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Novel Insight into the Role of LXRα in Metabolic Regulation via DNA Binding as a Heterodimer with PPARα and as a Homodimer

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NOVEL INSIGHT INTO THE ROLE OF LXRα IN METABOLIC REGULATION VIA DNA BINDING AS A HETERODIMER WITH PPARα AND AS A HOMODIMER

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

ANDREA M. KLINGLER
B.S. Biological Sciences, Wright State University, Dayton, OH 2013

2016
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Andrea M. Klingler ENTITLED Novel Insight Into the Role of LXRα in Metabolic Regulation via DNA Binding as a Heterodimer with PPARα and as a Homodimer BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Klingler, Andrea M. M.S., Department of Biochemistry and Molecular Biology, Wright State University, 2016. Novel Insight into the Role of LXRα in Metabolic Regulation via DNA Binding as a Heterodimer with PPARα and as a Homodimer.

Liver X receptor α (LXRα) plays a critical role in the maintenance of energy homeostasis within a cell through tight transcriptional regulation of genes involved in metabolism of lipids, glucose, and cholesterol. Although LXRα has been established to function as a heterodimer with the retinoid X receptor α (RXRα), recent studies have determined that LXRα also interacts directly with peroxisome proliferator-activated receptor α (PPARα). However, little is known regarding the functionality of this heterodimer, if any exists at all. This study determined that a heterodimer of PPARα and LXRα is capable of binding to candidate response elements in vitro with high affinity by electrophoretic mobility shift assays and quenching of intrinsic protein fluorescence. Additionally, LXRα exhibited high affinity binding to DNA in the absence of a heterodimer partner, suggesting homodimeric interaction. Transactivation assays indicated that overexpression of PPARα and LXRα significantly increased activity of the endogenous APOA1 promoter, and overexpression of LXRα alone resulted in increased activity of all of the promoters tested, often even more so than LXRα/RXRα. These results provide new insight into the scope of metabolic regulation by LXRα, and raise important questions and considerations when targeting these proteins for treatment of metabolic disorders.
# TABLE OF CONTENTS

## I. INTRODUCTION

- Nuclear Receptors as Transcription Factors
  - 2
- PPAR
  - 5
- LXR
  - 8
- PPAR and LXR: Cross-talk and Heterodimerization
  - 10

## II. GOALS AND HYPOTHESES

## III. MATERIALS AND METHODS

- Candidate Response Element Design
  - 14
- Protein Expression and Purification
  - 16
- Electrophoretic Mobility Shift Assays
  - 17
- Binding Assays
  - 20
- Candidate Response Element Validation and Location
  - 21
- Gene Selection and Construction of Luciferase Reporter Plasmids
  - 21
- Transactivation Assays
  - 28
IV. RESULTS 29-55

- PPARα/LXRα binds candidate REs in vitro .......................... 29
- PPARα/LXRα and LXRα alone bind candidate REs with nanomolar affinity .......................................................... 34
- Candidate REs occur naturally in the genome ...................... 43
- PPARα/LXRα and LXRα alone transactivate endogenous promoters .......................................................... 45

V. DISCUSSION 56-64

VI. LIST OF ABBREVIATIONS 65

VII. REFERENCES 66-75
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Schematic representation of nuclear receptor structure and function</td>
<td>5</td>
</tr>
<tr>
<td>2. Construction of luciferase plasmid inserts for APOA1 and CXCR5</td>
<td>25</td>
</tr>
<tr>
<td>3. Construction of luciferase plasmid inserts for SULT2A1 and SREBP-1c (L4RE only)</td>
<td>26</td>
</tr>
<tr>
<td>4. Construction of luciferase plasmid inserts for TNFRSF4 and TNFRSF18</td>
<td>27</td>
</tr>
</tbody>
</table>
| 5. Preliminary EMSAs of PPARα/LXRα with ABCG1 LXREs for buffer optimization.  
   (A) PPARα/LXRα binding to ABCG1 LXRE with Buffer 1 | 31 |
|   (B) PPARα/LXRα binding to ABCG1 LXRE with Buffers 2, 3, and 4 | 31 |
|   (C) PPARα/LXRα binding to ABCG1 LXRE with Buffer 5 | 31 |
| 6. EMSA of PPARα/LXRα with candidate response elements showing preference for DR4 element | 32 |
| 7. Supershift assay demonstrating presence of both PPARα and LXRα in complex with DNA | 33 |
| 8. Intrinsic fluorescence quenching of PPARα upon titration with candidate REs | 35 |
| 9. Intrinsic fluorescence quenching of PPARα upon titration with known |
10. Intrinsic fluorescence quenching of LXRα upon titration with candidate REs

11. Intrinsic fluorescence quenching of LXRα upon titration with known REs

12. Intrinsic fluorescence quenching of PPARα/LXRα upon titration with candidate REs

13. Intrinsic fluorescence quenching of PPARα/LXRα upon titration with known REs

14. Plotting PPARα/LXRα binding data on Hill coordinates suggests multiple binding sites

15. Genomic locations of the L4RE candidate response element relative to known PPARα and LXRα binding sites

16. Western blot showing overexpression of transiently transfected PPARα, LXRα, and RXRα

17. Transactivation of APOA1

18. Transactivation of CXCR5

19. Transactivation of SULT2A1

20. Transactivation of SREBP-1c (known PPRE and LXREs)

21. Transactivation of SREBP-1c (L4RE only)

22. Transactivation of TNFRSF4

23. Transactivation of TNFRSF18
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sequences of candidate PPARα/LXRα response elements</td>
<td>15</td>
</tr>
<tr>
<td>2. Composition of binding buffers for EMSA reactions</td>
<td>19</td>
</tr>
<tr>
<td>3. Primer sequences used in amplification of candidate target genes</td>
<td>24</td>
</tr>
<tr>
<td>4. Binding affinities of PPARα, LXRα, &amp; PPARα/LXRα for known &amp; candidate REs</td>
<td>42</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

Firstly, I would like to dedicate this work to the memory of my late mentor, Dr. Heather Hostetler, without whose incredible guidance, encouragement, and belief in my worth as a scientist none of this would have been possible, and who I could never thank enough for giving me a chance when no one else would.

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I. INTRODUCTION

Energy homeostasis is maintained in the body through tight regulation of the biochemical processes involved in metabolism. The importance of such regulation is evident in the instance of its failure, as is seen in metabolic disorders. As of 2012, 2.7% of US adults had been affected by cerebrovascular disease or stroke, 13.4% were affected by high cholesterol, 11.3% with heart disease, and 32.5% with hypertension. In addition, 11.9% of US adults were affected with diabetes as of 2010, and, as of 2012, 69% of US adults were overweight or obese, a condition which has been shown to contribute to the likelihood of the previously mentioned diseases and conditions. Since these conditions are among the top 10 causes of death in the United States, understanding the mechanisms behind the regulation of metabolic processes involved is of the utmost importance. Focus in this interest has been turned toward the proteins, known as nuclear receptors, which control elements of numerous pathways of metabolism, especially those which regulate levels of glucose, intracellular lipids, and cholesterol by acting as transcription factors and altering gene expression to achieve the desired effect. By teasing apart the regulatory mechanisms involved, it will be possible to achieve maintenance and/or prevention of these conditions more readily.
Nuclear Receptors as Transcription Factors

Nuclear receptors are ligand-activated transcription factors that alter the expression of genes in order to regulate cellular processes. They are distinct from membrane-bound receptors in that they are located in the interior of the cell, and thus are activated by small, hydrophobic molecules that are able to diffuse through the membrane into the cytosol, such as steroid hormones or other lipids. Nuclear receptors respond to changes in cellular metabolic requirements by binding a consensus sequence in genomic DNA in order to initiate transcription of target genes. To date, 48 individual nuclear receptor genes have been identified in the human genome, and they are further classified into six subfamilies based on sequence homology: the NR1 subfamily proteins are similar to the thyroid hormone receptor (TR), which includes the peroxisome proliferator-activated receptors (PPARs), the liver X receptors (LXRs), and the retinoic acid receptors (RARs). The NR2 subfamily is comprised of the retinoid X receptors (RXRs) and other receptors with similar properties, including hepatic nuclear factor 4 (HNF4). Hormone receptors such as the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) are grouped into the NR3 subfamily, with the remaining subfamilies having relatively few members of note. Additionally, identified receptors which cannot be easily classified are grouped into subfamily NR0. One example is the small heterodimer partner (SHP), which lacks a domain to bind DNA. Receptors are further classified into one of four types based on mechanistic action properties and their subcellular localization in relation to ligand binding, DNA binding and dimerization properties: Type I nuclear receptors include members of the NR3 subfamily, which bind their respective ligands and
form homodimers in the cytoplasm before translocating into the nucleus and then activate transcription at inverted repeat sequences known as hormone response elements (HRE). Type II nuclear receptors, which include members of the NR1 subfamily as well as RXR, are localized in the nucleus independently of ligand binding and bind direct repeat sequences as heterodimers. It is also noteworthy that the constitutive androstane receptor (CAR), a member of the NR1 subfamily, is ligand-independent and constitutively active. Type III receptors are similar to Type II in that they bind direct repeat sequences, but they have the requirement of forming homodimers, and include members of the NR2 subfamily, such as RXR. Finally, Type IV receptors bind only a single RE half-site, and are the only nuclear receptors that bind DNA as a monomer, although some are also capable of binding as a dimer.

Nuclear receptors exhibit a characteristic modular structure comprised of five domains linked to their function as ligand-activated, DNA-binding proteins. The N-terminal A/B domain of nuclear receptors is highly variable among family members, and has no three-dimensional crystal structure. This region has, nevertheless, been shown to be important as a site of post-translational modification [1] and carries out the protein’s ligand-independent transcriptional activation function [2]. DNA binding occurs at the highly conserved C region, or DNA-binding domain (DBD). This domain consists of two zinc-finger motifs, which exact the two functions necessary for successful DNA binding and transcriptional activation: recognition of and binding to RE sequences, and facilitation of dimerization between receptor partners. Located within the amino-terminal zinc finger is a region known as the proximal- or P-box, which consists of cysteine residues bonded to a zinc ion and surrounding residues, the sequence of which confers
response element sequence specificity. The carboxy-terminal zinc finger contains the distal-, or D-box region, which is involved in receptor dimerization. Separating the DBD from the ligand-binding domain is a flexible, less-conserved hinge region, which contains the receptor’s nuclear localization signal and allows for conformational changes in the surrounding domains. Activating ligands bind their respective receptor at the ligand-binding domain (LBD), which consists of alpha-helices and forms a hydrophobic “pocket” in which the ligand may fit. The size of the ligand binding pocket, intuitively, varies from receptor to receptor in order to accommodate each protein’s preferred ligand; for example, the ligand binding pocket of PPARα has a volume of 1177 Å³ [3], while that of LXRα is 700-800 Å³ [4]. Additionally, the ligand binding domain contains a dimerization interface and the ligand-dependent activation function. The C-terminal region of nuclear receptors is also a site of post-translational modification, and it acts as a “lid” over the ligand binding pocket upon ligand entry and binding.
Figure 1. Schematic representation of nuclear receptor structure and function.
Nuclear receptor proteins exhibit a characteristic modular structure consisting of five domains: N-terminal AF-1, DNA-binding domain (DBD), a flexible hinge region, ligand binding domain (LBD), and the C-terminal AF-2 domain. Type II receptors form permissive heterodimers with RXRα, and the DNA binding domains bind the half-sites of a response element to activate or repress transcription of target genes.

As previously mentioned, nuclear receptors alter gene expression by recognizing and binding their respective REs, which typically consist of hexameric repeats of direct, inverted, or everted orientation (DRx, IRx, or ERx, respectively). The nucleotide hexamers are separated by a small sequence of spacer nucleotides, generally 1 to 10 nucleotides in length and of variable sequence. The significance of the spacer nucleotides is not entirely clear. Further, nuclear receptors can use response elements to activate transcription regardless of whether the sequence is on the plus or minus strand. Response elements are located throughout the genome, primarily in promoter regions or other areas upstream of the coding sequences, although response elements have been characterized in
intrinsic regions as well. In particular, the ABCA1 gene has been shown to have a response element for LXRα in the promoter region, as well as another functional promoter and response element in the first intron. However, the role of this response element in eliciting a physiological effect was not determined [5]. Since extensive cross-talk exists between regulatory proteins, genes may have response elements for many different nuclear receptors. The hierarchy of control regarding genes containing multiple response elements is not necessarily the result of distance from the promoter, but is in fact based on several factors, such as tissue-specific expression of receptors, concentration of receptors and their respective ligands, and relative binding affinities. A well-known example of this, and one of particular relevance to this study, is the promoter region of SREBF1, which codes for the sterol regulatory element binding protein (SREBP-1c). This section contains two LXR response elements (LXRE) in addition to a PPAR response element (PPRE). Activation of its RE by LXRα results in upregulation of the gene, while PPARα activation represses SREBP-1c expression.

PPAR

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors that belong to the NR1 subfamily, which also includes the vitamin D receptor (VDR), farnesoid X receptor (FXR), and the RAR-related orphan receptor (ROR). Three isoforms of PPAR have been identified, which are coded for by separate genes: PPARα, PPAR(3/δ, and PPARγ. Owing to their tissue specificity, the three isoforms of PPAR bind distinct ligands and have roles in distinct processes. PPARα, cloned in 1990 [6] and
the first of the isoforms identified, is primarily found in the liver, heart, kidney, adrenal glands, and other tissues, and is a regulator of the body’s response to fasting, primarily as pertains to oxidation of lipids. PPAR(3/δ is ubiquitously expressed, and activated by synthetic compound GW501516, and also functions as a regulator of fatty acid oxidation, as well as electron transport chain uncoupling and thermogenesis. PPARγ is abundant in white adipose tissue as well as in macrophages, and its ligands include thiazolidinediones, a class of drugs used to increase insulin sensitivity in diabetic patients. The receptor functions in adipose tissue to regulate genes involved in adipogenesis and cell differentiation. As the predominant isoform present in highly metabolic tissues and a critical player in the body’s regulation of energy homeostasis, PPARα will be the focus of this study.

A type II nuclear receptor, PPARα forms a permissive heterodimer with the retinoid X receptor alpha (RXRα) to bind a direct-repeat 1 motif (DR1) in order to initiate transcription of its target genes. The nucleotide sequence of identified PPREs can be quite degenerate from the consensus AGGTCAnAGGTCA sequence, although PPARα is not quite as promiscuous as some other receptors, such as RXRα. Even so, PPARα has been observed binding to a known PXR/CAR binding site near the gene encoding cytochrome p450 (CYP2C8), which follows a DR-4 motif [7]. As previously mentioned, PPARα targets are generally involved in pathways comprising the body’s response to fasting, in particular the breakdown of fats for energy. Long-chain fatty acids (LCFA) and their thioester derivatives (LCFA-CoA) serve as high affinity endogenous ligands for PPARα [8], and as such the receptor is responsible for direct regulation of many key
enzymes in mitochondrial, microsomal and peroxisomal fatty acid oxidation, such as acyl-CoA oxidase (ACOX1). HMG-CoA synthase (HMGCS2), which is responsible for a step in the process of converting fatty acids to ketone bodies in order to be transported elsewhere to be used for energy, is another target. PPARα and its agonists have also been linked to regulation of genes involved in the transport of fatty acids, such as carnitine palmitoyltransferase (CPT1), which aids in the transport of fatty acids into the mitochondria for oxidation, as well as liver-type fatty acid binding protein (L-FABP), with which PPARα also interacts directly [9]. In addition to increasing expression of genes involved in lipid catabolism, PPARα also decreases expression of genes responsible for the synthesis of fatty acids, such as fatty acid synthase (FAS)[10] and acetyl-CoA carboxylase (ACC)[11], indirectly through repression of SREBP-1c[12]. PPARα also plays a role in regulation of cholesterol metabolism, inducing breakdown of lipoproteins through lipoprotein lipase (LPL)[13] and increase high density lipoproteins (HDL) through direct control of major components such as apolipoprotein AI [14], and in glucose metabolism through pyruvate dehydrogenase kinase 4 (PDK4)[15]. Additionally, PPARα functions in an anti-inflammatory capacity through negative cross-talk with pro-inflammatory transcription factors [16] and through binding and inducing breakdown of leukotriene B4 [17], a derivative of arachidonic acid involved in inflammation.

Many prevalent metabolic disorders arise from dysregulation of fatty acid and glucose metabolism, and are characterized by increased levels of free fatty acids, cholesterol, and inflammation. Since PPARα is a prominent regulator of fatty acid metabolism as well as a factor in negative regulation of inflammation, it is implicated in
many such disease processes, in addition to being an attractive therapeutic target. A number of synthetic and endogenous ligands have been identified for PPARα, beginning with compounds known as peroxisome proliferators that are carcinogenic to rodents and led to the receptor’s initial identification. As an important regulator of lipid metabolism, PPARα is the target of a class of hypolipidemic drugs known as fibrates, as well as the compound Wy-14,643, which are used to treat cardiovascular disease by correcting cholesterol and triglyceride levels. Additionally, a number of naturally occurring lipids have been shown to bind PPARα in vitro and affect transactivation in cells. The relatively large size allows the binding pocket to accommodate a diverse range of lipid conformations, from long-chain fatty acids such as palmitic (C16:0) and arachidonic (C20:4) acids, branched-chain fatty acids, and much larger fatty acyl-CoA thioesters, as well as those of very long-chain fatty acids [8, 18]. In fact, PPARα binds the CoA thioesters of many saturated long-chain and very long-chain fatty acids with higher affinity than the respective free fatty acids. Moreover, PPARα has been shown to bind non-lipid molecules as well; specifically, sugars, such as glucose [19].

**LXR**

Liver X receptor alpha (LXRα) is also a member of the NR1 subfamily, and, similarly to PPARα, is predominantly expressed in the liver, kidney, lung, intestine, macrophages and adipose tissue. A second isoform, LXRβ, has significant sequence homology to LXRα, but is expressed ubiquitously. Like PPARα, LXRα is a type II nuclear receptor and also forms heterodimers with RXRα. However, the LXRα/RXRα
heterodimer binds a direct-repeat 4 (DR4) response element known as a liver X receptor response element (LXRE), as opposed to the DR1 that is bound by PPARα. The sequence specificity of LXREs is greater than that of PPREs, but some degeneracy is still observed. The physiological role of LXRα, like that of PPARα, has been elucidated as that of an important nutrient sensor and regulator of metabolic processes, although LXRα modulates pathways which often work in the opposite direction of those involving PPARα, mainly cholesterol metabolism and fatty acid synthesis. Highly characterized LXRα target genes include sterol regulatory element-binding protein 1c (SREBP-1c) [20], which is a transcription factor responsible for regulating levels of fatty acids and cholesterol in the cell, and cholesterol transporters such as the ATP-binding cassettes A1, G1, G5, and G8 [21-23]. Additionally, LXRα is also a known regulator of apolipoprotein E (ApoE), carbohydrate response element-binding protein (ChREBP), and FAS. Accordingly, oxidized cholesterols such as 22(R)-hydroxycholesterol and other oxysterols have been shown to function as LXRα ligands. Further, LXRα has been identified as a glucose sensor [24], similarly to PPARα, and has an important anti-inflammatory role in preventing macrophage foam cell formation and decreasing atherogenesis. Thus, LXRα has been strongly implicated in disease states involving excess fats and cholesterol, such as hypercholesterolemia, atherosclerosis, cardiovascular disease, and hyperlipidemia. Genetic variation in the receptor is linked with increased risk of metabolic issues. Of particular note is a single nucleotide polymorphism (SNP) at -1830 bp that, in conjunction with mutations at -840 bp and -115 bp from the ATG start codon, is associated with increased risk of ischemic vascular disease [25]. In addition, risk of developing symptoms of metabolic syndrome is also influenced by genetic
variation in LXRα [26]. LXRα knockouts have resulted in hepatomegaly, increased atherogenesis and macrophage lipid accumulation, as well as symptoms resembling Tangier disease, which is related to the LXRα target gene ABCA1. As previously mentioned, LXRα is an important regulator of SREBP-1c, which is important in regulating cell cholesterol and fatty acid levels. This poses a problem when considering LXRα as a therapeutic target: while LXRα activation does decrease atherogenesis and increase cholesterol efflux, it also results in increased triglycerides. It is for this reason that, despite showing potential to alleviate symptoms of metabolic disorders, drugs targeting LXRα have not been approved for use.

**PPAR and LXR: Cross-talk and Heterodimerization**

Previous studies have indicated that significant cross-talk exists between the pathways regulated by PPARα and LXRα. Many genes upregulated by LXRα are in turn downregulated by PPARα, and vice versa. A prime example of this is that LXRα has been shown to repress PPARα/RXRα activation of ACOX, while conversely, PPARα represses LXRα/RXRα-mediated activation of SREBP-1c [27-29]. Agonists for PPARα, LXRα, and RXRα have been shown to have overlapping transcriptional programs as well, [30]. Further, a 2012 study by Boergesen et al.[31] observed an overlap of binding sites occupied by PPARα and LXRα in mouse liver. PPAR and LXR have been suggested to bind DNA in areas where a PPRE and an LXRE overlap, as was observed with the CYP7A1 gene [32], and further study by the same group determined PPARα/LXRα may be responsible for repression of CYP7A1 in humans, rather than LXRα/RXRα as is seen
in mice [33]. Despite their canonical mechanism involving heterodimerization with RXRα, interest in the field has recently turned toward direct interaction between PPARα and LXRα themselves. A study by Miyata et al. using human and mouse protein suggested interaction through two-hybrid screening and *in vitro* protein binding assays [34]. Also, the kinetics of interaction between the LBD of PPARα and LXRα have been investigated through surface plasmon resonance (SPR) and molecular dynamics simulations (MD) [35], concluding that the LBDs of the proteins are capable of interaction. Despite this, these and other studies have concluded that the heterodimerization of PPARα and LXRα results in a nonfunctional complex that is incapable of binding DNA. More recently, it has come to light that human and mouse PPARα behave differently in terms of ligand binding [36], and that it is necessary to examine this interaction using full-length, human proteins to properly elucidate its function. Such studies have been completed, showing that full-length, human PPARα and LXRα interact *in vitro* through fluorescent binding assays and circular dichroism spectroscopy, and *in vivo* through transactivation assays and co-immunoprecipitation [37]. More importantly, electrophoretic mobility shift assays determined that these proteins were able to bind DNA *in vitro*, suggesting that not only is the heterodimer capable of binding DNA, it may indeed be functional. Although none of the genes tested in the aforementioned re-ChIP experiments, such as SREBP-1c and HMGCS2, were bound simultaneously by both proteins [31], this does not rule out the possibility that a heterodimer could bind at these, or other, genomic locations, and possibly serve a regulatory function itself.
II. GOALS AND HYPOTHESES

The goal of this study is to examine if full-length, human PPARα/LXRα heterodimers to act in a functional capacity and regulate gene expression through high affinity binding to DNA. Furthermore, since a response element for PPARα/LXRα heterodimers has not been previously identified, this study aims to establish whether PPARα/LXRα heterodimers bind a specific type of response element, and if that response element occurs naturally in the human genome.

To that end, the specific aim of this thesis is that PPARα/LXRα binds a novel DNA sequence as a heterodimer with high affinity, and that the sequence occurs in genomic locations near relevant promoters that result in statistically significant transactivation upon binding by PPARα/LXRα.
III. MATERIALS AND METHODS

Candidate Response Element Design

It is well known that PPARα and LXRα, when in complex with RXRα, bind a DR1 and DR4 response element, respectively. However, there is no guarantee that the PPARα/LXRα heterodimer follows the binding characteristics of either individual protein, or that the AGGTCA_AGGTCA sequence is also the ideal site for this heterodimer to bind. Miyata et al. examined PPARα/LXRα binding to idealized sequences in the form of DR0-DR5 response elements by electrophoretic mobility shift [34], but none was observed with the hexameric sequences used. Thus, this study examined consensus sequences for known PPREs [38] and LXREs [39] for the most commonly observed half-sites, and those half-sites were used to construct candidate PPARα/LXRα response elements whose half-sites corresponded to those of a PPRE (P), an LXRE (L), or a hybrid of PPRE and LXRE (H). To determine the influence of the number of nucleotides separating the half-sites, sequences separated by 1, 2, 3, or 4 nucleotides were designed. Single-stranded oligonucleotide primers (Table 1) were purchased from IDT (Coralville, IA) and dissolved to 1 μg/μl stock solution in 1X TE. The forward and reverse primers were mixed in annealing buffer (10mM Tris, pH 8.0, 1mM EDTA, 50mM NaCl) and incubated at 95°C in a dry bath for 5 min. The aluminum block was then removed from heat and the oligonucleotide solutions were allowed to
return to room temperature over 2-3 hours. Concentration was determined from absorbance measurements at 260 nm using a Nanodrop spectrophotometer, and purity was assessed from ratios of absorbance measured at 260 nm/280nm and 260 nm/230 nm.

<table>
<thead>
<tr>
<th>Element</th>
<th>Sequence</th>
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<tr>
<td>P1RE</td>
<td>5’- gatcttc AGGGCA a AGGTCA gg -3’</td>
</tr>
<tr>
<td>P2RE</td>
<td>5’- gatcttc AGGGCA ag AGGTCA gg -3’</td>
</tr>
<tr>
<td>P3RE</td>
<td>5’- gatcttc AGGGCA agc AGGTCA gg -3’</td>
</tr>
<tr>
<td>P4RE</td>
<td>5’- gatcttc AGGGCA agct AGGTCA gg -3’</td>
</tr>
<tr>
<td>L1RE</td>
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</tr>
<tr>
<td>L2RE</td>
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</tr>
<tr>
<td>H4RE</td>
<td>5’- gatcttc AGGGCA cctg GGATCA gg -3’</td>
</tr>
</tbody>
</table>

Table 1. Sequences of Candidate PPARα/LXRα Response Elements. Oligonucleotide primers were used for in vitro assessment of protein-DNA interactions. Half-sites are presented in underlined capital letters, while spacer nucleotides and extraneous 5’ and 3’ sequence are in lower case.
Protein Expression and Purification

Full-length, recombinant human PPARα, LXRα, and RXRα were expressed and purified using plasmids constructed in the laboratory [37], which contained the cDNA for the appropriate protein inserted into the pGEX-6-P3 vector for expression in bacterial cells. These proteins were expressed with an N-terminal glutathione S transferase (GST) affinity tag separated from the start codon by a 6X histidine tag to aid in purification. Expression plasmids were transformed into Rosetta 2 cells (Novagen, Philadelphia, PA) and grown overnight in 100 ml (for PPARα and RXRα) or 40 ml (for LXRα) Luria Bertani (LB) broth media supplemented with 0.1mg/ml ampicillin, 0.2 mg/ml chloramphenicol, and 10% glucose at 30°C and 200 rpm. Overnight cultures were subcultured into 1 L prewarmed LB the following day and grown at 37°C until the desired OD600 (1-1.5) was reached, approximately 2-3 hours. At this point, 0.1 M IPTG was added to the cultures to induce protein expression, and incubated at 16°C for a further 4 hours. Following this, cells were pelleted in an Avanti-J26 XPI centrifuge at 8500 rpm for 10 minutes at 4°C. 100mM PMSF was added to each pellet to inhibit proteolysis, and the pellets were stored at -80°C until purification.

Pellets were resuspended in 10 ml 2X L&C buffer (40mM Tris, pH 8.0, 0.35mM NaCl, and 20% glycerol), 1mM DTT, 2mM EDTA, and 10 ml EDTA-free protease inhibitor cocktail (SIGMAFAST, Sigma-Aldrich, St. Louis, MO) dissolved in 2X L&C buffer. Cell membranes were disrupted by sonication at 50% amplitude for six 30 second intervals, with 30 seconds between each. Following sonication, the cell suspension was
centrifuged at 10,000 rpm for 20 minutes to remove cell debris and produce a cleared cell lysate, which was then circulated on 1 mL GST affinity columns with a flow rate of 0.1 ml per minute at 4°C.

Following complete circulation of the cleared lysates, the columns were washed once with 2 ml 1X L&C buffer (20mM Tris, pH 8.0, 0.175mM NaCl, and 10% glycerol), once with 5 ml 2X L&C buffer containing 10mM ATP and 50mM MgCl2, and lastly with 10 ml 1X L&C buffer containing 1mM DTT and 2mM EDTA to ensure removal of all but tagged, bound protein from the column. On-column cleavage of the His-GST tag was accomplished by circulating 1 ml 1X L&C buffer containing 1mM DTT, 2mM EDTA, and 120 μg PreScission protease (GE Healthcare Life Sciences, Pittsburgh, PA) for 4 hours, and untagged protein collected in 1 ml eluates until all protein was removed. Concentration of eluates was estimated by Bradford assay, and protein purity assessed by SDS-PAGE followed by Coomassie blue staining, and aliquots were stored at -80°C until use.

**Electrophoretic Mobility Shift Assays**

Two hundred ng of each purified protein were incubated in binding buffer with 40 ng double-stranded oligonucleotide for 30 minutes at room temperature, then cross-linked by UV irradiation (Stratagene, San Diego, CA) at 120mJ/cm². Samples were then mixed with 6X gel loading buffer included in the Molecular Probes kit (Invitrogen, Carlsbad, CA) and protein-DNA complexes were resolved on 6% native polyacrylamide gels in
0.5X TBE buffer with a 100 bp DNA ladder (Invitrogen, Carlsbad, CA) and a protein ladder (BioRad, Hercules, CA). To visualize protein-DNA complexes, gels were stained with SYBR green (diluted to 1X in TBE buffer) for 20 minutes while protected from light, rinsed, and imaged on a Fujifilm LAS-3000 cooled charge-coupled device camera. Gels were then stained overnight in SYPRO Ruby, destained in destaining solution (10% methanol, 7.5% acetic acid), and imaged. Relative band intensities were quantified as mean 16-bit grayscale density in ImageJ (https://imagej.nih.gov/ij/download.html). Since PPARα/LXRα binding has not been previously observed in fluorescent EMSA experiments, several compositions of binding buffer were examined to determine optimal conditions for the reaction (see Table 2 [22, 40, 41]). Buffers were prepared as 5X solutions, with the final concentration in the binding reaction being 1X. Similar experiments were conducted with PPARα only, LXRα only, RXRα only, PPARα/RXRα, and LXRα/RXRα. To confirm both PPARα and LXRα were present in the shifted complexes, supershift assays were carried out by adding 1 μg of anti-PPARα (sc-1985X, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-LXRα (PA1-9066, Pierce) antibody after cross-linking and allowing the mixture to equilibrate for 15 minutes. Supershifted complexes were resolved on 4% non-denaturing polyacrylamide gels, stained and imaged as described.
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**Table 2. Composition of binding buffers for EMSA reactions.** Concentrations given are for 1X solutions. Buffers were prepared as 5X solutions and diluted as necessary.
Binding assays

Fifty nM each of purified recombinant PPARα and LXRα were mixed in phosphate buffered saline (PBS) and titrated against increasing concentrations (0-200 nM) of annealed double-stranded oligonucleotide (P4RE, L4RE, H4RE, ACOX PPRE, or SREBP-1c LXRE). After 3 minute equilibration, quenching of intrinsic aromatic amino acid fluorescence at 24°C was measured by monitoring emission spectra (310 nm – 370 nm) using an excitation wavelength of 280 nm obtained on a PC1 photon counting spectrofluorometer (ISS, Champaign, IL). Changes in maximal fluorescence intensity following subtraction of PBS blank were plotted against oligonucleotide concentration. Binding affinity was estimated by nonlinear regression analysis using the ligand binding function of Sigma Plot (SPSS, Chicago, IL). Subsequent experiments were repeated using titrations up to 40 nM oligonucleotide. Similar experiments were also done to assess the binding affinities for PPARα and LXRα individually, using 100 nM protein concentration.
**Candidate Response Element Validation and Location**

Available data from PPARα and LXRα ChIP-seq experiments was obtained from supplemental files from [31] in BED format and checked for overlap using Microsoft Excel and bedtools (https://github.com/arq5x/bedtools2/releases). Local BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastDocs&DOC_TYPE=Download) was then used to align the resulting overlapping sites to the Hg19 human genome assembly. Additionally, LXRα ChIP-seq data from a monocyte-induced macrophage cell line [42] was obtained. Finally, local BLAST (optimized for short nucleotide sequences) was used to locate instances of the P4RE, L4RE, and H4RE candidate response elements in the genome, with word size set to 16 and percent identity set to 100. Output files in BED format for ChIP-seq data sets and RE searches were then used to generate tracks using Integrated Genome Browser (http://bioviz.org/igb/download.html).

**Gene Selection and Construction of Luciferase Reporter Plasmids**

NCBI BLAST was used to search the human genome for occurrences of the P4RE, L4RE, and H4RE sequences, and exact matches located within 5kb of an identified promoter [43,44] were considered. Three known targets of PPARα and LXRα were chosen for further study: apolipoprotein A1 (APOA1), a component of high-density lipoproteins known to be regulated by PPARα and LXRα [14,45]; cytosolic sulfotransferase 2A1 (SULT2A1), involved in sulfation of steroids (such as cholesterol) and bile acids in order to facilitate excretion and known to be regulated by PPARα and LXRα [46,47]; and sterol regulatory element binding protein 1c (SREBP-1c). Three
potential novel targets were chosen based on expression of both PPARα and LXRα in B and T lymphocytes [48-51] and the possibility that PPARα/LXRα heterodimers regulate a distinct subset of genes (as opposed to those already associated with either protein). The three were: C-X-C chemokine receptor 5 (CXCR5 or BLR1), involved in B cell migration and Burkitt’s lymphoma; tumor necrosis factor superfamily member 4 (TNFRSF4, also known as OX40), and tumor necrosis factor receptor superfamily member 18 or glucocorticoid-induced TNF receptor (TNFRSF18 or GITR). Separate fragments containing the L4RE and the promoters of APOA1, SULT2A1, and CXCR5 were amplified by polymerase chain reaction from genomic DNA isolated from HepG2 cells using primers indicated in Table 3. Fragments were cloned separately into the pGEM-T easy vector and subsequently transferred as one insert into the KpnI-SacI (for APOA1 and SULT2A1) or BglII-XhoI (for CXCR5) sites of the luciferase reporter plasmid pGL4.17 to generate APOA1-pGL4.17, SULT2A1-pGL4.17, and CXCR5-pGL4.17. TNFRSF4 was amplified using the indicated primers as a single 712 bp fragment containing both the L4RE and the promoter and cloned into the pGEM-T easy vector, then transferred to the KpnI-Sacl sites of pGL4.17 to generate TNFRSF4-pGL4.17. TNFRSF18 was amplified as a single 1321 bp fragment containing both the L4RE and the promoter using the indicated primers and cloned into the pGEM-T easy vector before being transferred to the KpnI-Xhol sites of pGL4.17 to produce TNFRSF18-pGL4.17. The promoter for SREBP-1c with and without known PPRE and LXRE sequences had been previously cloned into pGL4.17. A short fragment (355 bp) containing only the L4RE was amplified using the indicated primers and cloned into pGEM-T before being inserted into the existing
SREBP-1c-pGL4.17 plasmid using the *KpnI-EcoRI* sites to produce SREBP-1cShort-pGL4.17. Plasmids were verified by DNA sequencing performed by Retrogen.
Table 3. Primer sequences used in amplification of candidate target gene. Bases in upper case represent the genomic target sequence, while underlined lower case indicates restriction enzyme sites and extra bases at the 5’ end are in simple lower case.
Figure 2. Construction of luciferase plasmid inserts for APOA1 and CXCR5. Plasmids were constructed in two separate amplicons, one containing the promoter region and one containing the RE (position indicated in light grey). Inserts were cloned into the multiple cloning site of pGL4.17 at the designated restriction sites.
Figure 3. Construction of luciferase plasmid inserts for SULT2A1 and SREBP-1c (short). Plasmids were constructed from single amplicons containing only the RE (position denoted in light grey). Existing sequence was removed by restriction digest at the indicated sites, and replaced with new inserts.
Figure 4. Construction of luciferase plasmid inserts for TNFRSF4 and TNFRSF18. Plasmids were constructed from single amplicons containing both the 16-nucleotide L4RE sequence (indicated in light grey) and the gene promoter, and inserted into the multiple cloning site of the pGL4.17 vector at the designated restriction sites.
Transactivation assays

COS-7 cells were seeded in 24-well cell culture dishes and grown in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum for 8 hours at 37°C and 5% CO₂. Media was then replaced with low serum DMEM and cells were transfected with 400 ng luciferase plasmid (APOA1, CXCR5, SULT2A1, SREBP-1cShort, SREBP1cKnown, TNFRSF4, or TNFRSF18), 400 ng protein overexpression plasmid (PPARα, LXRα, RXRα, PPARα and RXRα, LXRα and RXRα, PPARα and LXRα, or empty vector, pSG5), and 40 ng of pRL-CMV (Renilla luciferase driven by a CMV promoter, used as an internal control for transfection efficiency) using Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA) and grown overnight. Transfection media was replaced with serum-free DMEM and allowed to grow another 20 hours before harvesting in lysis buffer (Promega, Madison, WI). Lysates were transferred to a 96-well microtiter plate and assayed for Firefly luciferase activity using the dual-luciferase reporter assay kit (Promega). Luminescence was measured using a BioTek Synergy plate reader (BioTek, Winooski, VT). Resulting data were normalized for transfection efficiency and the empty pSG5 vector samples for each gene were arbitrarily set to 1. Overexpression of PPARα, LXRα, and RXRα proteins in cell lysates was confirmed by Western blotting.
RESULTS

**PPARα/LXRα binds candidate REs in vitro**

Electrophoretic mobility shift assays were carried out using purified full-length human proteins and an LXRE from the ABCG1 gene, as well as candidate PPAR/LXR response element oligonucleotides following a DR1, DR2, DR3, or DR4 motif. When resolved on a native gel and stained with SYBR green fluorescent dye, bands corresponding to free oligonucleotide appear at the bottom of the gel, while bands that correspond to oligonucleotide in complex with protein are retarded in migration. Since PPARα/LXRα interaction with DNA has not been evaluated using fluorescently stained gel shifts previously, five different binding media were tested for determining optimal pH and salt concentration for PPARα/LXRα binding to DNA (Table 2); Buffer 5 composition allowed bands which corresponded to the position of the protein bands indicated by SYPRO Ruby stain (Fig. 5C), and was thus used for subsequent experiments. Buffer 1, 2, and 4 did not result in a mobility shift (Fig. 5A-B), and Buffer 3 only resulted in nonspecific shifts further in the gel (Fig. 5B). While faint shifted bands were observed in each lane, those corresponding to DR4 sequences were 2 to 3 fold more intense (Fig. 6), as quantitated by densitometry. When binding media contained only an individual protein, no binding to DNA was observed by PPARα alone, consistent with that full-length PPARα requires an interacting partner to bind DNA, while LXRα unexpectedly bound the DR4 oligonucleotides without a potential heterodimer partner present. Addition of an antibody against either protein further retarded band migration,
indicating that both proteins were present in the shifted complex. However, with addition of the PPARα antibody, a portion of material failed to be supershifted, suggesting the presence of a second complex consisting only of LXRα bound to DNA (Fig. 7).
Figure 5. Preliminary EMSAs of PPARα/LXRα with ABCG1 LXRE for buffer optimization. SYBR Green (left) and SYPRO Ruby (right) stains of EMSA experiments with Buffer 1 (A), Buffer 2 (B, lanes 1 and 2), Buffer 3 (B, lanes 3 and 4), Buffer 4 (B, lanes 5 and 6), and Buffer 5 (C). Buffer 5 alone demonstrated binding.
Figure 6. EMSA of PPARα/LXRα with candidate response elements showing preference for DR4 element. 200 ng each of PPARα and LXRα were incubated with 40 ng of candidate RE oligonucleotides and resolved on 6% non-denaturing polyacrylamide gels. Bands representing bound PPRE-like (P), LXRE-like (L), or hybrid (H) sequences separated by 4 nucleotides exhibited the highest band intensity.
Figure 7. Supershift assay demonstrating presence of both PPARα and LXRα in complex with DNA. The protein-DNA complex is shifted further upward in the gel with the addition of an antibody against PPARα or LXRα. However, the entire complex is not shifted with the PPARα antibody, but is entirely shifted with the LXRα antibody, suggesting the presence of a second complex consisting of only LXRα bound to DNA.
**PPARα/LXRα and LXRα alone bind candidate REs with nanomolar affinity**

Since EMSAs only provide a qualitative representation of protein-DNA interactions *in vitro*, it was necessary to examine the ability of PPARα/LXRα to bind DNA in a manner that would yield quantitative information at concentrations that are physiologically relevant in the cell. To that end, intrinsic protein fluorescence quenching experiments were conducted, wherein changes in fluorescence emission by aromatic amino acid residues in the protein as a function of DNA oligonucleotide concentration were measured and reflect conformational changes in the protein induced by DNA binding. The change in fluorescence emission was plotted as a function of the total concentration of DNA titrated allows for an estimation of binding affinity.

PPARα does not bind DNA without an interacting protein partner, which is consistent with the absence of shifted bands observed in EMSA experiments (Fig. 7). To confirm those results, 100 nM PPARα was titrated against increasing concentrations of the three candidate REs, as well as the known ACOX PPRE and SREBP LXRE. Intuitively, the only candidate RE to exhibit binding saturation was the P4RE, whereas the L4RE titration only resulted in a relatively weak change in fluorescence intensity without exhibiting saturation (Fig. 8). While the P4RE and H4RE titrations did result in a dramatic decrease in fluorescence intensity with a low apparent $K_d$ value (see Table 4), the resulting plot of fluorescence intensity against DNA concentration suggests that the sample failed to reach saturation within the range of DNA concentrations tested. This indicates that any interaction occurring was weak or non-specific at best. PPARα also failed to reach saturation when titrated with ACOX PPRE or SREBP LXRE (Fig. 9),
further establishing that PPARα binds DNA only in a weak or non-specific manner in the absence of a heterodimeric partner.

Figure 8. Intrinsic fluorescence quenching of PPARα upon titration with candidate REs. 100 nM PPARα was titrated against increasing concentrations of oligonucleotide, and the change in fluorescence intensity was plotted against DNA concentration to obtain apparent K_d. Data are represented as mean ± S.E., n≥3.
Figure 9. Intrinsic fluorescence quenching of PPARα upon titration with known REs. 100 nM PPARα was titrated against increasing concentrations of oligonucleotide, and the change in fluorescence intensity was plotted against DNA concentration to obtain apparent $K_d$. Data are represented as mean ± S.E., n≥3.

Alternatively, when examining 100 nM LXRα interaction with RE oligonucleotides, LXRα produced high affinity binding curves with a less intense change in fluorescence. LXRα bound all three candidate REs with affinities of less than 5 nM (Fig. 10), which clearly showed saturation, suggesting that LXRα is able to bind these REs with high affinity, and in the absence of a heterodimeric interacting partner. Further, LXRα bound the ACOX PPRE and the SREBP LXRE (Fig. 11), although the curve produced from the PPRE data was not as pronounced, and thus was bound with lower affinity.
Figure 10. Intrinsic fluorescence quenching of LXRα upon titration with candidate REs. 100 nM LXRα was titrated against increasing concentrations of oligonucleotide, and the change in fluorescence intensity was plotted against DNA concentration to obtain apparent $K_d$. Data are represented as mean ± S.E., n≥3.
Figure 11. **Intrinsic fluorescence quenching of LXRα upon titration with known REs.** 100 nM LXRα was titrated against increasing concentrations of oligonucleotide, and the change in fluorescence intensity was plotted against DNA concentration to obtain apparent $K_d$. Data are represented as mean ± S.E., n≥3.

The PPARα/LXRα mixture bound the P4RE, L4RE, and H4RE oligonucleotides with $K_d$ values in the low nanomolar range and indicative of high affinity binding (Fig. 12), which confirmed the results of the EMSA experiments. Of the three, the L4RE and H4RE bound with the highest affinity, around 2 nM, with the P4RE only slightly lower affinity at around 5 nM. Interestingly, binding to either the ACOX PPRE or the SREBP-1c LXRE was not detected (Fig. 13). Furthermore, transforming the binding data for the L4RE and the H4RE into a Hill plot yields a slope of <1, indicating at least two negatively cooperative binding sites are present (Fig. 14). Taken together with the overall shape of the curves, which seem to reach saturation early but climb again around the highest concentrations, this suggests a transition from high affinity binding to low affinity binding, presumably represented respectively by LXRα and PPARα.
Figure 12. Intrinsic fluorescence quenching of PPARα/LXRα upon titration with candidate REs. 50 nM each PPARα and LXRα (1:1, total 100 nM protein) was titrated against increasing concentrations of oligonucleotide, and the change in fluorescence intensity was plotted against DNA concentration to obtain apparent $K_d$. Data are represented as mean ± S.E., n≥3.
Figure 13. Intrinsic fluorescence quenching of PPARα/LXRα upon titration with known REs. 50 nM each PPARα and LXRα (1:1, total 100 nM protein) was titrated against increasing concentrations of oligonucleotide, and the change in fluorescence intensity was plotted against DNA concentration to obtain apparent K$_d$. Data are represented as mean ± S.E., n≥3.
Figure 14. Plotting PPARα/LXRα binding data on Hill coordinates suggests multiple binding sites. Plots were generated by calculating partial saturation (Y) from the change in fluorescence intensity, and plotting the log of (Y/1-Y) against the log of the oligonucleotide concentration. The slopes of the lines were 0.4795 for the L4RE, and 0.4901 for the H4RE, indicating negative cooperativity and, thus, multiple binding sites.
Table 4. Binding affinities of PPARα, LXRα, and PPARα/LXRα for known and candidate REs. Apparent Kₐ values (nM) were obtained for all experiments, n≥3. Lower values indicate higher affinity binding.

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<td>SREBP LXRE</td>
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Candidate REs occur naturally in the human genome

Chromatin immunoprecipitation (ChIP) is an effective way to gauge protein-DNA interactions in a cell-based environment, and is a powerful tool for determining sequence specificity when combined with high-throughput next generation sequencing (ChIP-seq). Previous ChIP-seq studies were used to address whether the L4RE, P4RE, or H4RE sequences were bound by PPARα and LXRα. Sequential ChIP-seq, which would show DNA sequences bound by PPARα and LXRα simultaneously, is very difficult to perform due to the small amount of bound DNA recovered from such experiments. Information from ChIP-seq data was limited to overlap between ChIP-seq experiments performed with antibodies against PPARα and LXRα individually.

2655 LXRα sites across the genome were obtained from [42], which were already mapped to Hg19. 1920 sites were present in the overlap from Hg19-mapped mouse data from [31], although they were not identical to those from the LXRα only data. The P4RE sequence yielded a single hit on chromosome 12, while the H4RE was not present at all in a search of Hg19. The L4RE, however, occurred 87,486 times, with e values ranging from 0.027 to 0.004, indicating that hits did not occur due to random chance (Fig.15, Bottom). The data sets for the overlap, LXRα-only, and L4RE sites do not overlap, although there are sites located in close proximity (Fig.15, Middle).
Figure 15. Genomic locations of the L4RE candidate response element relative to known PPARα and LXRα binding sites. **Top:** Integrated Genome Browser view of chromosome 12 of the Hg19 human genome assembly, with tracks generated from ChIP-seq data for LXRα alone, an overlap of ChIP-seq data for PPARα and LXRα, and the L4RE candidate response element. **Middle:** Expanded view of chromosome 17, with the position of the SREBF1 gene indicated with a vertical grey line, demonstrating a lack of overlap between the three sets. **Bottom:** Close view of a representative L4RE site demonstrating an exact match to the sequence used *in vitro*. 
**PPARα/LXRα and LXRα alone transactivate endogenous promoters**

Once the ability of PPARα/LXRα to bind the candidate RE sequences was established *in vitro* via EMSAs and binding experiments, the possible functional consequences of such binding in a cell-based environment were determined. To accomplish this, a BLAST search of the human genome for the candidate REs yielded many instances of an exact match to the L4RE sequence. The search was narrowed to only include sequences within 5 kilobases of a known or identified promoter element, of which 6 were chosen for further study. *APOA1, SULT2A1*, and *SREBF1* are all known to be regulated by either PPARα or LXRα, or by both. Additionally, occurrences near *CXCR5, TNFRSF4*, and *TNFRSF18* were also chosen in order to examine the possibility that a distinct subset of genes are regulated by the PPARα/LXRα heterodimer rather than being regulated by the PPARα or LXRα heterodimers, and also that genes expressed in different tissues (B cells and T cells, in this case) respond to regulatory stimulation from PPARα and/or LXRα differently. Portions of genomic DNA containing the target L4RE sequence and the endogenous gene promoters were cloned into the pGL4.17 promoter-less luciferase reporter plasmid (Fig. 2-4), and transactivation assays were performed to assess the ability of PPARα and LXRα, transiently overexpressed via pSG5 expression constructs, to alter RE activity in COS-7 cells. Overexpression of transfected proteins was determined immediately following each replicate by Western blotting, which showed a marked increase in protein levels in cells transfected with one or both overexpression plasmids (Fig. 16). However, the expression of both proteins does not increase so
strongly in cells which were co-transfected with both PPARα and LXRα expression plasmids.

Figure 16. Western blot showing overexpression of transiently transfected PPARα, LXRα, and RXRα. Lysates from COS-7 cells transfected with the indicated plasmids were run on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and probed with antibodies against PPARα, LXRα, and RXRα.
The APOA1-pGL4.17 plasmid was constructed from two separate amplicons, one 564 bp fragment containing the promoter machinery, and another 517 bp fragment which contained the L4RE sequence, omitting 4641 bp between them. Transactivation of this reporter remained at basal levels in cells transfected with the pSG5-PPARα plasmid alone, nearly identical to those cells transfected only with empty pSG5 vector. In contrast, cells transfected with pSG5-LXRα demonstrated significantly increased L4RE transactivation, approximately 3- to 4-fold higher than vector alone (Fig. 17). Although some RXRα is present in COS-7 cells, transactivation is not significantly increased from this level when RXRα is co-overexpressed, suggesting that this finding is not dependent on levels of that protein and is, in fact, a result of LXRα activity, potentially as a homodimer. Significantly increased transactivation was also observed when PPARα and LXRα were co-overexpressed, although the increase was not as strong as that from cells transfected with LXRα alone and appeared to indicate that the presence of PPARα inhibits LXRα activity. Taken together, these results suggest that not only could PPARα/LXRα heterodimers transactivate this L4RE and alter expression of the APOA1 gene, LXRα may also do so as a homodimer.
Figure 17. Transactivation of APOA1. Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p>0.05, ** = p>0.005, and *** = p>0.001. Data are represented as mean ± S.E., n≥4.
The construction of the CXCR5-pGL4.17 plasmid was similar to that of APOA1-pGL4.17, with a 965 bp fragment containing the TATA-less promoter [44] and a 379 bp fragment with the response element and only 891 bp omitted between them. This plasmid, however, was not as responsive to transactivation by exogenous PPARα and LXRα (Fig. 18). Only cells transfected with pSG5-LXRα alone demonstrated significantly increased luciferase expression, and only reaching about 2-fold higher expression levels than the vector control. Cells overexpressing PPARα alone showed a slight decrease in RE activity, while those transfected with both PPARα and LXRα increased transactivation only minimally. These data indicate that CXCR5 is not likely a target of these two proteins, but may be affected by LXRα alone.

Figure 18. Transactivation of CXCR5. Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p>0.05, ** = p>0.005, and *** = p>0.001. Data are represented as mean ± S.E., n≥4.
The SULT2A1 promoter was previously cloned into pGL4.17 by Mrs. Jeannette Manger, and a 565 bp fragment containing the L4RE was inserted into the existing plasmid in order to complete SULT2A1-pGL4.17. Transfection with pSG5-PPARα affected a minor decrease in RE activity, although not enough to be statistically significant. In a similar trend to that seen with APOA1 and CXCR5, RE activity increased significantly in cells transfected only with pSG5-LXRα, and co-transfection of both PPARα and LXRα overexpression plasmids also resulted in an increase in RE activity. However, like the change seen with PPARα alone, this difference was not determined to be statistically significant. Once again, it seemed that LXRα was able to affect transactivation of this promoter through the L4RE response element in the absence of PPARα as a heterodimer partner (Fig. 19).

**Figure 19. Transactivation of SULT2A1.** Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p>0.05, ** = p>0.005, and *** = p>0.001. Data are represented as mean ± S.E., n≥4.
**SREBF1** is a well-characterized target of both PPARα and LXRα, and its promoter region contains two known LXREs, as well as a PPRE through which PPARα may repress LXRα-mediated upregulation of the gene. The L4RE sequence was located approximately 2 kb upstream of the promoter, and thus, two luciferase plasmids were tested for SREBP-1c: a construct previously cloned in the lab termed SREBP-1c-known-pGL4.17, which contains the endogenous promoter with the known PPARα and LXRα response elements, as well as a plasmid containing a 355 bp fragment containing the L4RE located near the gene inserted upstream of the promoter, with the known REs removed, henceforth referred to as SREBP-1c-short-pGL4.17. When cells only overexpressing PPARα were examined, there was again no significant change from basal transactivation levels observed, with either SREBP-1c plasmid. Likewise, there was no significant change in transactivation when both proteins were co-overexpressed, indicating that the PPARα/LXRα heterodimer is not capable of transactivating the L4RE (Fig. 20), or the known PPRE and LXREs (Fig. 21), with the **SREBF1** promoter. However, cells transfected with LXRα alone again demonstrated significantly increased transactivation of the known REs, but not of the L4RE. These data are intriguing due to the fact that SREBP-1c, as a major regulator of fatty acid synthesis in the cell, is of concern when targeting LXRα for therapeutic purposes, and it is interesting to note that while PPARα/LXRα appears to be able to transactivate *APOA1*, an effect that would be beneficial for such an application, it does not transactivate **SREBF1** and may thus provide an avenue through which to target LXRα while bypassing the negative side effects from attempts which target the LXRα/RXRα heterodimer.
Figure 20. Transactivation of SREBP-1c (known PPRE and LXREs). Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p>0.05, ** = p>0.005, and *** = p>0.001. Data are represented as mean ± S.E., n≥4.
Figure 21. Transactivation of SREBP-1c (L4RE only). Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p > 0.05, ** = p > 0.005, and *** = p > 0.001. Data are represented as mean ± S.E., n ≥ 4.
*TNFRSF4* and *TNFRSF18* are members of the tumor necrosis factor receptor superfamily, and are alternatively known as OX40 and GITR, respectively. Both genes are located in close proximity to an incidence of the L4RE, and thus it was possible to amplify the promoter region and the L4RE within the same fragment to construct TNFRSF4-pGL4.17 and TNFRSF18-pGL4.17. PPARα overexpression resulted in a decrease in transactivation of both of these plasmids, *TNFRSF18* (Fig. 23) more so than *TNFRSF4* (Fig. 22), but neither was statistically significant. PPARα/LXRα co-overexpression also failed to raise transactivation significantly above that displayed by vector-treated cells. As previously shown, only cells overexpressing LXRα alone exhibited a statistically significant increase in transactivation.

![Figure 22. Transactivation of TNFRSF4.](image)

*Figure 22. Transactivation of TNFRSF4.* Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p>0.05, ** = p>0.005, and *** = p>0.001. Data are represented as mean ± S.E., n≥4.
Figure 23. Transactivation of TNFRSF18. Transiently overexpressed proteins are noted on the x-axis, and the y-axis represents normalized relative activity of the indicated RE. Asterisks denote statistical significance from pSG5 vector-treated cells as determined by student’s t-test, with * = p>0.05, ** = p>0.005, and *** = p>0.001. Data are represented as mean ± S.E., n≥4.

Taken together, the results of transactivation experiments suggested that, while PPARα/LXRα may be able to affect expression of APOAI via the L4RE, it is unable to do so with the other genes tested. Furthermore, LXRα was consistently able to transactivate these REs without an overexpressed heterodimer partner.
DISCUSSION

Energy homeostasis and metabolism are comprised of a host of interweaving processes governed by tight transcriptional regulation of the expression of relevant genes. PPARα and LXRα both play a vital role in this regulation through control of genes involved in the oxidation and synthesis of fatty acids, as well as efflux, transport, and metabolism of cholesterol. However, energy homeostasis is, by nature, complex, as are the mechanisms by which the pathways involved are modulated, and as such the full extent of the coordination between these two proteins is not entirely understood. This study aimed to broaden the understanding of the intricacies of metabolic regulation as it related to PPARα and LXRα by demonstrating that these two proteins, as a heterodimer, bind to DNA, specifically and with high affinity, and exact a physiological consequence.

Direct interaction between full-length, human PPARα and LXRα was successfully demonstrated by Balanarasimha et al., as was binding of the proteins to the ACOX PPRE and the SREBP-1c LXRE via electrophoretic mobility shift assays. Miyata et al. attempted to show PPARα/LXRα binding to DNA using idealized AGGTCA_AGGTCA response element sequences, separated by 0 to 5 nucleotides, but were unable to do so. It is important to consider that those and other studies were conducted using murine, tagged, and/or truncated versions of one or both proteins. Specifically, those experiments were conducted using murine PPARα and human LXRα. While valuable as a basis for future study, such experiments do not provide an accurate representation of the true DNA binding characteristics of the proteins. Incorporation of affinity tags and truncating
proteins may alter the structure of the proteins, which may cause changes in its function. Likewise, while there is significant sequence homology between mouse and human PPARα and LXRα, the functions differ slightly by species. For example, in mice, LXRα is a known regulator of cholesterol-7a-hydroxylase, or CYP7A1, but does not regulate the same gene in humans. Similarly, peroxisome proliferation seen in mice in response to PPARα ligands has not been observed in humans, and mouse and human PPARα have been shown to respond differently to binding of endogenous ligands. Thus, this study conducted EMSA and fluorescence quenching experiments using full-length, human PPARα and LXRα. The fact that even an idealized DR4 sequence did not bind raised the possibility that DNA binding by PPARα/LXRα heterodimers has a different DNA sequence specificity than RXRα heterodimers. Thus, this study designed candidate PPARα/LXRα response elements using half-sites from consensus data for PPREs and LXREs, which were degenerate from the idealized RE sequence.

This work demonstrated, in vitro, that PPARα and LXRα bind DNA as a heterodimer with a clear preference for a DR4 response element. While bands with low intensity were observed in lanes with the DR1, DR2, and DR3 elements, those in the DR4 lanes were significantly more intense. While binding reactions in which only PPARα was present did not result in a shifted complex, which is consistent with previously published data, shifts were observed, intriguingly, where only LXRα was present. Supershift assays were performed to assess and confirm the presence of both proteins in the shifted complex. Additional retardation in the gel was observed using either an antibody against PPARα or against LXRα, indicating that the shifted complex consisted of both PPARα and LXRα bound to DNA. While bands indicating binding to the H4RE
were slightly less intense than the P4RE or the L4RE, PPARα/LXRα heterodimers did not demonstrate specificity for any of these particular sets of half-sites. However, binding to all three candidate REs was more intense than binding to the ACOX PPRE or the SREBP-1c LXRE, supported by additional fluorescence quenching experiments. This failure to bind more degenerate REs suggests that PPARα/LXRα heterodimers may have greater sequence specificity with respect to its target sequences than PPARα/RXRα or LXRα/RXRα heterodimers.

Intrinsic fluorescence quenching experiments provided more insight into how the protein-DNA interactions observed in EMSAs occurs. A 1:1 mixture of PPARα and LXRα bound all three candidate REs with very high affinity, apparent \( K_d \) values of less than 5 nM. The affinity for the P4RE was slightly lower than for the L4RE or the H4RE. However, it is important to note that apparent \( K_d \) value alone is not an accurate representation of binding, as many factors may produce skewed apparent \( K_d \) values. For example, based on \( K_d \) value alone, PPARα appears to exhibit high affinity binding to candidate REs, as well as to ACOX and SREBP, in the absence of a heterodimer partner. Since such a conclusion is inconsistent with data obtained from EMSA experiments, it is also necessary to consider whether the concentration dependence for oligonucleotide-induced changes in protein fluorescence exhibit hyperbolic or sigmoid shapes. In the event of high affinity binding, such a plot would present as a steep hyperbolic curve that reaches saturation far to the left on the x-axis. In the case of PPARα-only samples, the curves obtained were relatively shallow, and did not appear to reach saturation within the range of concentrations tested. These data indicate that, instead of high affinity binding
suggested by estimated $K_d$ value alone, PPARα binds these candidate RE sequences, as well as ACOX and SREBP-1c, with a very low affinity or in a nonspecific manner.

This hypothesis is supported by the data for the PPARα/LXRα mixture. The binding curves generated from these data appear to reach saturation at low concentrations of DNA, but begin climbing again towards the higher concentrations. Since the curve does not fully plateau upon reaching saturation, the presence of multiple binding sites was possible. A Hill plot showed a slope of less than 1 supported the presence of at least two binding sites, which function in a negatively cooperative manner. While uncommon among nuclear receptors, binding of RXRα ligand 9-cis-retinoic acid to TR/RXR heterodimers decreases the affinity of 3,3,5-tri-iodothyronine (T3) for TR, resulting in negative cooperativity within the heterodimer [52]. Additionally, it has been proposed that, along with direct binding as with a PPARα/RXRα heterodimer, PPARα may also be able to affect gene regulation indirectly. A heterodimer partner binds DNA directly while PPARα binds weakly or nonspecifically [53]. Such a mechanism would explain the shape of the binding curves, with the initial saturation representing a high affinity site, presumably LXRα, and the subsequent increased change in intensity at higher DNA concentrations corresponding to a low affinity site representing PPARα.

These data show that LXRα, unlike PPARα, is capable of binding to DNA in the absence of a heterodimer partner. While murine LXRα has been shown to bind a cAMP-responsive response element (CNRE) as a monomer to regulate the renin gene, interaction with a DR4 LXRE was shown to be homodimeric [54]. To wit, binding to DNA by human LXRα in the form of a homodimer has not been previously
demonstrated. Shifted protein-DNA complexes were seen in LXRα-only EMSAs with all three candidate REs, as well as with the ACOX PPRE and SREBP-1c LXRE in previous work. Furthermore, the presence of an LXRα-DNA complex was suggested in supershift assays, where addition of a PPARα antibody resulted in a supershift. However, a second, unshifted band was also present. In contrast, the addition of a LXRα antibody produced only a single supershifted band. These results could be caused by antibody affinity and epitope accessibility, but both antibodies were tested on their respective RXRα heterodimers, and no second complex was observed. Fluorescence quenching assays confirmed the specificity of this binding, as well as the idea that the high affinity binding observed with PPARα/LXRα experiments was due to binding by LXRα. In the absence of PPARα, LXRα demonstrated very high affinity binding to all three candidate REs as well as to ACOX and SREBP-1c. It is possible that homodimeric LXRα exists in cells when levels of protein and/or ligand do not favor formation of a heterodimer with RXRα or PPARα, but such conditions are the focus of other projects in the laboratory and have yet to be elucidated.

In order to determine the functional significance of these findings, the occurrence of the three candidate RE sequences within the human genome was investigated. Exact matches from a BLAST search of the 16-nucleotide H4RE sequence were not obtained, and P4RE sequence were rare, with only a single site on chromosome 12. Results for the L4RE sequence, however, were surprisingly numerous. Around 87,000 results were obtained for the exact L4RE sequence used in the in vitro experiments in this study, with e-values ranging from 0.027 to 0.004, suggesting that they are not occurring simply due to chance. When compared to results from overlapped PPARα and LXRα binding sites
determined by ChIP-seq, many incidences of the L4RE were present within close range. Since the significance of the 4 spacer nucleotides is not entirely understood, it is possible that other sites bound by either PPARα/LXRα heterodimers or LXRα homodimers could be revealed with different nucleotides in those positions, for the P4RE and the H4RE in addition to the L4RE. It is also interesting to note that most of the LXRα sites from Feldmann et al. do not line up with the L4RE BLAST results, and do not also line up with the PPARα/LXRα overlaps. It is possible that, since the ChIP-seq experiments from Boergesen et al. were conducted in mouse liver and those from Feldmann were done in macrophage foam cells, LXRα’s DNA binding activity may be tissue-specific. While PPARα is present in macrophages, it is possible that LXRα may instead interact with the more prominent PPARγ in those cells. It has been previously suggested with TR, a nuclear receptor in the same superfamily as LXRα, that the sequence surrounding nuclear receptor response element half-sites are important for determining which proteins are necessary in order to affect gene expression [55]. Certain nucleotides immediately 5’ of the AGGTCA half-site render the binding site more optimal for binding by TR as a homodimer, while others will require RXRα in order to bind and affect gene expression. Given the frequency of the L4RE within the human genome and the relative scarcity of binding sites observed in ChIP-seq experiments, it is possible that a similar mechanism is at work with LXRα.

The ability of LXRα to influence the expression of genes as a heterodimer with PPARα or as a homodimer was tested via transactivation assays using firefly luciferase reporters and a selection of incidences of the L4RE sequence within 5 kb of an endogenous promoter. Such genomic locations were chosen to present a more focused
portrait of the consequences of homodimeric and heterodimeric DNA binding by LXRα compared to that of a viral promoter, such as TK or CMV. The design of the plasmids used in this study ensures that the effect seen on luciferase expression as driven by the gene promoter closely resembles that which may occur *in vivo*. Furthermore, COS-7 cells were chosen due to their low endogenous expression of PPARα, LXRα, and RXRα, despite not being human-derived cells. HepG2 cells, while a human-derived cell line widely used to study these proteins, express the proteins at very high levels causing significant experimental difficulties. All of the luciferase plasmids tested increased luciferase expression significantly when LXRα alone was overexpressed, while only one, APOA1, showed a statistically significant increase from the vector control when both PPARα and LXRα were overexpressed. In fact, overexpressing PPARα in addition to LXRα resulted in an overall decrease in transactivation compared to LXRα alone. It is possible that some observed transactivation in cells overexpressing LXRα was due to the exogenous LXRα interacting with endogenous RXRα, but overexpression of RXRα in addition to LXRα did not result in a corresponding increase in transactivation, suggesting that the majority of RE activity in those cells was due to LXRα alone. It is important to note that, when co-overexpressed with either PPARα or RXRα, the increase in LXRα expression noted in the immunoblot is not as profound as when it is overexpressed on its own. This may be the reason for this discrepancy, but is more likely to suggest a dependency on sufficient concentrations of LXRα. Furthermore, the presence of PPARα decreased transactivation of all reporters examined. It is therefore possible that LXRα homodimers function to resume the activity of LXRα/RXRα heterodimers in the event of a shortage of RXRα, or in response to a different cellular environment, while
PPARα/LXRα heterodimers function to counteract LXRα activity in some aspects while carrying it on, albeit at a lower level, in others.

These findings suggest that the role of LXRα in metabolic regulation needs to be included when considering clinical significance of these proteins. Although small molecule ligand effects were not within the scope of this study and when considering LXRα as a possible therapeutic target for metabolic disorders, understanding the mechanisms involved and pathways controlled by each form of the protein. LXRα as an agonist could be a powerful tool for treatment of disorders such as atherosclerosis, cardiovascular disease and hypercholesterolemia, but agonists may affect LXRα function differently in an environment that favors the formation of a PPARα/LXRα heterodimer rather than an LXRα/RXRα heterodimer, which may in turn function differently in an environment favoring LXRα homodimer formation. In the case of SREBP-1c, which is involved in the synthesis of both cholesterol and fatty acids, LXRα alone was capable of transactivating the gene’s endogenous promoter nearly as well as LXRα/RXRα heterodimers, while PPARα/LXRα did not produce significant transactivation of the gene. However, PPARα/LXRα did transactivate APOA1, although at lower levels than did LXRα alone or LXRα/RXRα, which plays an important role in mitigating atherosclerosis by facilitating removal of cholesterol from the body. It is possible, then, that the PPARα/LXRα heterodimer may function to suppress LXRα/RXRα activity, presumably as well as LXRα homodimer activity, in the presence of high levels of cholesterol and facilitate its breakdown and elimination. It follows, then, that if PPARα/LXRα is capable of activating other anti-atherosclerotic targets of LXRα, but not those involved in synthesis of fatty acids and triglycerides, and the conditions favoring
PPARα/LXRα heterodimerization were elucidated, it may be possible to determine a means to drive cellular metabolism toward its formation as a therapeutic measure against atherosclerosis and hypercholesterolemia while avoiding adverse side effects from LXRα’s involvement in fatty acid synthesis.

In conclusion, the role of nuclear receptors, and LXRα in particular, in regulation of metabolic function is extremely complex, and involves a great deal of cross-talk and direct interaction with other proteins as it recognizes and binds target DNA sequences to affect levels of gene expression. This study demonstrated LXRα binding *in vitro* as both a heterodimer with PPARα and, for the first time, as a homodimer, to a DR4 DNA sequence that naturally occurs in the human genome with great frequency. Furthermore, these data showed that a PPARα/LXRα heterodimer does not bind the known PPARα target in the ACOX gene, nor does it bind the LXRα target in SREBP-1c. Finally, the findings of this study determined that both PPARα/LXRα heterodimers and LXRα homodimers are capable of transactivating endogenous promoters in cells and suggest a novel role for these forms of LXRα in the overall maintenance of energy homeostasis. The effects of synthetic and endogenous ligands on DNA binding and transactivation by LXRα homodimers and PPARα/LXRα heterodimers, as well as that of mutant proteins that disrupt protein-protein and protein-DNA interactions, have yet to be investigated. Determination of further sequences which may be bound by LXRα homodimers or PPARα/LXRα heterodimers could be accomplished using a high-throughput systematic evolution of ligands by exponential enrichment (SELEX) experimental approach [57], and would be beneficial to understanding the critical role these proteins play in energy metabolism and homeostasis.
LIST OF ABBREVIATIONS

ABCA1 – ATP-binding cassette transporter A1
ACOX – Acyl-CoA oxidase
APOA1 – Apolipoprotein AI
ChIP – Chromatin immunoprecipitation
CYP7A1 – Cholesterol 7α hydroxylase
DBD – DNA binding domain
DRx – Direct repeat
DTT – Dithiothreitol
EDTA – Ethylenediaminetetraacetic acid
EMSA – Electrophoretic mobility shift assay
ER – Estrogen receptor
FAS – Fatty acid synthase
LBD – Ligand binding domain
LXR – Liver X receptor
LXRE – Liver X receptor response element
PPAR – Peroxisome proliferator-activated receptor
PPRE – Peroxisome proliferator response element
RE – Response element
RXR – Retinoid X receptor
SELEX - Systematic Evolution of Ligands by Exponential Enrichment
SREBP – Sterol regulatory element binding protein
TR – Thyroid hormone receptor
REFERENCES


