 140 experiments were performed in the Y190 yeast strain by Kenji Miyata) and were then tested for β-Gal activity with both qualitative overlay and quantitative liquid culture assays (refer to Table 3). The data obtained from the liquid culture assays, presented in Table 3, represent the average activity of three independent yeast transformations measured in triplicate (± SD).

Although very weak, an LXRα/RIP 140 interaction could be detected in both assays employed. Despite the fact that the interaction was so much weaker than that of the PPARα /RIP 140 interaction, investigations proceeded with interaction studies of LXRα and RIP 140 *in vitro*.

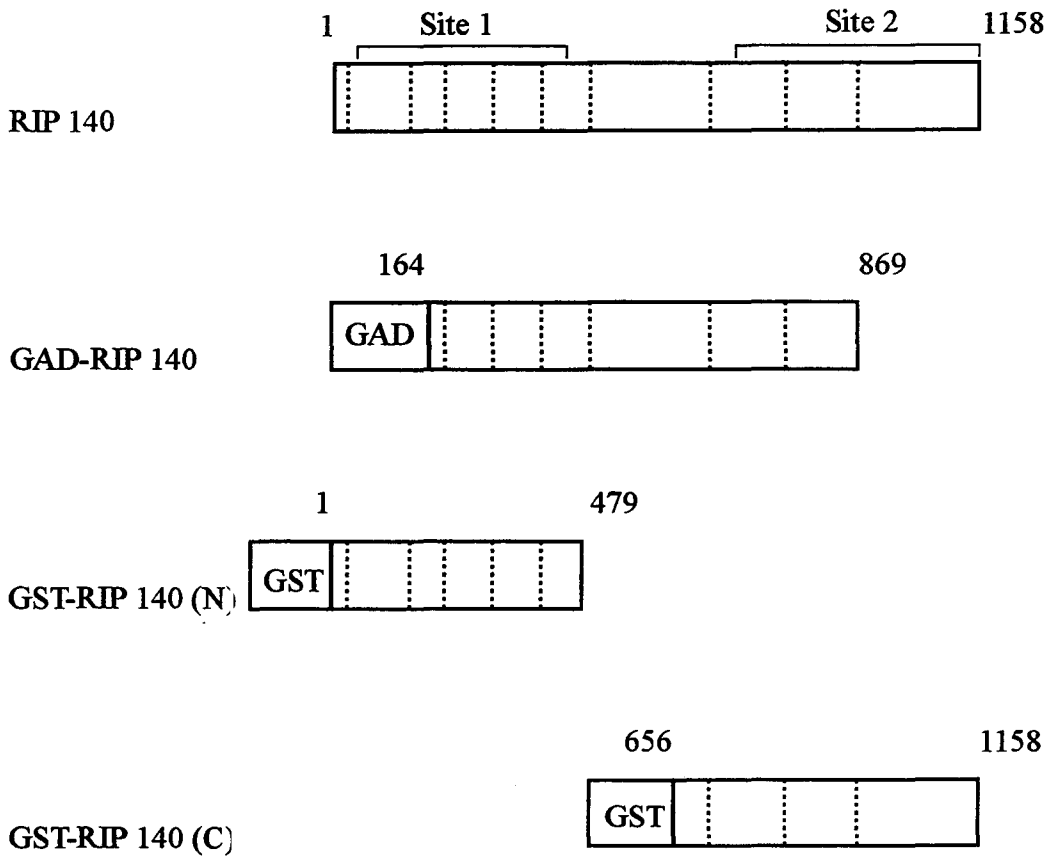


Figure 14: Schematic representation of the RIP 140 constructs utilized in the interaction and functional studies with LXRα. The RIP 140 LXXLL motifs are indicated by dashed lines and the two distinct RIP 140/nuclear receptor interactions sites, site 1 and site 2, are illustrated.

Table 3: RIP 140 Interacts with LXRα *in vivo*

GAL4 DNA Binding Domain Fusion (DBD)	GAL4 Activation Domain Fusion (AD)	β-Galctosidase Activity	
		Agarose Overlay	Liquid Assays
PCY2 yeast strain		-	0.00+/-0.00
Full Length GAL4		++++	772.48+/-240.15
GBT9 (DBD)		-	0.01+/-0.001
	GAD424 (AD)	-	0.01+/-0.001
GBT9 (DBD)	GAD424(AD)	-	0.01+/-0.001
LXRα		-	0.055+/-0.014
	RIP 140	-	0.03+/-0.012
	RXRα	-	0.038+/-0.013
GBT9 (DBD)	RIP 140	-	0.046+/-0.004
GBT9 (DBD)	RXRα	-	0.057+/-0.005
LXRα	GAD424 (AD)	-	0.055+/-0.004
[illegible]	[illegible]	[illegible]	[illegible]
LXRα	RXRα	++	1.938+/-0.60
[illegible]	[illegible]	[illegible]	[illegible]
PPARα(DMN1)	RXRα	++	5.21+/-1.21

3.3.2 LXRα Interaction Studies with RIP 140 *In Vitro*

The initial GST-protein binding studies, conducted as a collaborative effort with Hansa Patel and Kenji Miyata, were designed in order to examine whether or not RIP 140 interacted with LXRα *in vitro*. Furthermore, these studies were designed to examine whether or not RIP 140/LXRα interactions were ligand dependent as shown for the other nuclear receptors such as RXR. For these investigations LXRα-GST, PPARα-GST and RXR-GST (as a positive control) fusion proteins with *in vitro* translated full length L-^{35}[S]-methionine labeled RIP 140 (Cavaillès *et al.*, 1995). Experiments were performed in the presence or absence of vehicle (95% ethanol (E) for LXRα and DMSO (D) for

RXRα and PPARα) and/ or drug (7μM 22(R)-OH-CH (HC) for LXRα, 1μM 9-*cis* RA (RA) for RXRα and 100μM WY-14,643 (Wy) for PPARα).

As demonstrated in Figure 15A, RIP 140 did not bind to the GST beads under any of the conditions tested. RIP 140 did bind to GST-LXRα (20% of input, lane8) and neither the addition of vehicle nor 22(R)-OH-CH had any effect on the interaction (compare lane8,9 and 10). Interestingly, PPARα (8-10% of input, lane 2) also interacted with RIP 140 in a similar ligand-independent manner (compare lane 2, 3 and 4). In contrast, RIP 140 binding to RXRα was only minimal in the absence of ligand or in the presence of vehicle but was significantly increased with the addition of 9-*cis* RA (compare lanes 5, 6 and 7) as previously reported (L'Horset *et al.*, 1996). Thus, unlike results achieved with other nuclear receptors reported to date, LXRα (and PPARα) bound to RIP 140 in a ligand/activator-independent manner.

The second set of GST-protein binding assays were designed to investigate whether or not there existed a site1 or site2 preference of RIP 140 for LXRα (and PPARα) as observed for other nuclear receptors (L'Horset *et al.*, 1996). For these experiments, GST-RIP 140(N) (aa. 1-479) and GST-RIP 140(C) (aa 656-1158) were employed with *in vitro* translated L-^{35}[S]-methionine labeled LXRα, RXRα and PPARα in the presence or absence of vehicle and/or ligand as previously described. As illustrated in Figure 15B, none of the *in vitro* prepared LXRα, RXRα or PPARα proteins bound to the GST beads under any of the conditions tested. LXRα was shown to bind to both RIP 140 clones well, with a slight preference for site 2 (GST-RIP140 C), and the addition of vehicle

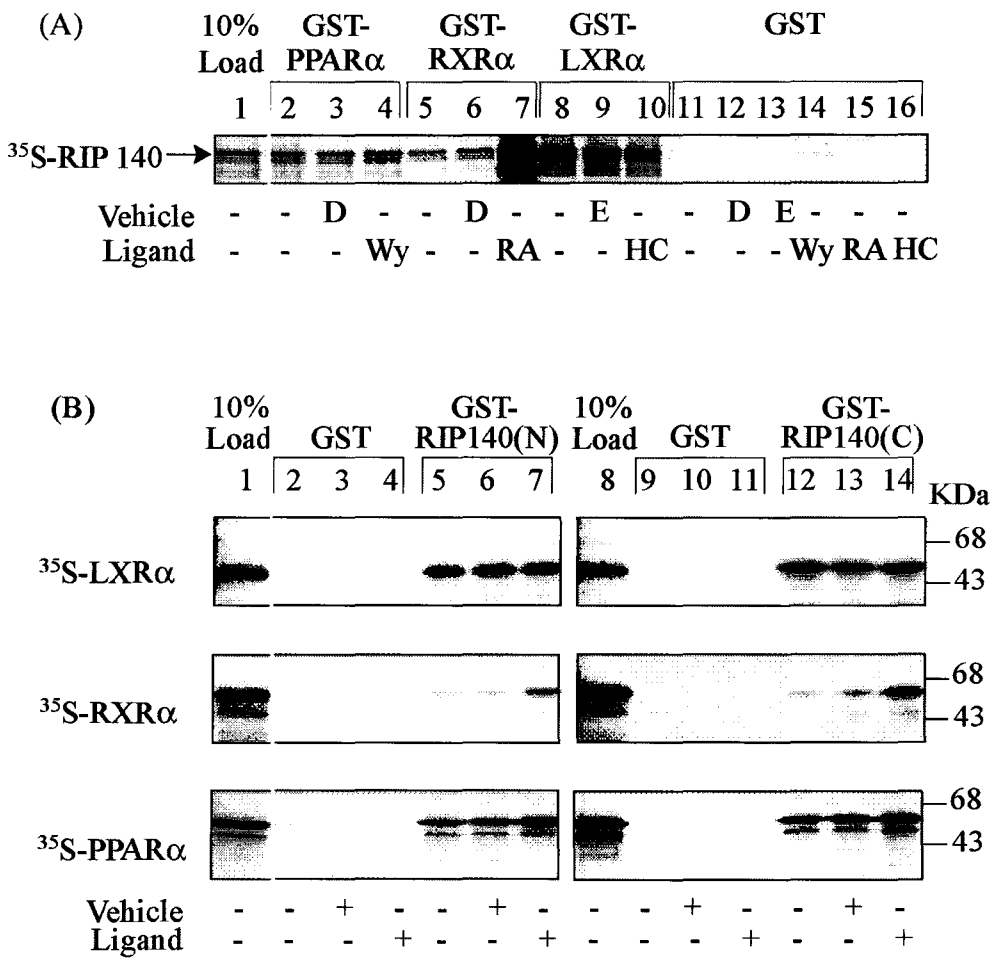


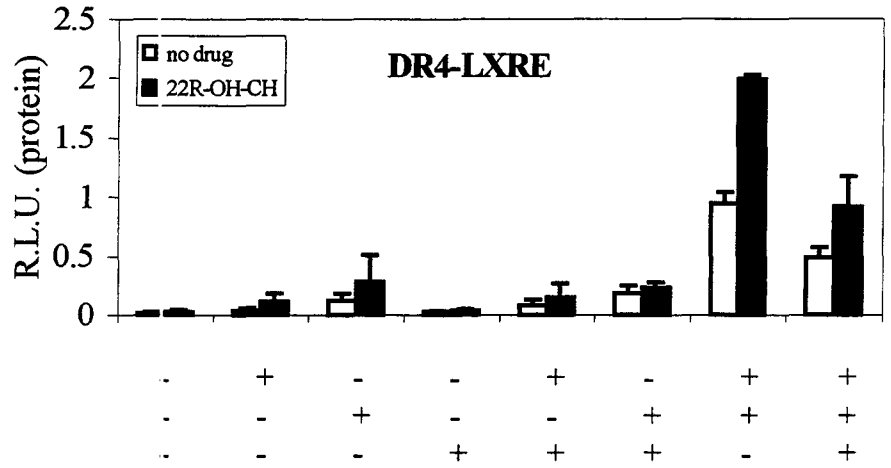
Figure 15: *In vitro* GST-binding studies which illustrate a specific interaction between LXRα, RXRα and PPARα with RIP 140. (A) in a ligand-independent manner for LXRα and PPARα and a ligand-dependent manner for RXRα. *In vitro* translated L-[^{35}S]-methionine labeled RIP 140 was incubated with GST-LXRα, GST-RXRα, GST-PPARα or GST coupled beads. (B) Ilustrates LXRα binds to both GST-RIP 140 (N) (site 1) and GST-RIP 140 (C) (site 2) with an apparent preference for site 2. *In vitro* translated L-[^{35}S]-methionine labeled LXRα, RXRα or PPARα were incubated with either GST-RIP 140 (N) or GST-RIP 140 (C) coupled beads. Both (A) and (B) studies were performed in the absence or presence of vehicle (95% ETOH (E) for LXRα and DMSO (D) for both RXRα and PPARα) or drug (7μM 22(R)-OH-CH for LXRα, 1μM 9-cis RA (RA) for RXRα and 100μM WY-14,643 (Wy) for PPARα). Subsequent to incubation, the beads were washed and the bound proteins were eluted and resolved on a 10% polyacrylamide SDS-PAGE gel. Adapted from Miyata *et al.*, 1998.

and/or 22(R)-OH-CH did not increase binding. PPARα was also shown to interact with both domains equally well; however, a modest 2-fold enhanced binding was observed for PPARα in the presence of Wy-14,643. In contrast, RXRα binding of RIP 140 was shown to be ligand-dependent; however, no difference in binding between site1 and site2 was detected in our system, which differs from a previous study that reports an RXRα preference for site1 (L'Horset *et al.*, 1996). This could be attributable to the fact that the clones employed in this study were slightly different than those used in the aforementioned report (the GST-RIP 140(C) encompassed one more LXXLL motif than the C-terminal/site2 clone employed in L'Horset *et al.*, 1996). Thus, in the system employed, LXRα (and PPARα) can bind to either site1 or site2 domains of RIP140, with an apparent preference for site2, in a ligand/activator-independent manner.

3.3.3 Transfection Studies of LXRα, RXRα and RIP 140 with the DR4-LXRE Response Element in the BSC40 Cell Line

Transient transfection experiments were designed in order to investigate the effect of RIP 140 on LXRα/RXRα-mediated transactivation *in vivo*. These transfection assays were performed in BSC40 cells in the presence of the pDR4(X2)luc luciferase reporter plasmid (1.25 μg) alone or in combination with LXRα, RXRα and RIP 140 (0.5 μg each) where the effect of exogenously added 22(R)-OH-CH was also examined. As demonstrated in Figure 16A, co-transfection of LXRα and RXRα resulted in a 60-fold and 100-fold induction in the absence and presence of 22(R)-OH-CH respectively. However, upon the addition of an equivalent amount of RIP 140 both ligand-independent and dependent activities were reduced by approximately half.

(A)



(B)

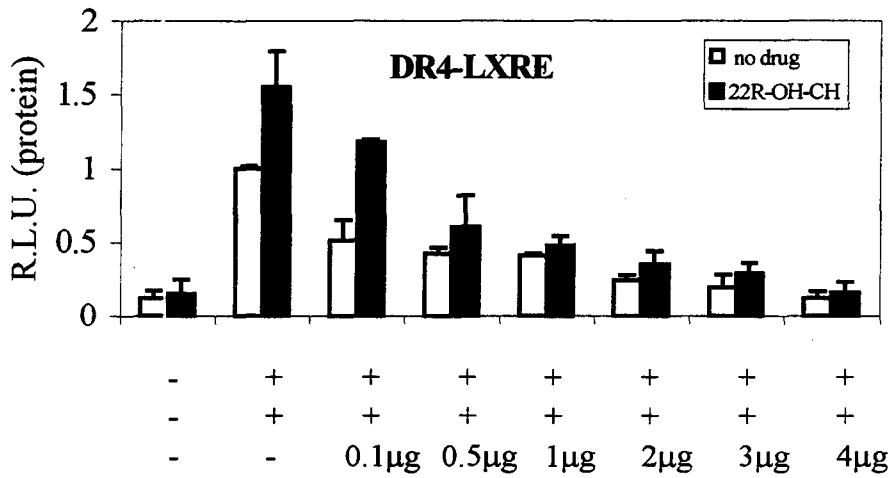


Figure 16: Transient transfection assays demonstrating that RIP 140 antagonizes transactivation mediated by LXRα/RXRα *in vivo* in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with either the pDR4(X2) luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for (A) LXRα, RXRα or RIP 140 (0.5 μg each) or (B) LXRα and RXRα (0.5μg each) with increasing amounts of the RIP 140 expression vector (0.1-4 μg). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) was added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

A second set of transfection experiments were carried out in order to determine the effect of RIP 140 titration, in increasing amounts, on LXRα/RXRα transactivation. Once again, transient transfections were performed in BSC40 cells in the presence of the pDR4(X2)luc luciferase reporter plasmid (1.25μg) alone or in combination with LXRα and RXRα (0.5 μg each) and increasing amounts of RIP 140 (0.1μg to 4μg). As illustrated in Figure 16B, RIP 140 inhibited LXRα/RXRα-mediated transactivation at all concentrations tested in a dose-dependent manner. These results are comparable to those found for PPARα (Miyata *et al.*, 1998) and for ER, at high RIP 140 concentrations (Cavaillès *et al.*, 1995) reported. This observed repression may be attributable to a number of factors, which are examined in the discussion.

3.3.4 Transfection Studies of LXRα, RXRα and RIP 140 with the LXRE-ΔMTV Response Element in the BSC40 Cell Line

In order to ascertain whether or not the reported RIP 140-mediated repression of reporter gene activity, described in Section 3.3.3, was DNA response element construct dependent, transient transfections were performed with the LXRE-ΔMTV reporter construct. These transfection assays employed the BSC40 cell line in the presence of the TK-LXRE3-Luc luciferase reporter plasmid (1.25 μg) alone or in combination with LXRα, RXRα and RIP 140 (0.5 μg each) where the effect of exogenously added 22(R)-OH-CH was investigated. These studies illustrate, in Figure 17, that transfection of LXRα alone results in a two-fold increase in activation potentials with the addition of 22(R)-OH-CH over activation levels achieved in the absence of drug, where the addition

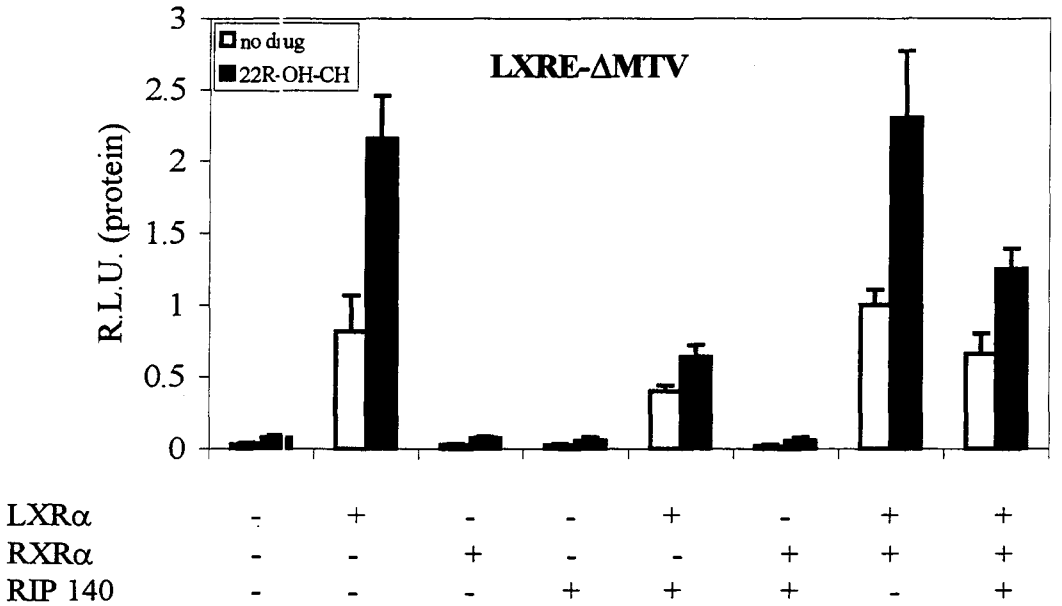


Figure 17: Transient transfection assays demonstrating that RIP 140 antagonizes transactivation mediated by LXRα/RXRα *in vivo* in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with the TK-LXRE3-Luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for LXRα, RXRα and RIP 140 (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) was added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of three separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

of exogenous RXRα had no significant effect on activity levels (and are therefore comparable to results reported in Section 3.1.1). Co-transfection of RIP140 and LXRα resulted in a substantial repression of transcriptional activity either in the absence or presence of 22(R)-OH-CH). Similarly, transfection of RIP140, LXRα and RXRα together also resulted in a repression of activity, although to a slightly lesser extent. Thus, regardless of the DNA response element construct employed, in the BSC40 cell line, RIP140 has a repressive effect on LXRα-mediated transactivation.

3.3.5 RIP 140 *In Vitro* Binding Studies with the LXRα A/B Domain Deletion RIPΔ3

Despite the fact that the LXRα A/B domain deletions, in comparison to full length LXRα, exhibited no significant differences in either EMSA nor transient transfection studies, the possibility that the region spanned by the LXRα deletion constructs may be important for co-factor binding remained. This possibility was addressed, for the co-factor RIP 140, *via* GST-binding assays, which employed both GST-RIP140(N) (Figure 18A) and GST-RIP140(C) (Figure 18B) fusion proteins. These fusion proteins were incubated with either *in vitro* translated L-[^{35}S]-methionine labeled RIPΔ3 (the construct with the largest section of the LXRα A/B domain deleted) or full length LXRα. These studies were carried out in the presence or absence 22(R)-OH-CH (final concentration of 7 μM) or an equivalent volume of vehicle (95% ETOH).

As illustrated in Figure 18A, both LXRα and RIPΔ3 bind to the GST-RIP 140 (N) protein fusion in a ligand-independent manner. Quantification of the amount of LXRα and RIPΔ3 that was retained by the GST fusion protein revealed that LXRα and RIPΔ3

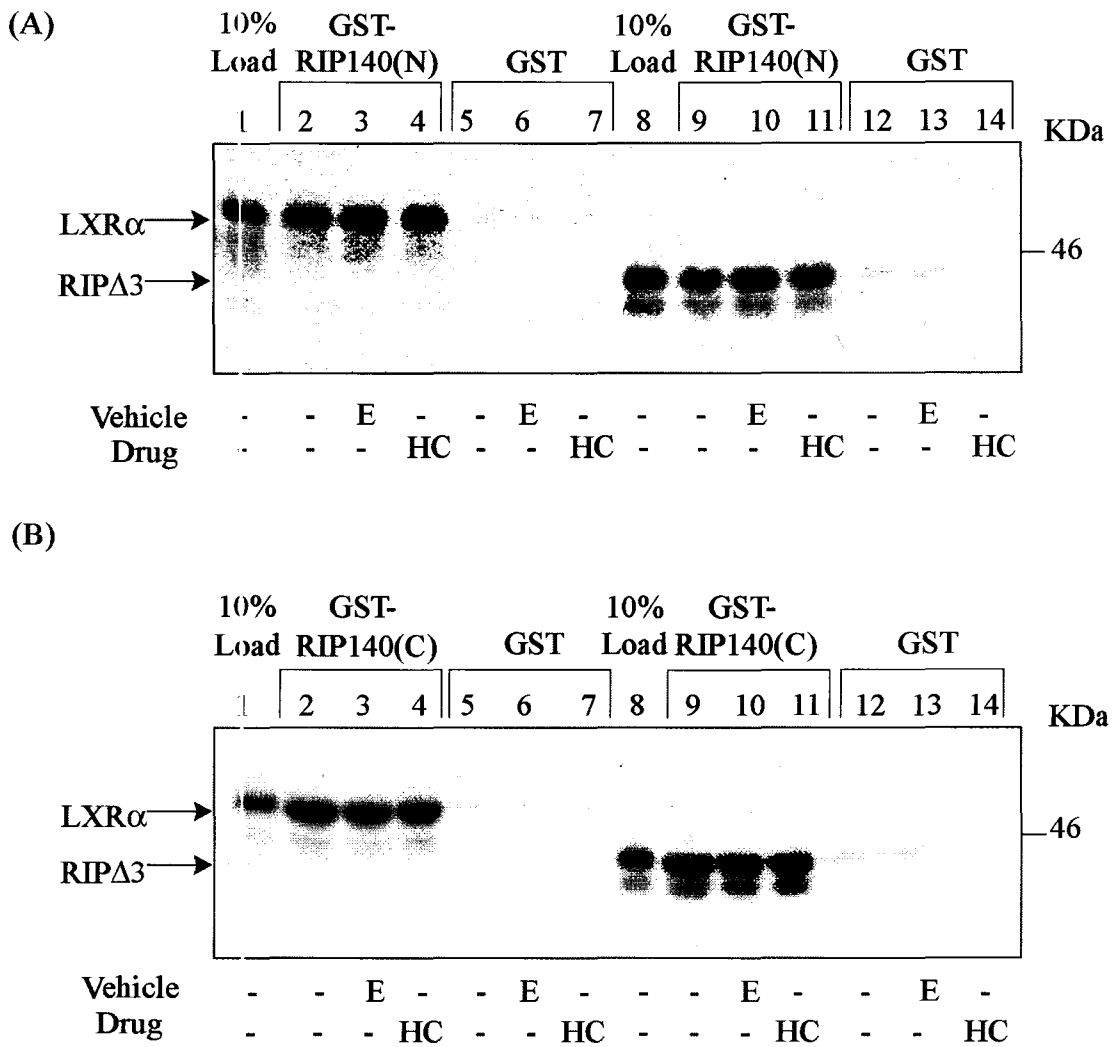


Figure 18: *In Vitro* GST-binding studies which illustrate a specfic interaction between either LXRα or the LXRα A/B domain deletion construct RIPΔ3 with RIP 140 in a ligand-independent manner. *In Vitro* translated L-[^{35}S]-methionine labeled LXRα or RIPΔ3 were incubated with GST and (A) GST-RIP 140 (N) or (B) GST-RIP 140 (C) coupled beads in the absence or presence of vehicle (95% ETOH (E)) or drug (7μM 22(R)-OH-CH (HC)). Subsequent to the incubation period, the beads were washed and the bound proteins were eluted and resolved on a 10% polyacrylamide SDS-PAGE gel.

bound with similar affinities; LXRα input binding percentages were 12% in the absence of vehicle and drug (lane 2), 12% in the presence of vehicle (lane 3) and 12% in the presence of 22(R)-OH-CH (lane 4) and RIPΔ3 input binding percentages were 10% in the absence of either vehicle or drug (lane 9), 12% in the presence of vehicle (lane 10) and 11% in the presence of 22(R)-OH-CH (lane 11). Similar results were achieved for LXRα and RIPΔ3 with the GST-RIP 140 (C) fusion protein (Figure 18B) studies; LXRα input binding percentages were 19% in the absence of vehicle and drug (lane 2), 17% in the presence of vehicle (lane 3) and 17% in the presence of 22(R)-OH-CH (lane 4) and RIPΔ3 input binding percentages were 17% in the absence of either vehicle or drug (lane 9), 15% in the presence of vehicle (lane 10) and 15% in the presence of 22(R)-OH-CH (lane 11). Further more binding of LXRα and RIPΔ3 to GST alone, under any of the conditions tested (both Figure 18A and 18B), was negligible (always less than 1% of input). Thus, taken together, the results indicate that the initial 63 amino acids of the LXRα A/B domain are dispensable for RIP 140 interaction.

3.3.6 Transfection Studies of RIPΔ3, RXRα and RIP 140 with the DR4-LXRE Element in the BSC40 Cell Line

Transient transfection studies of RIPΔ3 and RXRα with RIP 140 were designed in order to address the possibility that deletion of the LXRα A/B domain may effect RIP 140-mediated repression. These transfection assays were performed with the pDR4(X2)luc luciferase reporter plasmid (1.25ug) alone or in combination with RIPΔ3 (or LXRα) and RXRα and RIP140 (0.5ug each). The effects of absence of drug and

presence of both 9-*cis*RA and 22(R)-OH-CH together were also investigated. Figure 19 illustrates that no significant effectual difference between full length LXRα and RIPΔ3, when co-transfected with RXRα, either in the absence or presence of drug, as previously described in Section 3.2.2. Transfection of RIPΔ3 (or LXRα), RXRα and RIP 140 resulted in a significant decrease in both RIPΔ3/RXRα and LXRα/RXRα-mediated transactivation in both the absence and presence of 9-*cis* RA and 22(R)-OH-CH.

These transient transfection studies indeed demonstrate that deletion of the A/B domain of up to 63 amino acids (ripd3) of LXRα has no effect on transactivation mediated by RXRα/LXRα and that the addition of RIP140 resulted in a similar repressive/inhibitory effect.

3.3.7 *In Vitro* AF-2 Core Domain Deletion Studies of LXRα with RIP 140

An intact nuclear receptor E domain, including the AF- core domain, which comprises the putative helix 12, has been shown to be required for RIP 140 binding (Cavaillès *et al.*, 1995, Joyeux *et al.*, 1997, Collingwood *et al.*, 1997, Treuter *et al.*, 1998, Miyata *et al.*, 1998). Thus, in order to determine if the AF-2 domain of LXRα was necessary for RIP 140 interaction, the C-terminal 7 amino acids (aa 432-447) of the GST-LXRα fusion protein were deleted (refer to Figure 20A). This construct, GST-LXRαΔAF-2, was subsequently employed in an *in vitro* GST-binding assay with L-[^{35}S]-methionine labeled RIP 140 (refer to Figure 20B). These experiments demonstrate that despite cleavage of the AF-2 domain of LXRα, RIP 140 was still able to bind, although to a lesser extent; quantification of RIP 140 retention by GST-LXRα was 9% of input

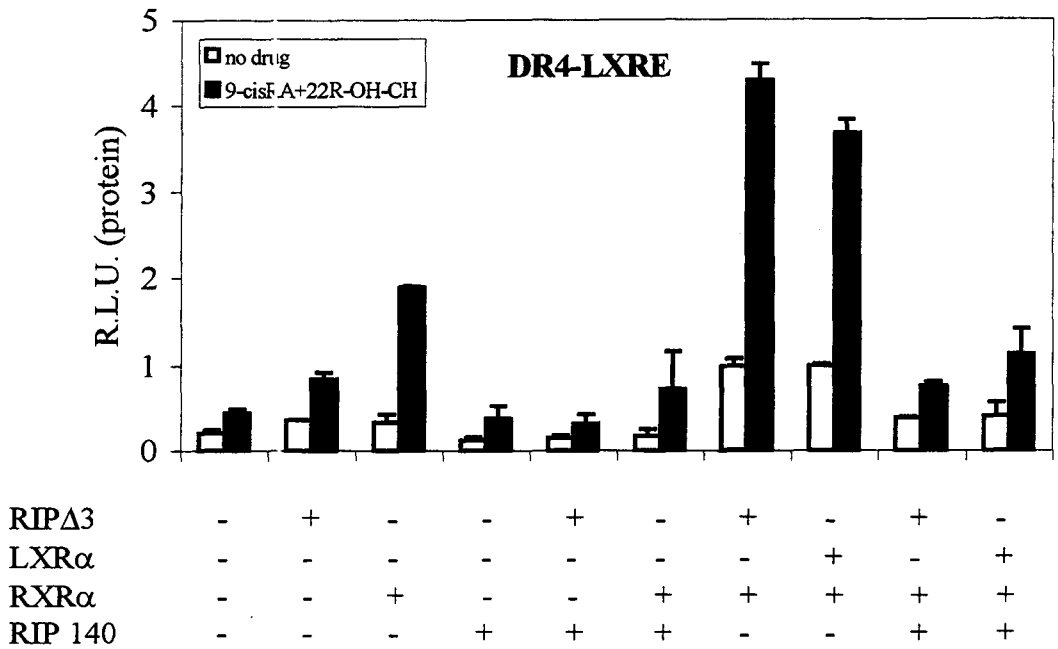


Figure 19: Transient transfection assays demonstrating that RIP 140 antagonizes transactivation mediated by both RIPΔ3/RXRα and LXRα/RXRα *in vivo* in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with the TK-LXRE3-Luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for RIPΔ3, LXRα, RXRα and RIP 140 (0.5 μg each). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and the RXRα ligand 9-*cis* RA (final concentration 1 μM) were added together. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

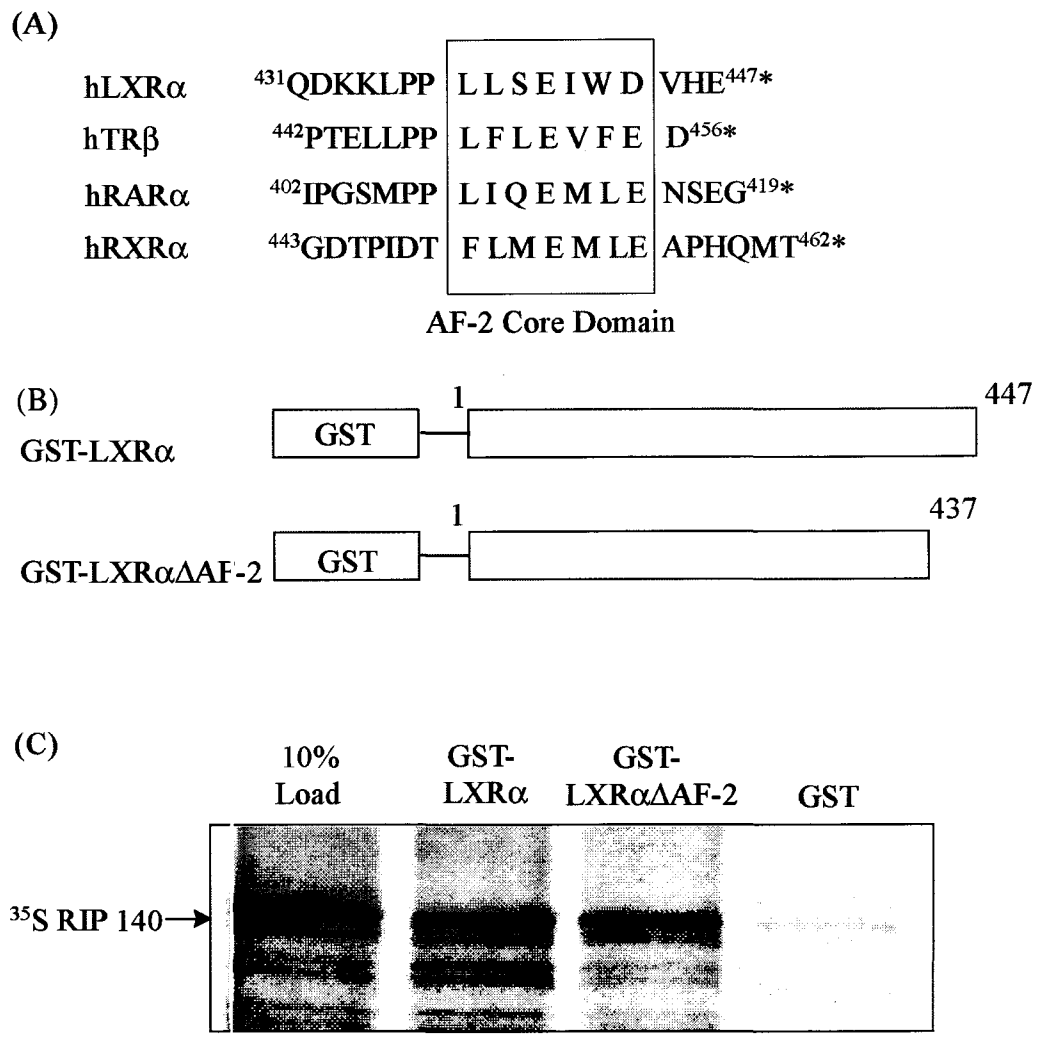


Figure 20: *In vitro* GST-binding study which illustrates a specific interaction between RIP 140 and both the full length LXRα and the LXRα AF-2 deletion GST fusion proteins. (A) Schematic representation of the AF-2 core domains of LXRα, TRβ, RARα and RXRα adapted from Willy and Mangelsdorf, 1997. (B) Schematic representation of the GST-LXRα and GST-LXRαΔAF-2 constructs. (C) *In vitro* translated L-[^{35}S]-methionine labeled RIP 140 was incubated with either GST, GST-LXRα or GST-LXRαΔAF-2 coupled beads. Subsequent to the incubation period the beads were washed and the bound proteins were eluted and resolved on a 10% polyacrylamide SDS-PAGE gel.

(lane 2) and 5% of input for GST-LXRαΔAF-2 (lane 3). Binding of RIP 140 to GST alone was negligible (less than 1% of input (lane 4)). Thus, these results indicate that RIP 140 requires the LXRα AF-2 core domain for efficient binding yet further suggest that other regions of LXRα (although not the N-terminal 63 amino acids) participate in binding. This is consistent with findings that an intact E domain is required for efficient RIP 140 binding (Treuter *et al.*, 1998).

3.3.8 *In Vitro* Binding Studies of LXRα with Both SRC-1a and SMRT Co-factors

GST-protein binding assays were designed to investigate whether or not LXRα interacted with either the co-activator SRC-1a or the co-repressor SMRT. Furthermore, ligand investigations of these potential interactions were also performed in order to determine not only if an LXRα/SRC-1a interaction occurs in a ligand-independent manner, but also if an LXRα/SMRT interaction can be abolished upon addition of ligand as observed for both nuclear receptor and/co-activator or co-repressor interactions respectively. These studies employed the GST-LXRα fusion protein and either the *in vitro* translated L-[^{35}S]-methionine labeled SRC-1a or SMRT proteins in the absence of drug and vehicle, the presence of vehicle (95% ETOH) or the presence of the LXRα activator 22(R)-OH-CH (final concentration 7 μM).

The results of the GST-binding studies with LXRα and SRC-1a are illustrated in Figure 21A. Analysis of the results reveal that SRC-1a binding to GST alone was negligible under any of the conditions tested (did not exceed 1% of input) (lanes 5, 6 and 7). However, SRC-1a did bind to GST-LXRα in the absence of vehicle and 22(R)-OH-

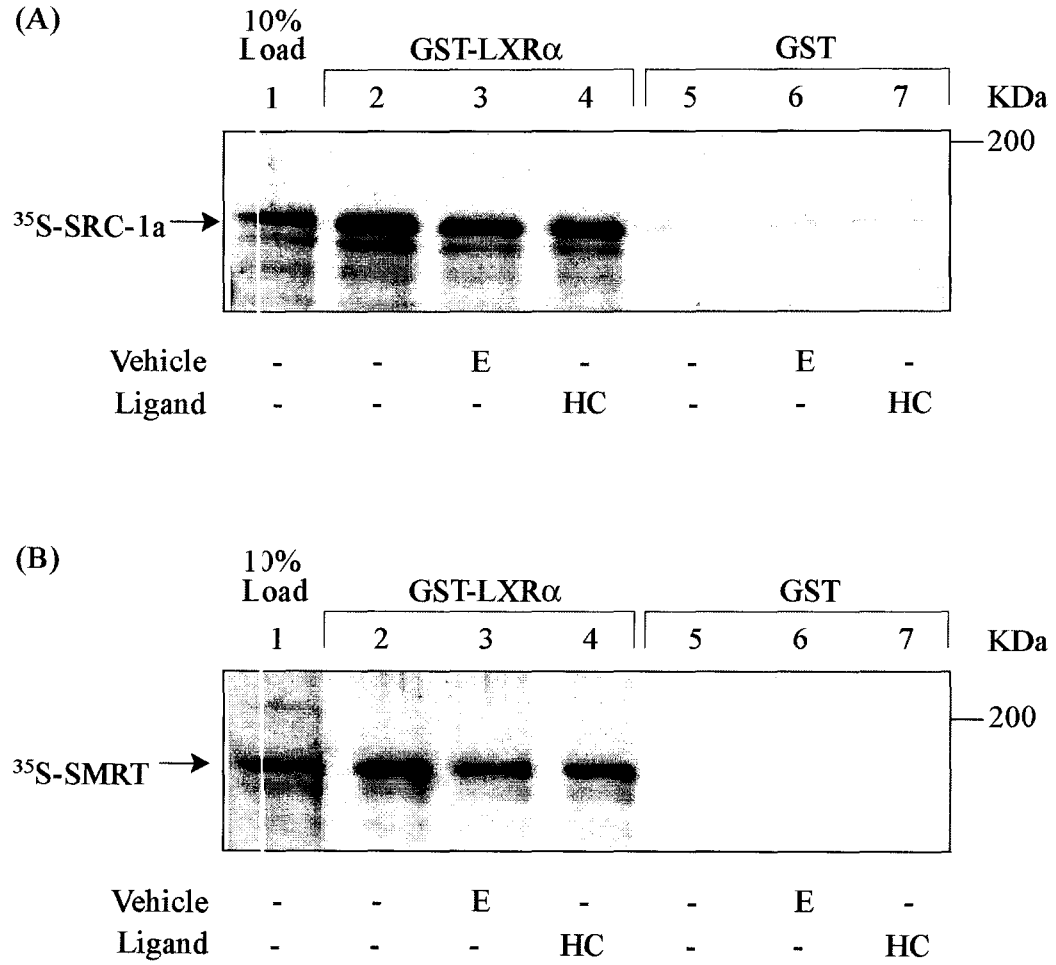


Figure 21: *In vitro* GST-binding studies that illustrate a specific interaction between LXRα and the co-factors SRC-1a and SMRT. (A) In vitro translated, L-[^{35}S]-methionine labeled SRC-1a or (B) SMRT were incubated with GST-LXRα or GST coupled beads either in the absence or presence of the LXRα activator 22(R)-OH-CH (HC) (7μM) or an equal volume of vehicle (95% ETOH (E)). Subsequent to the incubation period, the beads were washed and the bound proteins were eluted and resolved on a 10% polyacrylamide SDS-PAGE gel.

CH (23% of input) and in the presence of both vehicle and 22(R)-OH-CH (both 16% in lanes 3 and 4 respectively). Thus, the SRC-1a/LXRα interaction occurs in a ligand-independent manner and the interaction was not enhanced with the addition of 22R-OH-CH. Furthermore, vehicle (95% ETOH) appears to disrupt the interaction in the binding assay to a certain degree for which 22R-OH-CH is not able to compensate.

The results of the GST-LXRα and SMRT GST-binding studies, presented in Figure 21B, demonstrate that SMRT binding to GST alone was negligible regardless of the conditions employed (less than 1% of input in lanes 5, 6 and 7). However, SMRT did bind to the GST-LXRα fusion protein in the absence of both vehicle and 22(R)-OH-CH (14% of input in lane 2), in the presence of vehicle (95% ETOH) (10% of input in lane 3) and upon addition of 22(R)-OH-CH (11% of input in lane 4). Thus, the addition of 95% ETOH appears to disrupt the SMRT/LXRα interaction slightly and the addition of 22(R)-OH-CH did not have any significant effect on the interaction.

The observations reported here for the SRC-1a/LXRα and SMRT/LXRα interactions may attributable to a number of factors, which are examined in the discussion.

3.3.9 *In Vitro* LXRα AF-2 Core Domain Deletion Studies with SRC-1a and SMRT Co-factors

In order to determine if the AF-2 core domain of LXRα was necessary for either SRC-1a or SMRT binding, a series of GST-binding assays were performed. These studies employed both the GST-LXRα and GST-LXRαΔAF-2 fusion proteins with either

in vitro translated L-[^{35}S]-methionine labeled SRC-1a and SMRT in the absence of both vehicle and 22(R)-OH-CH.

The results of the investigations of GST-LXRαΔAF-2 with SRC-1a are presented in Figure 22A. Analysis of these results indicate that binding of SRC-1a to the GST-LXRαΔAF-2 fusion protein, in comparison to GST-LXRα binding, is significantly lower and not above background binding to GST alone (0.6% of input bound to the AF-2 deletion (lane 3), 0.6% of input bound to GST alone (lane 4) and 3% of input bound to GST-LXRα). These findings suggest that the AF-2 core domain of LXRα is essential for SRC-1a binding, as observed for interactions with other nuclear receptors.

SMRT interaction studies with either GST-LXRα or GST-LXRαΔAF-2 are presented in Figure 22B. Surprisingly, these results illustrate that binding of SMRT to the GST-LXRαΔAF-2 fusion protein was significantly higher than that achieved with the GST-LXRα fusion protein (20% of input (lane 3) and 13% of input (lane 2), respectively). Retention of SMRT by GST alone was negligible (less than 1% in lane 4). These findings suggest that the LXRα AF-2 core domain (helix 12) impedes LXRα/SMRT interactions.

3.3.10 Transfection Studies with LXRα, RXRα and SRC-1a with the DR4-LXRE Element in the BSC40 Cell Line

In order to determine the effect, if any, of SRC-1a on LXRα-mediated transactivation, transient transfection assays were designed. These transfection studies were performed in the BSC-40 cell line in the presence of the pDR4(X2)luc reporter

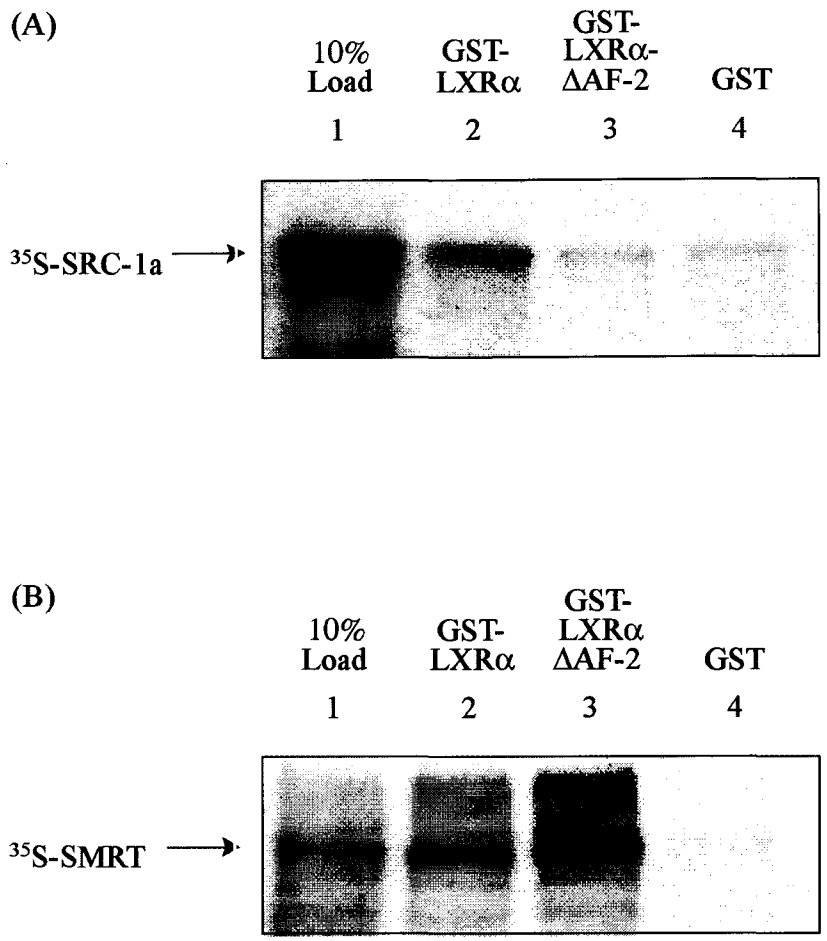


Figure 22: (A) *In vitro* GST-binding study that illustrates a loss of binding between the LXRα AF-2 deletion GST fusion protein and SRC-1a and (B) *In vitro* GST-binding study that illustrates a specific interaction between LXRα and the LXRα AF-2 deletion GST fusion proteins with SMRT. (A) In vitro translated, L-[^{35}S]-methionine labeled SRC-1a or (B) SMRT were incubated with GST-LXRα, GST-LXRαΔAF-2 or GST coupled beads in the absence of both vehicle and 22(R)-OH-CH. Subsequent to the incubation period, the beads were washed and the bound proteins were eluted and resolved on a 10% polyacrylamide SDS-PAGE gel.

plasmid (1.25ug) alone or in combination with LXRα, RXRα (0.5ug each) and increasing amounts of SRC-1a (0.001-2 μg). The effects of exogenously added 9-*cis* RA and 22R-OH-CH together were investigated. As demonstrated in Figure 23, SRC-1a, even at the lowest concentration of 0.001ug, in the presence of both 9-*cis* RA and 22(R)-OH-CH, significantly repressed LXRα/RXRα-mediated transactivation (approximately by two thirds). This repression was also observed in the absence of drug but to a lesser extent (approximately 50%). This surprising observation may be the result of a number of factors, which are thoroughly examined in the discussion.

3.3.11 Transfection Studies of LXRα, RXRα and SMRT with the DR4-LXRE Response Element in the BSC40 Cell Line

A preliminary transient transfection experiment was performed in order to investigate the effect, if any, of SMRT on LXRα-mediated transactivation. This transfection study were performed in the BSC-40 cell line in the presence of the pDR4(X2)luc reporter plasmid (1.25ug) alone or in combination with LXRα, RXRα and SMRT (0.5ug each). The effects of exogenously added 9-*cis* RA and 22R-OH-CH, alone or in combination were investigated. As demonstrated in Figure 24A, SMRT, in the absence and presence of both 9-*cis* RA and/or 22(R)-OH-CH, had no significant effect on LXRα/RXRα-mediated transactivation.

A second preliminary transfection experiment was carried out in order to determine the effect of SMRT titration, in increasing amounts, on LXRα/RXRα transactivation. Once again, transient transfections were performed in BSC40 cells in the presence of the pDR4(X2)luc luciferase reporter plasmid (1.25μg) alone or in combination with LXRα

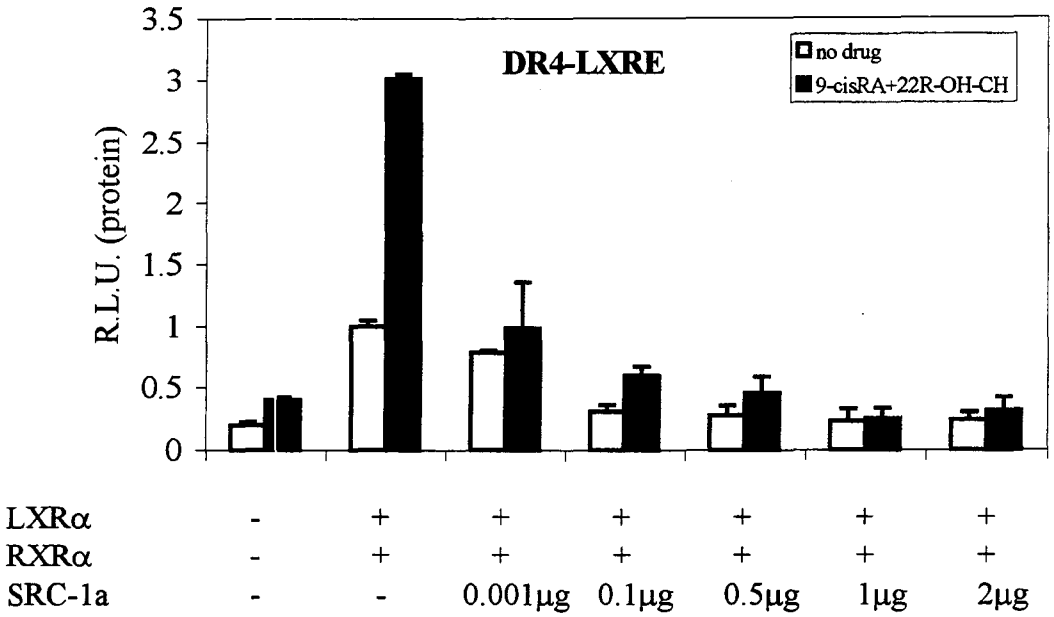
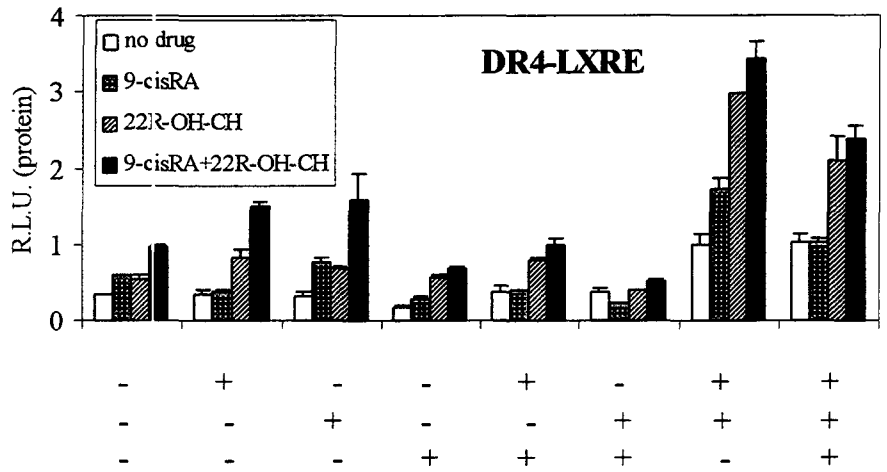


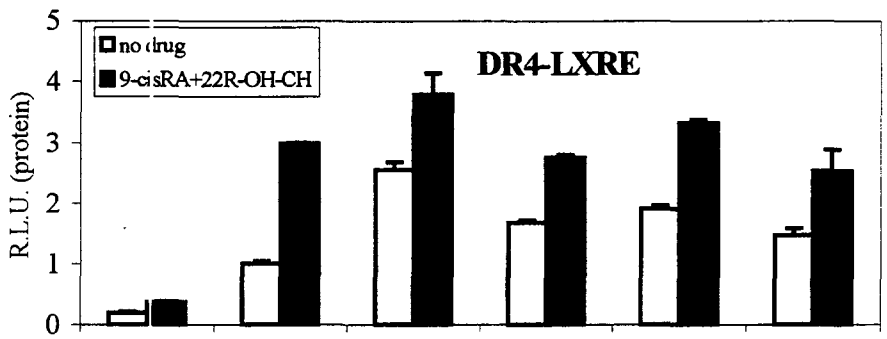
Figure 23: Transient transfection assays demonstrating that SRC-1a antagonizes transactivation mediated by LXRα/RXRα *in vivo* in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with the pDR4(X2)luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for LXRα and RXRα (0.5 μg each) with increasing amounts of the SRC-1a expression vector (0.001-2 μg). Where indicated, the LXRα activator 22(R)-OH-CH (final concentration 7 μM) and the RXRα ligand 9-*cis* RA (final concentration 1 μM) were added together. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent the average (±SD) of two separate experiments, performed in duplicate, and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

(A)



LXRα	-	+	-	-	+	-	+	+
RXRα	-	-	+	-	-	+	+	+
SMRT	-	-	-	+	+	+	-	+

(B)



LXRα	-	+	+	+	+	+
RXRα	-	+	+	+	+	+
SMRT	-	-	0.1μg	0.5μg	1μg	2μg

Figure 24: Preliminary transient transfection assays demonstrating that SMRT has no significant effect on transactivation mediated by LXRα/RXRα *in vivo* in the BSC40 cell line. BSC40 cells were transfected, utilizing the calcium phosphate method, with either the pDR4(X2) luc (1.25 μg) luciferase reporter plasmid alone or in combination with expression vectors for (A) LXRα, RXRα or SMRT (0.5 μg each) or (B) LXRα and RXRα (0.5μg each) with increasing amounts of the SMRT expression vector (0.1-2 μg). Where indicated, the LXRα activator 22(R)-OH-CH and RXR ligand 9-*cis* RA (final concentration 7μM and 1μM resepectively) were added. The lysates were assayed for luciferase activity in relative light units (R.L.U.) and adjusted to protein content. Values represent one experiment, performed in duplicate, where the error is represented as the range of values and normalized to the activity observed with LXRα/RXRα, in the absence of drug, which was set to one.

and RXRα (0.5 μg each) and increasing amounts of SMRT (0.1μg to 2μg). As illustrated in Figure 24B, SMRT had no significant effect on LXRα/RXRα-mediated transactivation in the presence of both 9-*cis* RA and 22(R)-OH-CH, although some minor fluctuations in activation levels are apparent. However, in the absence of drug, SMRT appeared to increase transactivation levels approximately two-fold, regardless of the SMRT concentration employed. The significance of this observation remains to be seen as further study is clearly required.

CHAPTER FOUR

DISCUSSION

4.1 Transient Transfection Studies of LXRα

Studies of the transactivation properties of LXRα *in vivo* were performed with either a DR4-LXRE or an LXRE-ΔMTV containing reporter construct (the pDR4(X2)luc and TK-LXRE3-Luc reporter constructs respectively). A direct comparison between the DR4-LXRE and LXRE-ΔMTV elements was not possible as they differ in DNA response element copy number, configuration and promoter context. Nonetheless, the effectual differences between the DR4-LXRE and the LXREΔMTV constructs were demonstrated *in vivo* in response to not only exogenously added LXRα and RXRα but also exogenously added 9-*cis* RA in a variety of cell lines.

The most dramatic difference observed for the DR4-LXRE and LXRE-ΔMTV constructs was demonstrated *via* studies with the LXRE-ΔMTV construct, which illustrated that transfection of LXRα resulted in transactivation levels that were comparable to that observed when LXRα was co-transfected with RXRα with certain exceptions (for the BSC40 cell line in the absence of drug and in the HepG2 cell line in the absence and presence of 22(R)-OH-CH, where a 50% increase in activation levels were achieved upon co-transfection with RXRα). In contrast, the DR4-LXRE construct, regardless of the cell line employed, did not result in any significant increase in transactivation levels above basal levels upon transfection with LXRα, whereas co-

transfection with RXRα resulted in a substantial increase. These observations may be attributable to the possibility that the LXRα/RXRα heterodimer preferentially binds to the LXRE-ΔMTV construct over the DR4-LXRE construct *in vivo*, although no difference in binding affinity has been observed *in vitro* for the DR4-LXRE and LXRE-ΔMTV response elements. Alternatively, the endogenous RXRα stores may be sequestered by binding to other nuclear receptors and/or co-factors which form non-functional complexes with the DR4-LXRE element not formed in the presence of the LXRE-ΔMTV element.

The mammalian cell lines employed in this study harbor endogenous nuclear receptors and co-factors, which can interact with LXRα, RXRα and the reporter constructs and thus provide a potential explanation for the difference observed upon transfection of RXRα with the reporter constructs. Studies with the DR4-LXRE reveal that transfection of RXRα results in transactivation levels that are considerably higher than those achieved with the LXRE-ΔMTV construct, regardless of cell line or drug conditions investigated. Thus, in this scenario, RXRα heterodimerizes with endogenous nuclear receptors, such as TR, which co-operatively bind to and subsequently activate the DR4-LXRE reporter construct. In contrast, binding of the RXRα/endogenous nuclear receptor complex may or may not be possible on the LXRE-ΔMTV element and those that are capable of binding, such as the LXRα/TR complex, may not be functional.

Another significant difference exhibited by the two LXRE constructs is their variant response in the presence of retinoids. Investigation of LXRα/RXRα-mediated

transactivation *via* the DR4-LXRE element revealed that this reporter construct lacked retinoid responsiveness, as illustrated upon addition of 9-*cis* RA, in both the COS-1 and HepG2 cell lines; however a slight response was observed in the BSC40 cell line. In comparison, the LXRE-ΔMTV proved to be retinoid responsive when transfected with LXRα regardless of the cell lines employed. Co-transfection of LXRα and RXRα, however, resulted in retinoid responsiveness for only the HepG2 cell line, whereas addition of 9-*cis* RA had no effect on transactivation mediated by LXRα/exogenous RXRα in the BSC40 cell line and a repressive effect in the COS-1 cell line. Similar studies have demonstrated that the LXRα/RXRα heterodimer on the DR4-LXRE lacks retinoid responsiveness, as illustrated upon addition of the retinoid methoprene acid, while the LXRE-ΔMTV response element is responsive, in the CV-1 cell line (Willy *et al.*, 1995). While the observations, reported in this study, may be due to the different promoter contexts, number of LXRE copies or configurations of these reporter constructs, these variables were shown to be irrelevant for the CV-1 cell line (Willy *et al.*, 1995). This transcriptional response mediated by the LXRα/RXRα heterodimer has been shown to require only the activation domain of LXRα and not that of RXRα (Willy and Mangelsdorf, 1997), which has been attributed to the phantom ligand effect (Schulman *et al*, 1997). This effect has been described as a unique form of allosteric control and is thought to occur as a result of the binding of ligand to one of the heterodimeric partners (RXRα in this case), resulting in a linked conformational change in the second heterodimer partner (LXRα) and the concomitant dissociation of bound co-repressors and subsequent association of co-activators. Ultimately, these studies indicate that retinoid

signaling, through the LXRα/RXRα heterodimer, appears to occur in an LXRE construct and cell line-specific manner and indicates that different cell lines may posses various factors, such as other nuclear receptors, co-factors and agonists/antagonists, which can either interfere with or enhance retinoid activation.

The transient transfection studies of LXRα further reveal that LXRα displays constitutive activity regardless of the cell line or LXRE reporter construct employed. This constitutive activity may be attributable to the presence of a functional AF-1 domain within the A/B domain of LXRα as reported for other nuclear receptors (Nagpal *et al.*, 1993, Wilkinson and Towle, 1997, Werman *et al.*, 1997, Metzger *et al.*, 1995, Sjöberg and Vennström, 1995, and reviewed in Schoonjans *et al.*, 1996). Alternatively, such activity may be due to the presence of endogenous LXRα activators/ligands as described for LXRα in the CV-1 cell line (Forman *et al.*, 1997). While these two possibilities are not mutually exclusive, three main factors have been presented in this study and in the Forman and colleagues (1997) study, which, taken together, indicate that the latter possibility is the more probable of the two. First, LXRα/RXRα-mediated constitutive activity of both the DR4-LXRE and LXRE-ΔMTV reporter constructs was not significantly enhanced upon the addition of the LXRα activator 22(R)-OH-CH, in either the COS-1 or HepG2 cell lines, indicating the presence of endogenous ligand. In the case of the BSC40 cell line, a modest 2-fold increase in activation is observed upon addition of 22(R)-OH-CH and may be attributable to sub-optimal levels of endogenous LXRα ligand. Second, LXRα A/B domain deletion studies, as outlined in Section 3.2, failed to

demonstrate a role for the LXRα A/B domain in transactivation and thus, further indicated the absence of a functional AF-1 domain within the N-terminal 63 amino acids of LXRα, although the C-terminal 34 amino acids of the A/B domain have yet to be investigated. Third, studies performed by Forman and colleagues (1997), report that the observed constitutive activity is mediated by the LBDs of both the LXRα and RXRα receptors. Furthermore, transfection studies, performed in the CV-1 cell line, presented in this report further indicate that this activity is dependent upon MVA biosynthesis and, in turn, upon the presence of MVA itself or MVA metabolites (including 22(R)-OH-CH), which may function as endogenous LXRα ligands.

4.2 LXRα A/B Domain Deletion Studies

4.2.1 EMSA Analysis

The EMSA studies with the LXRα and the LXRα A/B domain deletions demonstrated that the N-terminal 63 amino acids of LXRα are dispensable for LXRα/RXRα heterodimer complex formation with either the DR4-LXRE or the LXRE-ΔMTV elements. Thus, the observed decrease in complex formation observed for the SM1/RXRα heterodimer, on either of the LXREs, is attributable to the non-native N-terminal extension piece, which may induce a conformational change in LXRα that alters DNA binding specificity and/or RXRα heterodimer formation ability. While the components of the Shift B complex are not known it is possible that this shift is a ternary complex consisting of LXRα/RXRα and an endogenous co-factor in the rabbit reticulocyte lysate. Thus, although the biological significance of Shift B is questionable,

its formation appears to be specific and dependent upon not only the presence of LXRα and RXRα but also an intact LXRα A/B domain.

4.2.2 Transfection Studies of LXRα and the LXRα A/B Domain Deletions

The transient transfection studies of LXRα and the LXRα A/B domain deletions, regardless of the LXRE construct or cell line employed, failed to demonstrate a functional role for the N-terminal 63 amino acids of the LXRα A/B domain in transactivation. Furthermore, considering the limitations of the experiments performed, these studies imply that a functional AF-1 domain is absent in this region of the LXRα A/B domain. This observation may be attributable to a number of factors; first, the LXRα A/B domain does not participate in LXRα-mediated transactivation. Second, the LXRα A/B domain does participate in transactivation, however, function resides in the 34 amino acids that remain uninvestigated within the A/B domain of LXRα. Third, detection of a functional LXRα A/B domain, which may or may not reside in the regions spanning the deletion series, could be cell line or cell type-specific as observed for a number of other nuclear receptor A/B/ AF-1 domains (Berry *et al.*, 1990, Nagpal *et al.*, 1992, Nagpal *et al.*, 1993, Metzger *et al.*, 1995, Ikonen *et al.*, 1997, Wilkinson and Towle, 1997). In this scenario, the A/B domain effect may be masked by the presence of endogenous LXRα ligands (MVA metabolites) and thus detection may require a different cell line or cell type. Furthermore, the cell lines employed may lack co-factors, which would facilitate a potential AF-1/AF-2 synergism and/or AF-1 activity or, alternatively, possess co-factors that inhibit this activity/interaction. As well, although there is no

evidence that LXRα is phosphorylated (although a putative PKC recognition sequence has been located in the LXRα/RLD-1hinge region) it remains a possibility that the cells lines employed are unable to modify or, alternatively do modify, the LXRα A/B domain (or other regions) rendering the A/B domain inactive as reported for other nuclear receptors (Kato *et al.*, 1995, Rochette-Egly *et al.*, 1997, Adams *et al.*, 1997). Fourth, a number of studies with other nuclear receptors have demonstrated that A/B (AF-1) domain activity may be DNA response element and promoter context-specific (Berry *et al.*, 1990, Nagpal *et al.*, 1992, Nagpal *et al.*, 1993, Metzger *et al.*, 1995, Sjöberg and Vennström, 1995, Ikonen *et al.*, 1997, Wilkinson and Towle, 1997). Thus, it may be possible that LXRα A/B activity may require naturally occurring promoter contexts and/or DNA response elements, such as the CYP7a promoter and DNA response element.

Ultimately, further study of the LXRα A/B domain is required in order to investigate the possibility that the remaining 34 amino acids of the LXRα A/B domain may play a role in LXRα-mediated transactivation. Furthermore, the question as to whether or not LXRα possesses a functional AF-1 domain, within the A/B domain, remains to be properly addressed. While the studies presented here indicate the absence of such a domain, definitive identification would require that transient transfection assays be performed that employ a plasmid construct containing the yeast GAL4 DBD upstream of the isolated LXRα A/B domain and a reporter plasmid containing the yeast GAL4 response element(s). If indeed the A/B domain possesses a functional AF-1 domain, the LXRα A/B/GAL4 fusion construct would activate transcription of the GAL4-reponsive

reporter gene as previously demonstrated for PPARγ, RAR, RXR and TR (Nagpal *et al.*, 1993, Werman *et al*, 1997, Wilkinson and Towle, 1997).

4.3 Interaction and Functional Studies of LXRα and RIP 140

4.3.1 LXRα Interaction Studies with RIP 140 *In Vitro*

In vitro GST-binding studies confirmed the LXRα/ RIP 140 specific interaction, initially illustrated *via* the yeast dihybrid system. Furthermore, these studies revealed that the LXRα/RIP 140 interaction occurred in a ligand/activator-independent manner, as illustrated for the PPAR/RIP 140 interaction (Miyata *et al.*, 1998, Treuter *et al.*, 1998). Furthermore, LXRα demonstrated an ability to bind either subdomain of RIP 140 (site 1 and site 2), with an apparent preference for site 2, in a ligand/activator-independent manner. In contrast, previous reports have demonstrated that interactions between RIP 140 and other nuclear receptors, including RXR (also illustrated in this study), RAR, TR and ER, occur in a ligand-dependent or enhancing manner (Cavaillès *et al.*, 1995, L'Horset *et al.*, 1996). These observations may be indicative of the existence of various mechanisms that dictate the manner in which RIP 140 interacts with various nuclear receptors. Alternatively, structural differences may exist between the unliganded LBD, specifically the positions of helices 3, 4, 5 and 12, of LXRα (and PPAR) and other nuclear receptors (Renuad *et al.*, 1995, Bourget *et al.*, 1995), which constitutes a constitutively active, although potentially unstable, conformation, thereby facilitating RIP 140 ligand-independent interactions. In this scenario, the presence of ligand may serve to stimulate the displacement of bound co-repressors, which results in the subsequent exposure of a co-activator interaction site and may also serve to stabilize the

active conformation (Miyata *et al.*,1998, Treuter *et al.*, 1998) (refer to Figure 25 for proposed model).

4.3.2 Transfection Studies of LXRα, RXRα and RIP 140

Transient transfection studies of LXRα, RXRα and RIP 140, revealed that RIP140, regardless of the LXRE construct or cell line employed, antagonized LXRα/RXRα-mediated transcriptional activation *in vivo*. This RIP 140-mediated repression of transcriptional activity has also been reported for both RAR (Chakravarti *et al.*, 1996) and PPAR (in both mammalian and yeast cells) (Miyata *et al.*, 1998, Treuter *et al.*, 1998). These observations, however, are in contrast to previous reports, which indicate that RIP 140 acts as a *bona fide* co-activator when tethered directly to a promoter (Cavaillès *et al.*, 1995). As well, unpublished data from our laboratory indicates that RIP 140 activates PPARα-mediated transactivation in yeast cells. Furthermore, transfection studies of RIP 140 and ER, as well as studies performed in yeast, demonstrate an increase in activation; however, only minimal activation was achieved and was subsequently repressed as RIP 140 concentrations are increased (Cavaillès *et al.*, 1995, Joyeux *et al.*, 1997). The observed RIP 140-mediated repression of transcriptional activity may be attributable to the sequestration of downstream effector molecules by RIP 140. Alternatively, RIP 140 may function as a very weak co-activator of LXRα; however RIP 140 may compete with stronger co-activators, including SRC-1 and CBP/p300, for nuclear receptor binding and therefore, antagonism of LXRα transactivation is observed in the system employed. Nonetheless, despite the similarities between RIP 140 and other co-activators, a true 'co-activator' role for RIP 140 has come under question for a number of reasons; first, RIP

140 has demonstrated an inability to functionally substitute for SRC-1 (Torchia *et al.*, 1997). Second, RIP 140 has been shown to inhibit the interaction between the N-terminal and C-terminal regions of the AR receptor, and thus may inhibit AF-1/AF-2 mediated synergism (Ikonen *et al.*, 1997). This observation is contrasted by reports of other co-activators, such as SRC-1 and CBP/p300, which facilitate this interaction resulting in transcriptional activation (Kraus *et al.*, 1995, Ikonen *et al.*, 1997, Oñate *et al.*, 1998, Webb *et al.*, 1998). Third, RIP 140 fails to interact with not only CBP (Kamei *et al.*, 1996) but also to basal transcription factors such as TBP and TFIIB (Cavaillès *et al.*, 1995), which indicates an inability to function as a co-activator/bridging molecule with the transcription initiation complex. Thus, these concepts, in conjunction with the observations reported in this study, indicate that RIP 140 acts to attenuate the transcriptional response of nuclear receptors by preventing functional interactions with other, more potent co-activators.

4.3.3 Interaction and Functional Studies of RIP 140 and RIPΔ3

The *in vitro* GST-binding studies of RIP 140 with RIPΔ3 demonstrated that the N-terminal 63 amino acids of the LXRα A/B domain were dispensable for RIP 140 interaction. Similarly, transient transfection studies of RIPΔ3, RXRα and RIP 140 illustrated that deletion of up to 63 amino acids of the LXRα A/B domain had no effect on RIP 140-mediated repression of LXRα/RXRα transactivation. Taken together, these observations may indicate that RIP 140, in its inability to interact with the N-terminal domain and therefore its inability to facilitate a functional interaction between the N- and

C-terminal regions of LXRα, serves to attenuate the transcriptional response of nuclear receptors modulated by other, more potent, co-activators.

4.3.4 LXRα AF-2 Core Domain Deletion Studies with RIP 140

In vitro GST-binding studies illustrated the ability of RIP 140 to bind to the LXRα AF-2 domain (helix 12) deletion construct, although to a lesser extent than that achieved with full-length LXRα. These results indicate that RIP 140 requires the LXRα AF-2 core domain for efficient binding, however, it is apparent that other regions of LXRα participate in RIP 140 binding (which do not include the N-terminal 63 amino acids of the LXRα A/B domain). These findings are consistent with a previous study, which reported that while the AF-2 domain of PPAR was necessary for both RIP 140 and SRC-1 interaction, other regions of the PPAR LBD contributed to these interactions (Treuter *et al.*, 1998). Ultimately, these observations are also consistent with a current model that describes the formation of a compacted nuclear receptor LBD, *via* interactions with helices 3, 4, 5 and 12, which constitutes a hydrophobic groove that binds the LXXLL motifs of co-activators, which is thought to occur consequence of ligand binding (Nolte *et al.*, 1998, Darimont *et al.*, 1998). However, as previously described for LXRα and PPAR, this may not always be the case as the AF-2 domain of LXRα may exist in an active, though potentially unstable, conformation, which is subsequently stabilized by binding of ligand (refer to Figure 25).

Future studies of the RIP 140 interaction with LXR will undoubtedly include the identification of other regions within LXRα that are important for binding. Further LXRα AF-2 deletion studies are also required in order to determine the effect of the

addition of the LXRα potent activator, 22(R)-hydroxycholesterol, which may also be facilitated by the identification of LXRα ligands.

4.4 LXRα Interaction Studies with SRC-1a and SMRT *In Vitro*

In vitro GST-binding assays revealed that LXRα was able to interact with the co-activator SRC-1a in a ligand/activator-independent manner, where the addition of 22(R)-OH-CH had no apparent enhancing effect as previously demonstrated for the SRC-1/PPARγ interaction (Zhu *et al.*, 1996) and the p300/PPARα interaction (Dowell *et al.*, 1997). These observations may be explained with the aforementioned model, where the LXRα LBD helices 3, 4, 5 and 12 exist in an active (unstable) conformation and the presence of ligand serves not only to stabilize the active conformation but also to expose a co-activator interaction site by displacing bound co-repressors. The *in vitro* GST-binding assays, which investigated the LXRα/SMRT interaction, demonstrated that this interaction occurred in the absence of ligand/activator as observed for other nuclear receptor/co-repressor interactions. However, the addition of the LXRα activator, 22(R)-OH-CH, did not abolish nor reduce the LXRα/SMRT interaction. Furthermore, 22(R)-OH-CH failed to enhance either the RIP 140/LXRα or the SRC-1a/LXRα interaction. Taken together, these observations indicate that the potent LXRα activator, 22(R)-OH-CH, may not function as a 'true' LXRα ligand and that a derivative of this compound (or other MVA metabolites) may serve as an LXRα ligand. Nonetheless, it is apparent from these *in vitro* studies that 95% ethanol disrupts both SRC-1a/LXRα and SMRT/LXRα (and RIP 140/LXRα to a lesser extent) interactions and, therefore, it remains a possibility

that the presence of ethanol masks any 22(R)-OH-CH enhancing or inhibiting effect respectively.

4.5 LXRα AF-2 Core Domain Deletion Studies with SRC-1a and SMRT

In vitro GST-binding studies illustrated the inability of SRC-1a to bind to the LXRα AF-2 domain (helix 12) deletion construct. These results indicate that the LXRα AF-2 core domain is essential for efficient SRC-1a binding; however, it remains a possibility that other regions of LXRα participate in this interaction as reported for other nuclear receptor interactions including PPAR (Treuter *et al.*, 1998), TR (Jeyakumar *et al.*, 19970, and ER (White *et al.*, 1997, Henttu *et al.*, 1997). Ultimately, these observations are consistent with the concept that mutation and/or deletion of the AF-2 domain (helix 12) may destabilize the compact LBD conformation resulting in the disruption of the co-activator LXXLL-motif interacting hydrophobic core (Nolte *et al.*, 1998, Darimont *et al.*, 1998).

In contrast, in *vitro* interaction studies of SMRT and both LXRα and the LXRα AF-2 domain deletion GST fusion proteins, surprisingly demonstrated that the SMRT/LXRα AF-2 deletion interaction was significantly greater in affinity than the SMRT/LXRα interaction, as previously reported for the RAR/SMRT interaction (Wong *et al.*, 1998). This observation is consistent with the concept that co-repressor binding occurs when the LBD exists in an extended conformation (helix 12 is extended away from the other helices 3, 4, and 5), which usually occurs in the absence of ligand (Chen *et al.*, 1996, Lavinsky *et al.*, 1998). Thus, this study mimics this effect *via* deletion of the LXRα AF-2 domain, where otherwise the LXRα LBD may exist in a constitutively active (unstable)

conformation, thereby impeding the SMRT/LXRα interaction. Furthermore, these observations are also consistent with a model for LXRα where addition of ligand, which may not alter the position of helix 12 directly, may serve to induce a conformational change, which displaces bound co-repressors such as SMRT. Nonetheless, these results also indicate that other regions of LXRα are important for SMRT interaction, which may include the hinge region (domain D) and other areas of the LBD, as reported for other nuclear receptor/co-repressor interactions (Hörlein *et al.*, 1995, Kurokawa *et al.*, 1995, Chen and Evans, 1995).

Clearly, future studies would include the investigation of the effect of 22(R)-hydroxycholesterol on both the LXRα/SCR-1a and LXRα/SMRT interactions, the identification of other regions within LXRα that participate in binding and the effect of other LXRα ligands, yet to be identified, on these LXRα/co-factor interactions.

4.6 Transfection Studies of LXRα, RXRα and SRC-1a

Transient transfection studies of LXRα, RXRα and SRC-1a, revealed that SRC-1a, antagonized LXRα/RXRα-mediated transcriptional activation *in vivo*, despite its classification as a co-activator. While this observation initially seems surprising, this result may be attributable to several factors; first, the SRC-1a clone employed in these studies lacks the bHLH/PAS domain of full length SRC-1 (F-SRC-1) and thus may be incapable of binding in a functional manner as previously reported for the AR receptor where the SRC-1a clone prevented the N- and C-terminal domains of AR from interacting, thereby repressing AR-mediated activation (Ikonen *et al.*, 1997). In contrast, F-SRC-1 was shown to facilitate this interaction resulting in a potentiation of AR-

mediated transcriptional activation (Ikonen *et al.*, 1997). Second, the observed repression may be DNA response element or promoter context-specific as observed for the ER receptor (Kalkoven *et al.*, 1998). This study demonstrated that ER-mediated transcriptional activation was only slightly increased by F-SRC-1a (in comparison to the F-SRC-1e isoform) on one ERE/promoter context while another ERE/promoter context resulted in severe repression of ER-mediated activation. Thus, the repression reported in this study may be promoter context/DNA element-specific and a naturally occurring promoter context (possibly the rCYP7A promoter) and/or LXRE-CYP7a response element may be necessarily employed in order to achieve potentiation of activation by SRC-1. Third, it has been demonstrated that SRC-1 isoforms are functionally distinct and, therefore, it is possible the SRC-1e isoform (or other isoforms)may preferentially activate LXRα as described for the ER receptor (Kalkoven *et al.*, 1998). Thus, further investigation of the role that SRC-1 plays in LXRα-mediated transactivation may include transient transfection studies that employ full length SRC-1a, different LXREs and promoter contexts, various cell lines and other SRC-1 isoforms.

4.7 Transfection Studies of LXRα, RXRα and SMRT

Preliminary transient transfection studies of LXRα, RXRα and SMRT revealed that, SMRT had no significant effect on LXRα/RXRα-mediated transactivation, despite the fact that LXRα and SMRT were shown to interact with one another in GST-binding assays (Section 3.3.8). This observation has been previously reported by Zamir and colleagues (1997), where it was demonstrated that although SMRT 9and N-CoR) may be capable of binding to various nuclear receptors, including PPARγ and the orphan receptor

RevErb, in solution, SMRT may not function as a transcriptional repressor as these receptors are unable to bind these co-repressors when bound to their respective DNA response elements. Thus, further study of a potential LXR/SMRT functional interaction would require investigation of the ability of LXRα/RXRα heterodimers to recruit SMRT when bound to various LXREs. Furthermore, continuation of the transient transfection studies are required in order to not only definitively address the possibility that SMRT may modulate LXRα-mediated transactivation but also to determine the significance of the observed increase in ligand-independent transcriptional activity upon transfection of LXRα, RXRα and SMRT in the titration experiment.

4.8 Conclusion

In summary, the results presented in this study have provided insight into not only how LXRα functions as a transcriptional regulator of target genes, with respect to LXRα A/B and LBD (E domain) function, but also the complex mechanism by which co-factors, such as RIP 140, SRC-1a and SMRT modulate LXRα activation. Furthermore, this study indicates that current models, which describe the mechanism by which co-activators and co-repressors respectively facilitate or inhibit the transcriptional activation mediated by nuclear receptors, may require specific modifications in order to adequately delineate LXRα-mediated transactivation, as depicted in Figure 25. Figure 25A presents a simplified version of the current model, which illustrates that, in the absence of ligand, nuclear receptors, whose AF-2 domain (helix 12) exists in an inactive, extended conformation, associates with the co-repressor complex, which blocks an interaction between these receptors and the basal transcription machinery and causes the

deacetylation of core histones, thereby repressing the basal activity of target genes. The binding of ligand results in a conformational change that causes the disassociation of the bound co-repressor complex and results in a shift of the AF-2 domain form an extended to a compacted conformation with other helices of the LBD, which results in the formation of a co-activator interaction surface (actually an LXXLL, hydrophobic binding pocket). Recruitment of the co-activator complex follows, *via* its LXXLL motifs, which facilitates both interactions between the nuclear receptors and basal transcription machinery and acetylation of core histones, resulting in the transactivation of target genes. A proposed model depicting the mechanism by which co-factors modulate LXRα-mediated activity is presented in Figure 25B. While quite similar to the aforementioned model, this LXRα model differs in that the AF-2 domain is in an active, yet unstable, conformation, which nonetheless associates with a co-repressor complex, resulting in the repression of LXRα-mediated transactivation. The addition of ligand, in this case, serves to not only disassociate the co-repressor complex, but also to stabilize the existing AF-2 domain in its active, compacted conformation. Subsequent association of a co-activator complex, yet to be fully delineated for LXRα, follows, which results in the strong transcriptional activation of LXRα target genes. Alternatively, RIP 140 may be recruited, which may result in weak transactivation, thus attenuating the transcritpional response of LXRα by preventing functional interactions with other, more potent co-activators. However, how RIP 140 interacts with the basal transcription machinery has yet to be described, as studies have demonstrated that RIP 140 fails to interact with CBP, TBP and TFIIB.

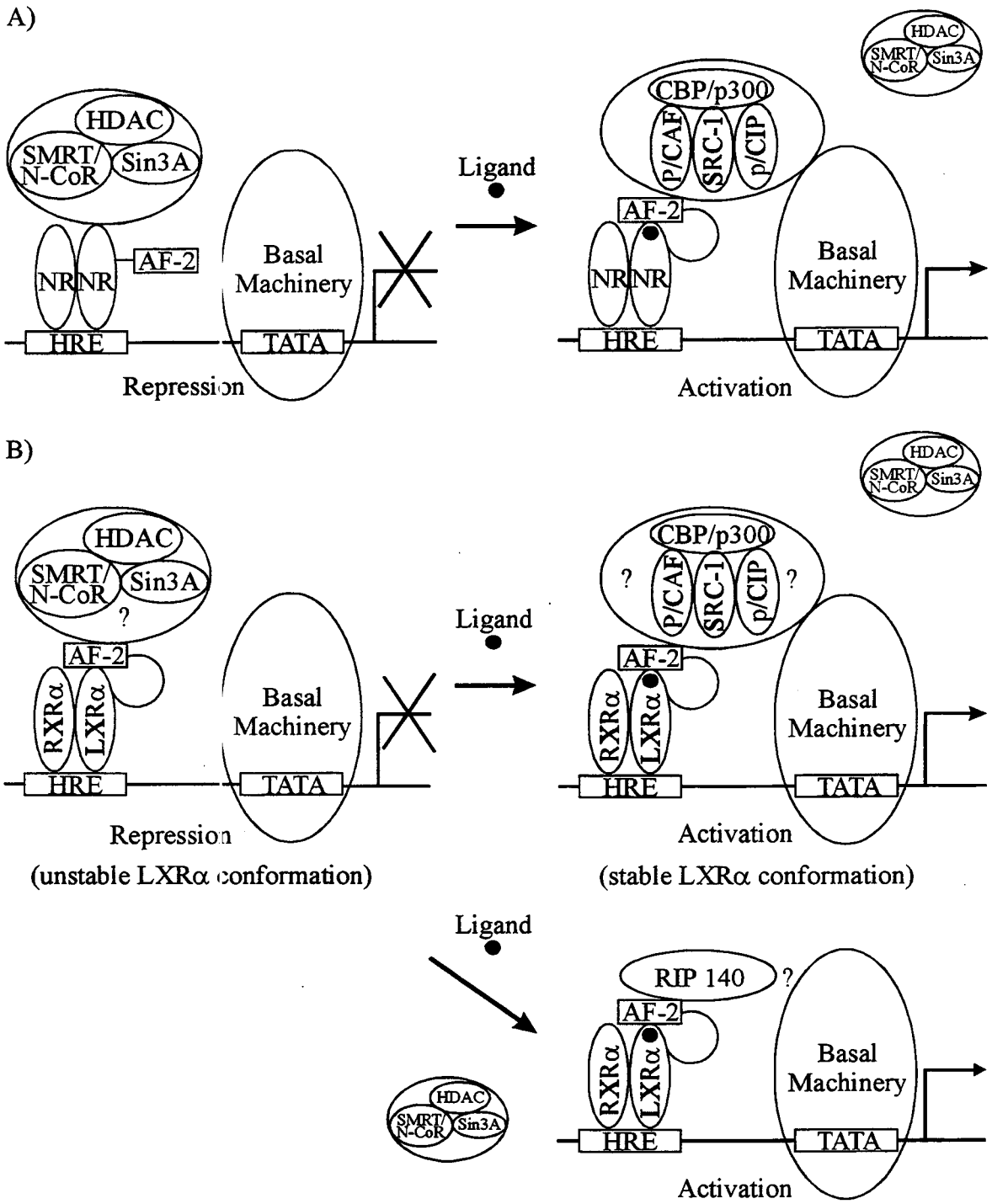


Figure 25: Model depicting the mechanism by which co-factors modulate the transcriptional activation mediated by A) members of the nuclear hormone receptor superfamily or B) LXRα. Adapted from Chen et al., 1996, Schulman et al., 1996, Korzus et al., 1998 and Westin et al., 1998. Refer to text for details.

Nonetheless, this proposed model for LXRα-mediated transactivation , is consistent with the observation that, in a cell free system, co-activators (and co-repressors) are capable of binding to LXRα in a ligand-independent manner as the LXRα AF-2 domain exists in a conformation which constitutes a co-activator interaction surface/LXXLL hydrophobic binding pocket. Furthermore, this model may also explain the observation that deletion of the LXRα AF-2 domain enhanced SMRT binding, as this may have exposed other SMRT/co-repressor interaction sites blocked by the LXRα AF-2 domain in its compacted, active, though unstable, conformation. Clearly, LXRα crystallographic studies, in conjunction with the studies presented here and those suggested throughout the discussion, will facilitate the delineation of the mechanisms by which LXRα-mediated transactivation is modulated and, in turn, how LXRα mediates transcriptional control of LXRα target genes. Ultimately, these LXRα investigations further our understanding of how nuclear receptors achieve specificity and diversity, *via* differences in DNA response elements, promoter contexts, ligands and co-factor binding, in the activation of target gene expression.

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