Title
Transcriptional modulation of hepatic lipoprotein assembly and secretion: coordinate regulation of the liver-fatty acid binding protein and microsomal triglyceride transfer protein genes

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Transcriptional Modulation of Hepatic Lipoprotein Assembly and Secretion:
Coordinate Regulation of the Liver-Fatty Acid Binding Protein and Microsomal
Triglyceride Transfer Protein Genes

A dissertation submitted in partial satisfaction of the
Requirements for the degree of Doctor of Philosophy in Biology
by
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2006
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Chair

University of California, San Diego
San Diego State University
2006
DEDICATION

This dissertation is dedicated to all those who choose to fight the momentum of obstacles and assert their will to evolve their own life path.
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The text and figures presented in the RESULTS section (Chapter III) are, in part, reprints of the material as it appears in:


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ABSTRACT OF DISSERTATION

Transcriptional Modulation of Hepatic Lipoprotein Assembly and Secretion: Coordinate Regulation of the Liver-Fatty Acid Binding Protein and Microsomal Triglyceride Transfer Protein Genes

by

Nathanael J. Spann

Doctor of Philosophy in Biology

University of California, San Diego, 2006
San Diego State University, 2006

Professor Roger A. Davis, Chair

Hepatic production of apolipoprotein (apo) B-containing lipoproteins provides a means to transport essential lipids and fat-soluble nutrients to peripheral tissues for utilization and storage. Liver-fatty acid binding protein (L-FABP) and microsomal triglyceride transfer protein (MTP) bind fatty acids and
glycerolipids, respectively and facilitate their transfer into the VLDL assembly and secretion pathway.

Sequence analysis reveals that the proximal promoter regions of \(\text{L-FABP}\) and \(\text{MTP}\) contain similar \(\text{DR1}\) elements, suggesting the transcription of these two genes may be coordinately regulated. The inability of \(\text{L35}\) hepatoma cells to express \(\text{L-FABP}\) and \(\text{MTP}\) was attributed to transcriptional repression via binding of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (\(\text{COUP-TFII}\)) to the proximal \(\text{DR1}\) elements in both promoters. The high expression of \(\text{L-FABP}\) and \(\text{MTP}\) by \(\text{FAO}\) hepatoma cells correlated with occupation of the proximal \(\text{DR1}\) elements by a heterodimeric complex consisting of the fatty acid activated nuclear receptors peroxisomal proliferator activated receptor \(\alpha\) (\(\text{PPAR}\alpha\)) and retinoid X receptor \(\alpha\) (\(\text{RXR}\alpha\)). Treatment of \(\text{L35}\) cells with \(\text{PPAR}\alpha\) and \(\text{RXR}\alpha\) agonists induced the transcription and expression of both genes via their \(\text{DR1}\) elements, and restored the ability of \(\text{L35}\) cells to secrete apo B. This was associated with coordinate alterations in the relative expression levels of the \(\text{DR1}\)-associated factors, as well as alteration of complexes occupying the \(\text{DR1}\) elements.

Expression levels of the coactivator peroxisome proliferator-activated receptor \(\gamma\) coactivator-1\(\beta\) (\(\text{PGC-1}\beta\)) correlates with \(\text{L-FABP}\) and \(\text{MTP}\) expression in both hepatoma cells and in the liver. RNAi-mediated reduction in the cellular content of \(\text{PGC-1}\beta\) inhibited the \(\text{PPAR}\alpha\)-\(\text{RXR}\alpha\) agonist induction of both genes in \(\text{FAO}\) cells. Adenoviral over-expression of \(\text{PGC-1}\beta\) induced both \(\text{L-FABP}\) and \(\text{MTP}\) mRNA levels in a manner dependent upon \(\text{PPAR}\alpha\)-
RXRα association with the proximal DR1 regions. The relative cellular concentrations of PPARα, RXRα and PGC-1β (increased in FAO cells) relative to COUP-TFII (increased in L35 cells) determines whether the activation-competent PPARα-RXRα complex or repressive COUP-TFII complex occupies the proximal DR1 elements of both the L-FABP and MTP promoters; in turn, determining the relative rates of transcription of both genes, allowing the liver to efficiently utilize fatty acids for VLDL assembly and secretion.
I. Introduction

1. Lipoprotein Assembly and Secretion

Essential lipids and fat-soluble nutrients are transported through the plasma to peripheral tissues, for anabolic and energy requirements, in the form of lipoproteins (1, 2). Each lipoprotein particle is composed of a surface monolayer of phospholipid, free cholesterol and various apolipoproteins, which surrounds a neutral lipid core of triglycerides and cholesterol esters (3). This unique amphipathic structure allows efficient transport of lipoprotein particles throughout the circulatory system. Three distinct gene products: apo B (4-6), L-FABP (7) and MTP (8, 9) are essential for lipoprotein assembly and secretion.

The concerted efforts of the lipid-transfer enzymes, L-FABP and MTP, facilitates the transfer of four major lipid classes (free cholesterol, phospholipids, triglycerides and cholesterol esters) to the nascent apo B via a two-step process (10, 11). Upon efficient lipidation of apo B, a secretion/transport-competent lipoprotein particle is formed and subsequently distributed through the circulation.

Apo B-containing lipoproteins are synthesized by the intestine and liver, allowing utilization of exogenous (diet derived) and endogenous (de novo synthesized) lipids, respectively. Exogenous lipids, such as dietary fatty acids and cholesterol, absorbed by the intestine are packaged into triglyceride rich lipoproteins referred to as chylomicrons. In addition to the major protein
component apo B, secreted chylomicrons contain a variety of apolipoproteins (apo AI, AIV, apo CI, II, III, and apo E) allowing for both the appropriate site-specific lipid utilization and efficient removal of metabolized chylomicron from the plasma. The exogenous fat is withdrawn from the chylomicron particle either for storage in adipose tissue or for oxidation in other tissues. Dietary cholesterol, mainly in the form of cholesterol ester, is then delivered to the liver as the remaining remnant particle is taken up via receptor-mediated endocytosis.

Endogenously produced triglycerides and cholesterol from the liver are transported to the peripheral tissues in the form of apo B-containing lipoprotein particles called very low density lipoproteins (VLDL). When the rate of hepatic de novo lipogenesis is reduced (i.e. fasting), fatty acids supplied by adipose tissue can provide sufficient substrate for the glycerolipid (triglyceride and phospholipid) synthesis and thus for VLDL assembly and secretion (12). Similar to chylomicrons, VLDL particles also contain apo C and apo E, however, they do not possess either apo AI or apo AIV. An initial enzyme-mediated triglyceride removal converts VLDL into intermediate-density lipoproteins (IDL), with subsequent removal further converting this particle into low-density lipoproteins (LDL). The liver and peripheral tissues can take up circulating LDL via receptor-mediated endocytosis. Unlike chylomicrons, which are rapidly cleared from plasma (~5-10 minutes on average), LDL particles can persist in the bloodstream for prolonged periods (averaging 2-3 days) before removal from circulation (13). This characteristic of liver-derived
apo B-containing lipoproteins allows for their accumulation and eventual oxidation in the plasma, leading to their proatherogenic potential.

Since plasma levels of apo B-containing lipoproteins directly correlate with the susceptibility to coronary artery disease, elucidation of the molecular mechanisms regulating their output has garnered much attention. It is now evident that control of hepatic VLDL output is a highly coordinated and complex process with regulation of VLDL assembly and secretion occurring on multiple levels: (1) translational and post-translational apo B synthesis and degradation, (2) hepatocyte lipid biosynthesis and availability, and (3) the process of lipid incorporation into the lipoprotein particle, in which the efficiency is regulated by the activities of key enzymes such as L-FABP and MTP.

1.1 Apolipoprotein B

A critical component of VLDL is the large amphipathic protein apolipoprotein B (apoB), which is necessary for the facilitation of systemic lipoprotein transport and maintenance of particle structural stability through its simultaneous association with both the hydrophilic outer shell and the hydrophobic core (14, 15). Apo B exists predominantly in two forms: the full-length apo B100 and smaller apo B48. In humans, apo B100 is expressed in the liver and is present on VLDL and its metabolites IDL and LDL (16). ApoB48 is expressed exclusively in enterocytes and is associated with intestinally derived chylomicrons and their metabolites, chylomicron remnant
particles (17). In several species (e.g. rodents) apo B48 is also expressed in the liver, where it is assembled into VLDL particles similar to those containing the full length apo B100 (18, 19). Apo B48 acquired its name as it is composed of approximately 48% of the N-terminus of apo B100. The occurrence of apo B48 is a result of a post-transcriptional editing of intestinal (also liver in some species) apo B mRNA that results in a stop codon about halfway along the ribonucleotide (20, 21). The editing process involves the activity of the cytidine deaminase Apobec-1 (22), which facilitates conversion of the glutamine codon 2153 (CAA) into a premature stop codon (UAA) (21). The ability of some species to secrete apo B48 from the liver is attributed to high levels of hepatic Apobec-1 expression (18, 19).

Since apo B100 provides necessary roles in the maintenance of lipoprotein particle integrity and in regulating plasma levels of LDL via its interaction with receptors responsible for LDL clearance from the plasma (23), its function is closely related to its structure. Models of lipoprotein associated-apo B100 depict it as a multidomain belt surrounding the particle (24), with the conformation and subsequent activity of the various domains dependent on particle lipid composition (25). The N-terminal α-helix (α1), which displays sequence homology with the ancient lipid transport protein lipovitellin (26), is responsible for its interaction with MTP (27) allowing neutral lipid transfer into the nascent apo B (28). Other functional domains consist of alternating hydrophobic β-sheets (β1 and β2) and hydrophilic α-helices (α1 and α2) extending through to the C-terminus of apo B100 (29). This frequent
alternation of hydrophilic and hydrophobic sequences allows multiple points of interaction with both the polar surface monolayer and the lipid-laden hydrophobic core leading to the ability of apo B100 to efficiently maintain lipoprotein structure (30). Both the β-sheets and α-helices have been shown to bind lipids, yet it is the β-sheet domains that bind lipid irreversibly allowing the permanent anchoring of apo B100 to the lipoprotein particle (29). While the α-helices are capable of binding lipids, this interaction is transient (29). Given that these same helices are the contact points for MTP (31), it is plausible that once apo B100 has acquired sufficient quantities of neutral lipid, conformation changes in these α-helices may result in dissolution of MTP-apo B association allowing the subsequent secretion of the mature lipoprotein particle.

Removal of apo B100-containing particles from the plasma, via the LDL receptor (LDL-R) is mediated by the LDL-R interacting domain of apo B100, which was mapped to amino acid residues 3359 through 3367 residing within the C-terminal β2-sheet (32). This sequence is absent in apo B48 and as a consequence apo B48-containing lipoproteins are taken up by either the chylomicron-remnant receptor or LDL-R via apolipoprotein E (33), which is added to the lipoprotein particle in the circulatory system during lipolysis (34). LDL-R binding to VLDL associated-apo B100 is inhibited by a region in the C-terminus of apo B100, which masks the LDL-R binding site only when the particle contains sufficient lipid to be classified as VLDL (35). Upon lipolytic removal of core lipid, which converts VLDL to LDL, apo B100 undergoes a conformation change allowing its association with the LDL-R (36). Additionally,
It has been reported that the more recently discovered VLDL receptor, which is highly abundant in heart, muscle and adipose tissue, can mediate the uptake of triglyceride-rich lipoproteins into peripheral fatty acid active tissues (37). The VLDL receptor is expressed at minimal levels in the liver, yet adenovirus-mediated transduction of this receptor into the livers of LDL receptor knockout mice greatly enhanced the hepatic clearance of lipoprotein-associated lipids from the plasma (37). Thus, the regulation of plasma VLDL at this level ensures that the core lipids will be utilized efficiently by the periphery.

There have been several mutations identified in the apo B100 gene with consequences ranging from premature truncation of protein synthesis to various amino acid substitutions causing compromised protein function and subsequent alterations in plasma lipid levels (38). Three naturally occurring mutations that result in decreased binding affinity for the LDL-R, have been identified. A missense mutation deemed R3500Q results in amino acid substitution of glutamine for arginine at residue 3500 causing the genetic disorder designated as familial defective apo B100 (FDB) (39). FDB is characterized by the occurrence of hypercholerolemia (25) and premature atherosclerosis (40) due to reduced uptake and catabolism of LDL by the liver (41). Another mutation occurring at residue 3500 (R3500W) is the substitution of arginine for tryptophan (42). Individuals with this mutation demonstrate a phenotype similar to individuals harboring the R3500Q mutation, characterized by hyperlipidemia and a higher incidence of atherosclerosis (43). Yet another mutation in the LDL-R binding domain of apo B100 is an exchange at residue
3531 (R3531C) which results in decreased binding affinity and subsequent hypercholesterolemia in these subjects (44). The phenotype of R3531C individuals is much less severe than those harboring mutations at residue 3500, apparently due to the relatively higher binding affinity of R3531C apo B100, which is about 3 times that of the other mutations that have been discovered thus far (44).

A study utilizing apo B truncation mutants demonstrated a strong correlation between apo B length and VLDL secretion efficiency (45). Various missense and frameshift mutations in the apo B gene, which lead to production of truncated forms of full-length apo B, result in the genetic disorder familial hypobetalipoproteinemia (FHβL) (46). FHβL is characterized by substantially decreased plasma levels of both apo B and LDL cholesterol (47), with a large number of subjects demonstrating varied degrees of hepatic steatosis (46, 48). Furthermore, some subjects with more extreme cases of FHβL manifest deficits in intestinal absorption of dietary fats and fat soluble vitamins, which can lead to severe neurodevelopmental abnormalities (47). Several mouse models, bearing different truncated forms of apo B, have been developed in attempts to better understand the pathophysiology of FHβL (9, 49, 50). The phenotype exhibited by these mouse models closely resembles that of their human counterparts, characterized by low plasma levels of apo B-containing lipoproteins and severe neurodevelopmental complications.

In contrast to human apo B-deficient states such as familial abetalipoproteinemia or FHβL (51), targeted disruption of the apo B gene in
mice leads to embryonic lethality (6). This phenotype could be rescued by complementation with a human apolipoprotein transgene (6). It has been suggested that the embryonic lethality is due to absence of lipoprotein assembly in the yolk sac which results in an inability to deliver lipid nutrients that are essential for embryonic development (52). Human fetal development ensues even in the face of apo B-deficiency as maternal lipoproteins are provided directly from the placenta, thus lipoprotein assembly and secretion is not a prerequisite for human fetal survival.

1.1.1 Apo B and VLDL Assembly

Formation of secretion-competent VLDL particles occurs in a two-step process (53). The assembly process begins in the rough endoplasmic reticulum (RER) as an MTP-mediated transfer of neutral lipid to apo B100 occurs as the apolipoprotein is being translated (54). This cotranslational lipidation, which allows for appropriate folding of apo B100 around the hydrophobic lipid core, results in the formation of a primordial lipoprotein particle called pre-VLDL (53). While formation of the pre-VLDL particle allows apo B100 to be retained within the ER for further maturation of the lipoprotein particle, it is underlipidated and unable to be secreted at this stage in the assembly process (55). As it is translocated through the lumen of the ER pre-VLDL then undergoes further lipidation through an MTP-dependent addition of bulk triglyceride, resulting in the formation of a mature VLDL particle (10). The
newly formed VLDL is then transported to the Golgi before it is packaged into secretory vesicles and released from the cell (14).

It is a general consensus that the secretion of hepatic apo B is not regulated at the level of transcription as apo B is synthesized constitutively (56). In fact, a number of studies have shown that apo B is synthesized in excess and a large amount of this newly synthesized apo B is targeted for intracellular degradation (15, 57, 58). As it is well documented that modulation of apo B100 production occurs both co- and postranslationally (59), the complexity of the secretory pathway reveals several checkpoints at which the level of hepatic apo B100 can be regulated. It has been demonstrated that multiple pause and restart processes occur during translocation of apo B through the ER, processes dependent upon specific sequences in the apo B protein (60). Failure to proceed through any of the various checkpoints, with the appropriate lipid load and conformation, leads to degradation of apo B via proteasomal and non-proteasomal pathways (61, 62). By modulating the degradation of apo B at any of the aforementioned checkpoints, VLDL assembly and secretion can be regulated by: (1) the availability of sufficient lipids to form both the surface monolayer (phospholipids and free cholesterol) and the neutral lipid core (cholesterol esters and triglycerides); (2) the activities of the lipid-transfer enzymes L-FABP and MTP.
1.2 Lipoprotein Lipids

Plasma VLDL consists of 90% lipid, of which 56% is triglyceride, 19% is phospholipid, and 17% unesterified cholesterol and cholesterol esters (3). The availability of the various lipid components, for incorporation into the lipoprotein particle, can modulate both the number of newly synthesized apo B molecules that are channeled into the secretory pathway and the general characteristics of the apo B-containing particle, in regards to lipid composition, size and secretion efficiency (63).

1.2.1 Phospholipid

Phospholipids are the major lipid component of the VLDL surface monolayer. While various phospholipids such as phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, phosphatidylserine, and phosphatidyl inositol can exist in plasma lipoproteins, the predominant phospholipid (roughly 60-75% depending on the species) of the surface monolayer is phosphatidylcholine (PC) (64). This suggests that regulation of VLDL assembly and secretion might occur through modulation of PC levels. The majority of PC utilized for lipoprotein synthesis is formed in the liver mainly through the CDP-choline pathway (~70-80% of synthesized PC) (65) in which the rate-limiting step is controlled by the enzyme cytidylyltransferase (CT). The activity of this enzyme allows for conversion of dietary choline into CDP-choline which is then combined with diacylglycerol (DG) to form PC (65). Choline deficiency is associated with an acute onset of hepatic triglyceride
accumulation, which is concomitant with a reduction of the various VLDL-associated lipids in the plasma (66). Studies in rats fed a choline-deficient diet revealed that PC becomes rate-limiting for VLDL synthesis in the appropriate context. In these animals, the diet-induced reduction in PC synthesis led to a significant decrease in the secretion of apo B-containing lipoproteins (67). The decreased VLDL secretion demonstrated by choline-deficient rat hepatocytes was a result of post-translational degradation of apolipoprotein B in the Golgi apparatus (68). In a more recent study, using rabbit hepatocytes, investigators determined that choline deficiency led to a co-translational degradation of apo B due to decreased efficiency of translocation across the ER membrane (69).

In the liver PC can also be synthesized from phosphatidylethanolamine (PE) by methylation via the PE methylation pathway regulated by activity of the microsomal enzyme PE \(N\)-methyltransferase (70). Inhibition of this enzyme/pathway by bezafibrate led to a reduction in VLDL secreted from primary rat hepatocytes (71). Additionally, drug-mediated inhibition of the PE methylation pathway \textit{in vivo} has been associated with decreased plasma triglyceride concentrations in rats (72); while mice engineered to express an inactive form of the PE \(N\)-methyltransferase gene demonstrated markedly decreased plasma lipid levels (in relation to wild type mice) when fed a choline-deficient diet (73). It has since been demonstrated that inhibition of the PE methylation pathway in cultured rat hepatocytes disrupts the late stage
of VLDL assembly by impairing the lipidation of apoB with bulk triglyceride (74), a step that is necessary for maturation of the VLDL particle (75).

These studies demonstrate not only the need for phospholipid in lipoprotein assembly but also suggest a specificity involved in terms of the phospholipid that is utilized in VLDL assembly, as PE could not replace the requirement for PC in the formation of secretion-competent lipoproteins. In accordance with this notion other studies revealed that monoethanolamine replacement of choline, as the phospholipid head group, did not allow for assembly and secretion of apo B-containing lipoproteins (76, 77). In fact, phosphatidyl monoethanolamine can block the translocation of apo B across the ER membrane (78, 79). The PE-mediated reduction in VLDL output was not due to an impairment of the assembly process as buoyant lipoprotein particles were formed (80). The reduction was a result of changes in ER membrane phospholipid composition which lessened the efficiency with which apo B was translocated into the ER lumen, thus causing the subsequent degradation of the immature particle (76).

1.2.2 Cholesterol and Cholesterol Esters

The supply of cholesterol, for incorporation into lipoproteins, is an important variable when considering the regulation of VLDL assembly and secretion. Cholesterol feeding has been shown to stimulate VLDL-triglyceride secretion from perfused rat liver (81), while addition of cholesterol to human hepatocytes increased the rate of secretion of VLDL-apo B (82). However, the
addition of cholesterol to the medium of human hepatoma (HepG2) cells had no effect on the levels of secreted apo B (83). These results were corroborated by findings from another study showing that cholesterol feeding had no effect on apo B secretion from isolated rat hepatocytes (84). The conflicting results of these studies, regarding the effects of cholesterol on apo B secretion, may have been due to either context- or species-specific differences causing altered susceptibility to cholesterol becoming rate-limiting in this process.

The rate-limiting step in cholesterol synthesis is controlled by the enzyme Hydoxymethylglutaryl-CoA reductase (HMG-CoA reductase), and studies in perfused rat liver demonstrated that stimulation of this enzyme was associated with increased secretion of both cholesterol and triglyceride (85). In contrast, exposure to the HMG-CoA reductase inhibitor lovastatin caused a marked reduction in the amount of triglyceride secreted into the perfusate, an effect that could be reversed by the addition of cholesterol to the diet of the lovastatin-fed rats (86). Since cholesterol levels play a role in the modulation of fatty acid and triglyceride biosynthetic pathways, it was possible that the effects of lovastatin on VLDL secretion were due to changes in the output of these pathways. However, it was later revealed that the lovastatin-mediated decrease in VLDL secretion was not due to alterations in triglyceride synthesis (81). It is important to note that none of the studies mentioned demonstrated changes in the levels of secreted apo B, yet led to alterations in the size and
lipid compositions of the lipoprotein particles that were being synthesized by the liver.

Levels of intracellular cholesterol can dictate hepatic expression of the LDL receptor (87). The LDL receptor is not only responsible for clearance of circulating apo B from the plasma (23), but recent evidence suggests a role in regulating hepatic secretion of apoB via modulation of its degradation prior to secretion (88, 89). Recent studies in LDL receptor deficient mice suggest that HMG-CoA reductase inhibitors, such as lovastatin, decrease VLDL secretion by increasing the intracellular degradation of apo B through a process that is LDL receptor-dependent (90).

While free cholesterol is an important component of the VLDL outer shell, the cellular content of free cholesterol is maintained relatively constant; thus it is unlikely to be a direct regulator of apo B-containing lipoprotein assembly and secretion (91). The majority of VLDL-cholesterol is present in the esterified form, known as cholesterol ester, which is deposited into the lipid core of the nascent lipoprotein particle (92). Increases in intracellular levels of cholesterol ester have correlated with decreased rates of apo B degradation (93, 94). Incubation of HepG2 with 25-hydroxycholesterol resulted in an increased cholesterol ester content and enhanced secretion of apo B (83). Additional studies using HepG2 cells have demonstrated that apo B secretion rates strongly correlate with the total mass of intracellular cholesterol ester (95).
The formation of CE is dependent upon the activities of several enzymes, yet the rate-limiting step is promoted by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), whose activity facilitates the esterification of free cholesterol to CE (96). Two different genes coding for cholesterol-esterifying enzymes have been identified, ACAT1 which is expressed ubiquitously (97) and ACAT2 which is expressed primarily in liver and intestine (98). Consistent with its distinct tissue-specific distribution, disruption of ACAT2 via generation of ACAT2 null mice resulted in a near absence of cholesterol esterification activity in both the liver and intestine (99). While overall plasma concentration of apo B in these animals remained unchanged, the size and composition of the secreted apo B-containing lipoproteins were significantly altered. In contrast, treating hamster hepatocytes with an ACAT inhibitor caused a marked reduction in apo B100 secretion (100). This effect was only seen when the cells were treated simultaneously with oleate, suggesting that in these animals cholesterol ester may only be rate-limiting for apo B secretion when fatty acids (the major lipid component of VLDL) are in excess. Further support of this hypothesis came from a study using non-human primates in which fatty acids (which normally stimulate VLDL secretion) failed to induce VLDL production when the animals were treated to a highly selective inhibitor of ACAT activity (101).
1.2.3 Triglyceride

As an energy currency triglyceride is the most concentrated biological fuel available, harboring about 9 kcal/g in comparison to carbohydrate whose energy content equates to roughly 4 kcal/g. In addition, triglycerides are biologically inert, avoiding the deleterious effects of their unesterified form, fatty acids, which can be extremely cytotoxic even at low intracellular concentrations. Together these characteristics make triglyceride ideal for energy storage, thus the transport of triglyceride throughout the body is crucial for maintenance of whole body energy homeostasis. However, their extreme hydrophobicity creates problems for its transport through the polar medium of the circulating bloodstream. To overcome this obstacle many vertebrates have evolved transport mechanisms via incorporation of large amounts of triglycerides into transport-competent lipoproteins, allowing efficient utilization of both exogenous (dietary) and endogenous (hepatic synthesis) triglyceride as an energy source.

Since triglyceride is the major lipid component of apo B-containing lipoproteins it stands to reason that its availability would play a major role as a regulator of lipoprotein assembly and secretion. Indeed, the regulation of hepatic triglyceride synthesis exerts a strong influence on VLDL production (102). Triglyceride synthesis is achieved through multiple acylation reactions for which fatty acids and glycerol-3-phosphate serve as substrates. There is a breadth of evidence suggesting modulation of lipoprotein secretion by fatty acids on multiple levels.
It is well established that increased availability of free fatty acids can effect VLDL assembly and secretion by modulation of the phospholipid and triglyceride biosynthetic pathways. Fatty acid stimulation of rat liver can lead to increased triglyceride synthesis and secretion from both liver slices (103) and perfused livers (104). In primary hepatocytes from both rats and mice, oleate increased the secretion of the VLDL lipids, triglycerides and phospholipids, without altering the secretion of apoB (90, 105, 106). Treatment of primary cultures of hamster hepatocytes with oleate stimulated both the synthesis and secretion of triglycerides and cholesterol esters, again with apo B secretion rates remaining unchanged (107).

In the study mentioned above, which utilized primary hamster hepatocytes, treatment of the cells with an inhibitor of fatty acid synthesis resulted in a decrease in triglyceride and apo B secretion by 90% and 50%, respectively (107). This effect was partially reversed by treatment of the cells with oleate (107). The combined data indicate that under basal conditions in hamster hepatocytes triglyceride availability is not rate-limiting for apo B secretion, but in accordance with the aforementioned studies in other species, it can still greatly influence the composition of the secreted lipoprotein particle. However, decreasing the intracellular pool of available triglyceride, by decreasing fatty acid synthesis, created a context in which fatty acids then became rate-limiting for apo B secretion; thus, leading to the oleate-stimulated apo B secretion likely by providing the needed substrate for triglyceride synthesis. Another study determined that increased triglyceride synthesis
caused the oleate-mediated stimulation of apo B secretion by HepG2 (human hepatoma) cells (108). Taking into consideration the results of Arbeeny et al., it is plausible that the differential effects of oleate on apo B secretion, demonstrated in the studies mentioned above are due context-specific differences in the basal rates of triglyceride synthesis and subsequent availability of VLDL-triglyceride. In support of this idea, oleic acid inclusion in the perfusate did not stimulate apo B secretion in livers from chow fed rats (109), yet resulted in significant stimulation of apo B secretion by livers from rats fed a high carbohydrate diet (which is associated with increased triglyceride synthesis) (110). Another study showed that in contrast to what occurs in rat hepatocytes, in HepG2 cells triglyceride from cytosolic stores could not be provided to apo B in quantities sufficient to protect it from degradation (111), thus in this context making triglyceride availability rate-limiting.

Other groups have demonstrated similar increases in the secretion of both apo B and triglycerides by human (111) and rat (112) hepatoma cells treated with oleic acid. However, these reports attributed the increased secretion of apo B, not to increased lipid biosynthesis and availability, but solely to decreased intracellular degradation of apo B.

There is evidence that the ability of fatty acids to modulate the secretion and composition of VLDL is influenced by fatty acid structure. In cultured rat hepatocytes, the efficiency with which individual fatty acids promoted VLDL triglyceride secretion was reduced with chain length and degree of saturation.
Guinea pigs fed a diet high in unsaturated fat secreted a greater number of apo B-containing lipoproteins in comparison to guinea pigs fed a diet high in saturated fat, however the particles had a lower triglyceride content (113). The authors reasoned that unsaturated fats (such as oleate, 18:1) might promote the incorporation of triglyceride into the nascent VLDL particles via a yet undiscovered mechanism (i.e. stimulation of enzymes such as MTP and L-FABP). Interestingly, a study using a human intestinal cell line demonstrated that MTP showed a preference for transferring unsaturated fatty acids into lipoproteins (114).

1.3 MTP

MTP is a heterodimeric protein found in the lumen of the ER of cells that are capable of secreting apo B, predominantly hepatocytes and intestinal enterocytes (115). Lower levels of MTP expression have also been detected in other tissues such as myocardium (116), yolk sac (9), and kidney (117), correlating with their ability to assemble apo-B containing lipoproteins. The assembly of apo-B containing lipoproteins has an absolute requirement for the various activities of MTP (118), which include the transfer of neutral lipids to the nascent apo B (119) and the ability of MTP to facilitate the proper folding and translocation of the apolipoprotein through the ER (120) (61). Abrogation of one or more of these concerted MTP-dependent processes leads to co-translational degradation of nascent apo B by the proteasome (57, 121-124).
The heterodimeric MTP complex consists of a unique 97-kDa subunit and a 55-kDa multifunctional protein identified as protein disulfide isomerase (PDI) (119). PDI, a ubiquitous protein which is present at high levels in the ER lumen (125), acts as a multifunctional chaperone catalyzing the formation, reduction and isomerization of disulfide bonds to assist in the appropriate folding of newly synthesized proteins (125, 126). However, this aspect of its activity is not necessary for the function of the MTP complex, in regards to its lipidation and stabilization of apo B (127). Instead, it appears that PDI serves to maintain the larger subunit in soluble form thus preserving its catalytic function (128). It has also been suggested that PDI may act as a chaperone by targeting MTP to the ER (118).

The 97-kDa subunit of MTP, which confers all of the lipid transfer activity to the heterodimer (129), is a homolog of the ancient lipid-binding protein lipovitellin (130). Lipovitellin functions in the transport of lipids from the liver to the developing oocyte of egg-laying animals (131). MTP is capable of transferring a wide variety of lipid classes (including those found in apo B-containing lipoproteins) between phospholipid membranes, demonstrating a preference for the transfer of neutral lipids such as triglycerides and cholesterol esters (132).

1.3.1 MTP and Apo B Secretion

The necessity for MTP in the production of apo B-containing lipoproteins was initially revealed by studies of patients with the genetic
disorder abetalipoproteinemia, in which there is a genetic defect in the MTP gene (8). This autosomal recessive disease is characterized by exceedingly low levels of plasma cholesterol, triglyceride and apo B due to an inability to secrete apo B lipoproteins from the liver and intestine (133-135). Individuals with this disease demonstrate impaired intestinal fat absorption and deficiencies in the peripheral delivery fat-soluble vitamins such as vitamins A and E, leading to neuro-ophthalmalogic complications (135). If initiated early in the course of the disease, therapeutic replenishment of such vitamins can ameliorate most of the complications exhibited by abetalipoproteinemic subjects (136). Several mutations within the MTP gene have been identified, most occurring as substantial deletions within the coding region of the gene (137). The wide variety of naturally occurring mutations have been associated with the broad spectrum of symptoms and degrees of severity exhibited by affected individuals (134).

Various lines of evidence from both cell culture systems and in vivo have confirmed the hypothesis that MTP is both essential and rate-limiting for the production of apo B-containing lipoproteins. Co-expression of apo B and MTP was sufficient to convert non-lipoprotein secreting cell lines into lipoprotein secretion competent cells, an effect that was dependent on the presence of MTP (138, 139). Adenoviral-mediated overexpression of MTP in human hepatoma cells caused marked induction in both MTP activity and apo B secretion rates (140). The increase in MTP was associated with a down-regulation of the proteasome-mediated degradation of apo B (140).
Overexpression of MTP in mice resulted in the stimulated secretion of VLDL triglycerides and apo B (141). In contrast, small molecule inhibitors of MTP lipid transfer activity selectively blocked apoB secretion when administered to hepatoma cells (142-144). These compounds did not effect the secretion of other lipoproteins such as apo AI (143, 145).

The essential role of MTP has been further investigated in genetically modified mice. Complete elimination of MTP function in homozygous null mice resulted in mid-gestational embryonic lethality accompanied by neurodevelopmental defects and an absence of hematopoiesis (9). The authors suggested that lethality was caused by the absence of lipoprotein secretion from the yolk sac, leading to an inability to deliver essential lipids to the developing embryo (9). Heterozygous knockout mice, which demonstrate a nearly 50% reduction in MTP expression and activity levels, exhibit parallel reductions in both plasma apo B100 (25%) and plasma cholesterol (20%) levels (9).

Two independent groups have generated viable mouse models by using Cre-LoxP mediated strategies to generate liver-specific MTP knockout mice (146, 147). Both lines of mice exhibited near complete reductions in plasma apo B100 levels and an absence of VLDL and LDL synthesis. Some differences were seen when comparing plasma lipid profiles of these mice. Raabe and colleagues observed a substantial reduction in total plasma triglyceride levels (146), while no significant changes in plasma TG were seen in the knockout mice created by the other group (147). The reason for the
apparent discrepancy in plasma triglyceride levels is not known; however, the mice demonstrating unaltered triglyceride levels showed greater levels of plasma apo B48 (147), relative to the apo B48 levels in mice created by Raabe and colleagues (146). This could be explained by an increased intestinal output of apo B-containing lipoproteins allowing plasma triglyceride levels to remain relatively constant in these mice; however, the validity of this possibility has yet to be explored.

Various MTP inhibitors have been developed that demonstrate in vitro inhibition of the transfer of triglycerides between lipid vesicles mediated by MTP (144). Both rabbits and mice treated with the MTP inhibitor, Implitapide, demonstrated decreases in secretion rates of VLDL-associated lipids (triglycerides and cholesterol) and apo B (148, 149). The Pfizer compound CP-346086 caused a lowering in plasma cholesterol and triglyceride levels in both experimental animals and humans (150). Similar decreases in plasma lipid levels were seen in guinea pigs administered the MTP inhibitor JTT-130 (151).

1.3.2 Substrate Regulation of Hepatic MTP Expression

Substrate-driven “feed-forward” transcriptional regulation is a common mechanism that allows both homeostasis and efficient and appropriate utilization to occur concomitantly (152). MTP preferentially transports triglyceride into apoB at a ratio of nearly 2:1, relative to its transport of cholesterol ester (94). Thus, it is likely that MTP expression would be regulated in a substrate-driven manner by triglycerides or their unesterified
form, fatty acids. In fact, fatty acids not only provide substrate for lipids for membrane biogenesis (phospholipids) and storage (triglycerides), they are important regulators of intracellular signaling pathways (153) and activators of gene transcription/expression by stimulating the activity of various nuclear receptors (154-156).

Fatty acid-mediated stimulation of MTP expression has been demonstrated in both cell culture systems and in vivo. Treatment of human hepatoma cells with oleic acid led to an induction of both MTP mRNA and protein, effects associated with increased gene transcription (157). The transcriptional induction of MTP by oleic acid resulted in increased MTP lipid transfer activity (157). Additionally, oleic acid stimulation of apo B secretion in rat hepatoma cells revealed a coordinate increase in MTP mRNA (158). In two separate reports in which hamsters were fed a high fat diet, hepatic MTP mRNA levels were increased by ~60%, an effect associated with increased plasma levels of VLDL-associated lipid (159, 160). In the report by Lin et al the increase in hepatic MTP mRNA demonstrated a dose dependence, as the level of MTP mRNA induction paralleled the percentage of fat in the provided diet (159). High-fat diet feeding in rats caused a ~50% increase in hepatic levels of both MTP mRNA and lipid transfer activity (161). In contrast, high-fat feeding of developing swine led to modest but statistically insignificant increase in hepatic MTP levels (162).

These contrasting results, concerning the induction of MTP by dietary fat, were dependent on the type of fat contained in the diet. It has been
reported that degree of MTP modulation is not only regulated by the amount of fat (159), but is dependent on specific fatty acid composition (160). In hamsters, diets enriched with saturated fat (such as tripalmitin and trimyristin) caused robust induction of MTP, while diets enriched in unsaturated fat (such as trilinolein and triolein) resulted in relatively modest changes (160). A diet enriched with primarily unsaturated fat was utilized in the study with swine, thus the lack of significant changes in MTP expression exhibited by these animals was likely due to the fat composition of the diet (162).

It has been demonstrated that the high fatty acid flux into the liver occurs in insulin resistant states (163). It has been suggested that the fatty acid flux contributes to an overproduction of VLDL by providing increased lipid substrate for lipoprotein assembly and secretion, thus causing a reduction in the degradation of apo B as it is directed into the secretory pathway (2). Recent evidence from a study using an animal model for insulin resistance has shown that the influx of fatty acids into the liver causes increased levels of hepatic MTP protein and activity, resulting in hepatic overproduction of VLDL (164). This suggests that the fatty acid induction of MTP, which facilitates the increase in hepatic VLDL output, contributes to the pathology associated with insulin resistance.
1.4 L-FABP

There are many functionally distinct pathways competing for the utilization of fatty acids by the liver. These include cellular uptake, esterification in the production of other lipids (e.g. triglycerides, phospholipids and cholesterol esters), β-oxidation, storage and export mainly in the form of VLDL lipids. The delivery of fatty acids into one or more of these pathways is a dynamic process, as changes in pathway-specific flux must occur rapidly and selectively in order to maintain energy and substrate homeostasis. An important component of such accommodation likely occurs following fatty acid uptake across the plasma membrane, where tissue-specific regulation of fatty acid binding has been attributed to distinct members of a large multigene family of small lipid-binding proteins. These intracellular fatty-acid binding proteins (FABPs) belong to a superfamily of cytosolic lipid-binding proteins that are abundantly expressed in vertebrate tissues. They are low molecular mass proteins (14-15kDa) that exhibit distinct tissue-specific expressions and are classified (named) according to the tissue in which they were most highly expressed. Some prominent members include heart (H-FABP), adipocyte (A-FABP), intestinal (I-FABP), and liver fatty acid binding proteins (L-FABP) (165, 166)

L-FABP is expressed at high levels in differentiated hepatocytes and enterocytes, with lower levels of expression in the kidney and colon (165, 167). L-FABP interacts with a broad range of ligands, including long-chain fatty acids (saturated, unsaturated, and branched-chain fatty acids), fatty acyl-
CoAs, phospholipids, cholesterol, bile acids and other lipophilic compounds protein, suggesting a role in multiple lipid metabolic pathways and thus in the maintenance of cellular lipid homeostasis (168). The importance of L-FABP is underscored by the fact that L-FABP mRNA constitutes 1.6% of translatable RNA in rat liver (167), and it has been shown to account for 3-5% of the cytosolic protein mass in rat hepatocytes (169).

Similar to other lipid binding proteins (MTP, apoB, vitellogenin) L-FABP demonstrates a characteristic β-barrel structure, which is formed by two orthogonal five-stranded β-sheets (170). In the unbound form the β-barrel is in a closed formation, but upon exposure to ligand two short α-helices (near the terminal region of the structure) undergo a conformational change allowing for both the entry and eventual exit of the newly available ligand (171). Most members of the FABP superfamily contain a single binding site allowing binding of one fatty acid molecule per protein (168). Extensive study using in vitro binding and crystallographic analyses indicate that L-FABP, which contains a considerably larger binding pocket (relative to other family members), is capable of binding two molecules of fatty acids simultaneously via the presence of two different ligand-binding sites (172, 173). The first binding site binds fatty acids with high affinity (~10nM), while the second site demonstrates a lower affinity (~100-500nM) for fatty acids (174). In rat liver, the concentration of L-FABP has been reported to be around 70 nmol/g, whereas the concentrations of unesterified fatty acids are roughly in the range of 50-100nmol/g (175). This suggests that L-FABP binds most of the
unesterified fatty acids and allows for their appropriation to intracellular sites of utilization as needed. In fact, it has been demonstrated that as much as 60% of cytosolic long chain fatty acids are bound by L-FABP (169, 176).

1.4.1 L-FABP and Hepatic Lipid Metabolism

Several observations are consistent with the proposal that L-FABP plays a prominent role in promoting fatty acid uptake and intracellular transport in the liver. Immuno-cytochemical localization studies have revealed that L-FABP can localize to the plasma membrane (177). Many members of the FABP family have been deemed "membrane active," in that in vitro systems have demonstrated their ability to exchange fatty acids with membrane structures via collisional and diffusional transfer mechanisms (178, 179). Photobleaching studies using fluorescent fatty acids indicated that their intracellular transport/diffusion were severely compromised in cultured hepatocytes lacking L-FABP (180). Transfection of mouse fibroblasts with L-FABP cDNA led to a ~50% increase in fatty acid uptake (181). Transfection of L-cell fibroblasts with L-FABP resulted in 2-fold increases in the cellular uptake of (182) and cis-parinaric acids (183). Rat hepatoma cells stably expressing L-FABP revealed increased uptake of palmitic acid (184). The rate of palmitic acid uptake in hepatocyte monolayers was determined primarily by the cytosolic concentration of L-FABP (185). In contrast, decreasing L-FABP content in human hepatoma cells by using antisense RNA resulted in a dose dependent decrease in the uptake of oleic acid (186).
Two independent groups have created lines of L-FABP null mice to address the validity of these findings in vivo (7, 187). Primary hepatocytes isolated from L-FABP null mice demonstrated a ~70% reduction in the uptake of oleic acid (7). Both studies found markedly reduced fatty acid uptake from the plasma in the null mice compared to their wild type counterparts (7, 187).

Studies with L-FABP null mice have not only revealed the importance of L-FABP in fatty acid uptake, but have verified its extensive role in hepatic lipid metabolism through its modulation of both intracellular fatty acid trafficking and multiple lipid metabolic pathways. One group reported altered lipid composition in livers of L-FABP null mice; 2-3 fold changes were observed for a broad range of lipids, including cholesterol, cholesterol esters, phospholipids and triglycerides (187). Primary hepatocytes isolated from L-FABP null mice demonstrated reduced fatty acid oxidation rates when treated with palmitate (188). Similarly, L-FABP null mice exhibited reduced fatty acid oxidation rates when fed either standard chow, ketogenic or diabetogenic diets (188). L-FABP null hepatocytes exhibited reduced rates of incorporation of fatty acids into esterification pathways, as cellular contents of diacylglycerols, triglycerides and phospholipids were significantly decreased (7). During fasting, hepatic fatty acid flux is increased as fatty acids are released from adipose stores of triglyceride. A study investigating the response by L-FABP null mice to fasting-induced changes in hepatic fatty acid flux revealed decreased fatty acid uptake, fatty acid oxidation, and esterified lipid levels as compared to the wild type control animals (7).
Fatty acid esterification and oxidation occurs in the endoplasmic reticulum (esterification), and mitochondria and peroxisomes (oxidation), respectively. Localization studies have indicated that L-FABP interacts with the cytoplasmic side of both the endoplasmic reticulum and mitochondrial outer membranes thus facilitating the transfer of fatty acids to these sites of utilization (189, 190). Recent immunoelectron microscopy data has revealed that L-FABP is similarly localized to peroxisomes (191). L-FABP can stimulate the activity of various enzymes involved in these processes both \textit{in vitro} (192) and in transfected cell lines and primary hepatocytes (190, 193-195). Transfection of fibroblasts with L-FABP resulted in increased intracellular concentrations in fatty acids, esterified lipids, and phospholipids (181). The change in lipid levels was associated with altered membrane fluidity (181). The authors suggested that L-FABP could regulate fatty acid utilization pathways via direct modulation of enzyme activity by changing membrane structure and fluidity.

Since L-FABP expression stimulates phospholipid and triglyceride biosynthetic pathways and given that lipoprotein assembly and secretion is highly dependent upon lipid biosynthesis and availability, L-FABP is likely to play a crucial regulatory role in apo B-containing lipoprotein synthesis. Targeted deletion of the L-FABP gene in mice potentiates hepatic cholesterol accumulation (196), an effect commonly associated with abrogation of hepatic lipoprotein output. Another report using L-FABP null mice documented increased hepatic levels in the various VLDL-associated lipids including
cholesterol, cholesterol esters, and phospholipids (187). L-FABP demonstrated a dose dependent induction of apo B secretion in rat hepatoma cells (184). Furthermore, L-FABP null mice have been characterized by decreased hepatic VLDL secretion (7).

In vitro studies have shown that L-FABP has high affinity and binding capacity for fatty acid oxidation products. The combined findings of these reports suggest that L-FABP is likely to be an effective endogenous cytoprotectant against oxidative stress. This hypothesis is supported by the finding in L-FABP stably transfected human liver cells in which they showed decreased cellular content of reactive oxygen species upon oxidative stress and reduced hypoxia-induced cellular damage (197).

1.4.2 Substrate Regulation of Hepatic L-FABP Expression

L-FABP is the predominant facilitator of substrate into fatty acid utilization pathways in the liver, thus in periods of fatty acid flux (from both exogenous and endogenous sources) L-FABP expression must be modulated accordingly, to ensure that its cellular concentration does not become rate-limiting for these processes. Given this obvious hypothesis, fatty acid regulation of L-FABP expression has been extensively explored and verified in cell culture systems and in vivo.

Mice fed a long-chain fatty acid (30% sunflower oil) enriched diet exhibited a 50% increase in hepatic L-FABP mRNA (198). Feeding mice a phytol enriched diet, resulted in a phytanic acid-mediated increase (4-fold) in
the hepatic level of L-FABP protein (199). Uptake of phytol by the liver results in its conversion to phytanic acid, a process that appears to take place in both the mitochondria and microsomes (200). The phytanic acid is then transported by L-FABP to the peroxisome for initiation of the oxidation process (199). Additionally, incubation with phytanic acid stimulated L-FABP promoter activity in human hepatoma cells (199). This induction was dependent on a functional DR1 element located in the proximal region of the L-FABP promoter (199). Treatment of rat hepatoma cells with oleate triggered increases in L-FABP mRNA (7-fold) and protein (4-fold), effects that were associated with a 17-fold induction of L-FABP gene transcription (201). Various long-chain fatty acids induced L-FABP mRNA 4-6 fold in primary rat hepatocytes (201). Livers from both rats (202) and swine (203) fed high-fat diets demonstrated increases in both L-FABP protein and activity. Thus, the fatty acid flux into the liver induces transcription and expression of L-FABP allowing a cellular content sufficient to efficiently deal with the increased demand for appropriate substrate utilization. This substrate regulation of L-FABP is consistent with findings detailing the regulation of other rate-limiting enzymes of multiple fatty acid metabolic pathways (204, 205).

2. Purpose and Scope of Dissertation

Hepatic overproduction of apo B-containing lipoproteins can lead to various metabolic and pathologic disorders including hyperlipidemia, diabetes, obesity, and cardiovascular disease. In turn, much effort has been directed
toward understanding the detailed mechanisms involved in the multilevel regulation of the assembly and secretion process of these lipoproteins. Identifying regulatory mechanisms by which hepatic VLDL synthesis is modulated, both negatively and positively, has been of great interest as it provides putative targets for the future development of new therapeutic approaches for maintaining appropriate plasma lipid levels.

The basis of this study centers around the use of two unique rat hepatoma cell lines, L35 and FAO, which demonstrate distinct gene expressions that correlate with their ability to synthesize secretion-competent apo B lipoproteins (206, 207). L35 cells completely lack the expression of the lipid transfer enzymes L-FABP and MTP, resulting in their inability to assemble and secrete lipoprotein particles. They uniquely demonstrate near in vivo levels of expression and activity of cholesterol-7α-hydroxyls (CYP7A1), an enzyme catalyzing the initial and rate-limiting step of cholesterol conversion to bile acids (208). The combined gene expressions divert cholesterol away from the lipoprotein secretory pathway and into the cholesterol catabolic bile acid biosynthetic, which is the major route by which the hepatocyte is protected against intracellular cholesterol accumulation. The L35 phenotype has allowed elucidation of the molecular mechanisms regulating both the inactivation of MTP expression (207) and stimulation of CYP7A1 expression and activity (207-209).

FAO cells, which have been used to study hepatic lipoprotein synthesis and secretion (210), are characterized by high expression of both L-FABP and
MTP, and a robust ability to assemble and secrete apo B-containing lipoproteins. In contrast to the L35 cell line, FAO cells do note express detectable levels of CYP7A1 (206, 208).

The main goal of this dissertation was to further elucidate the molecular mechanisms regulating apo B secretion via determining the causative factors dictating the coordinate expressions of L-FABP and MTP in the L35 (coordinate repression) and FAO (coordinate activation) cell lines. The following specific aims will be addressed in the body of this work: (1) determine the cis-element of the L-FABP gene that confers the cell-type specific expression, (2) determine the mechanism by which this cis-element causes inactivation of L-FABP in L35 cells, (3) define the mechanism dictating the high expression of both L-ABP and MTP in FAO cells, and (4) assess the relevance of the coordinate regulatory mechanisms in vivo.

Briefly, it was first determined that the cell-type specific expression patterns of L-FABP and MTP are coordinately determined through similar DR1 elements located in the proximal promoters of both genes. As shown previously with the MTP gene (207), the lack of L-FABP expression exhibited by L35 cells is due to the occupation of the proximal DR1 element by the "orphan" nuclear receptor COUP-TFII, which causes transcription inactivation of L-FABP gene. In the FAO cell line, the proximal DR1 elements of both the L-FABP and MTP promoters are occupied by PPARα-RXRα heterodimers, causing the high transcription and expression of both genes exhibited by these cells. Activation of PPARα and RXRα in L35 cells changes their phenotype to
something similar to that of FAO cells. Agonist activation of L35 cells changes the expression ratio of the DR1-associated factors favoring increased relative content of PPARα and RXRα (with decreased COUP-TFII) and subsequent displacement of COUP-TFII by the PPARα-RXRα complex. In turn, L-FABP and MTP expressions are induced, causing a restored ability to assemble and secrete apo B-containing lipoproteins. The coactivator PGC-1β was necessary for the PPARα-RXRα-mediated activation of L-FABP and MTP, and PGC-1β stimulation of both genes is PPARα-RXRα-dependent. Finally, L-FABP and MTP are coordinately activated by PPARα in vivo and coordinate inactivation of both genes confers mice protection from hepatic steatosis.

Deciphering of the detailed mechanisms dictating the coordinate expressions of L-FABP and MTP in the cell culture model systems provides insights as to the importance of such regulatory networks in vivo; thus, providing new targets for the therapeutic modulation of hepatic production of apo B lipoprotein synthesis.
II. METHODS

1. Cell Culture

L35 rat hepatoma cells were obtained as described (211). FAO rat hepatoma cells were FAO cells were obtained as a gift from Franz Simon (University of Colorado). HepG2 human hepatoma cells and BNL CL.2 mouse embryonic hepatoma cells were purchased from American Type Culture Collection (Manassas, Va). All cells were grown at 37°C in an atmosphere of air with 5% CO₂.

L35 cells were passed and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4% fetal bovine serum and 4% enhanced calf serum for 24 hours. The medium was changed to DMEM containing 0.5% fetal bovine serum and 1% Nutridoma HU. FAO and H35 cells were cultured in DMEM containing 4% fetal bovine serum and 4% enhanced calf serum. BNL cells were cultured in DMEM containing 10% fetal bovine serum. All media and serum were purchased from Irvine Scientific.

2. Transient Transfection and Luciferase Assay

Cells were cultured and transfected as described above. Cells were transfected using LipofectAMINE reagent (Invitrogen) according to manufacturer's protocol, with minor modifications. One day prior to transfection, L35 and FAO cells (2 x 10⁵) were seeded on 12-well plates. On the day of transfection, cells were transfected 0.8μg of promoter/luciferase reporter construct and 6ng of pRL-CMV plasmid as an internal control for
normalization of L-FABP and MTP promoter activities for differences in transfection efficiency. The normalized pRL-CMV activities are reported relative to activity of the empty vector (sans insert) from parallel experiments. Varying doses of COUP-TFII expression vector were added as indicated in the figure legends. The total DNA concentration for each assay was maintained as a constant by the addition of the empty expression vector pCR 3.1 (Invitrogen). Upon transfection, cells were incubated for 48 hours and harvested using passive lysis buffer (Promega). Luciferase activities were measured using the Dual-Luciferase Reporter Assay system buffer (Promega, Madison, WI).

The L-FABP and MTP promoter reporter assays using the PPAR\(\alpha\) and RXR\(\alpha\) agonists, WY-14,643 (WY) and 9-cis retinoic acid (cRA) respectively (A.G. Scientific Inc.), were performed as described in figure legends. Both WY-14,643 and 9-cis retinoic acid were dissolved in dimethyl sulfoxide (DMSO, 0.15% v/v) and used at working concentrations of 10\(\mu\)M (WY) and 1\(\mu\)M (cRA). Briefly, upon transfection cells were treated for 48 hours with agonists or DMSO alone as indicated. Cells were harvested and promoter/luciferase activity assays were performed as described above.

3. Reporter Gene Constructs and Expression Vectors

The wild type and mutant rat MTP reporter vectors (-135/+66) were as described previously (207). To generate the wild type rat L-FABP reporter vector (-141/+66), genomic DNA was isolated and purified from both L35 and
FAO cells using the DNeasy tissue kit (Qiagen). The promoter fragment was generated by PCR, using both L35 and FAO genomic DNA, and the following primers with the indicated restriction enzyme sites, forward 5'(KpnI)-GAA CAA ACT TCT GCC GGT ACC ATT CTG ATT TTT A-3' and reverse 5'(BglII)-TTC ATG GTG GCA ATG AGA TCT CCT TTC CAC AGC TGA-3'. The promoter fragments from both L35 and FAO were then cloned into KpnI and BglII sites of the empty luciferase reporter vector PGL3Basic (Promega). The resulting rat L-FABP promoter/reporter constructs were verified by DNA sequencing. The amplified region from L35 and FAO genomic DNA displayed no difference in the DNA sequence. The proximal sequences from the rat L-FABP and MTP were sequenced using the reporter constructs and aligned as shown in Figure 1 (Results section).

To generate the mutant L-FABP reporter vector a specific mutation in the proximal DR1 sequence was generated using the QuikChange site-directed mutagenesis kit (Stratagene). *In vitro* mutagenesis was carried out using the rat L-FABP-141-luciferase reporter vector as the template and two oligonucleotide primers (mutated bases underlined), each complementary to opposite strands of the vector (forward, 5'-AAT CGA CAA TCA CTG TG[C] TAT GGC CTA TAT TT-3'; reverse, 5'-AAA TAT AGG CCA TAG CAC AGT GAT TGT CGA TT-3'). The site-specific mutant construct was verified by DNA sequencing. The expression plasmid for COUP-TFII was a gift from Dr. Ming-Jer Tsai (Baylor Medical University).
4. Preparation of Nuclear Extracts

Nuclear extracts from L35 and FAO cells were prepared as described previously (207). Briefly, cells were trypsinized and harvested by centrifugation, washed with 1x phosphate-buffered saline, and resuspended in a hypotonic buffer (10mM HEPES, pH7.9 at 4°C, 25% glycerol, 1.5 mM MgCl2, 10mM KCl, 0.2 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol). After a 10-min incubation on ice, cells were lysed with use of a Dounce homogenizer. The nuclei were pelleted by centrifugation and resuspended in low salt buffer (20mM HEPES, pH7.9 at 4°C, 25% glycerol, 1.5 mM MgCl2, 0.02mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol). Subsequently, the high salt buffer (20mM HEPES, pH7.9 at 4°C, 25% glycerol, 1.5 mM MgCl2, 1.2mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol) was added dropwise with stirring. The resulting suspension was rocked gently for 30min to allow extraction of nuclear proteins. The nuclei were centrifuged again for 30min and the resulting supernatant was dialyzed for 1h against dialysis buffer (20mM HEPES, pH7.9 at 4°C, 20% glycerol, 100mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 0.5mM dithiothreitol).

5. Electrophoretic Mobility Shift Assays

All oligonucleotides used for EMSAs were synthesized by IDT. The following oligonucleotides (sense strands) were used in gel mobility shift assays: MTP-DR1, 5’-TGA CCT TTC CCC TAT AGA TAA ACA CTG TTG-3';
mutant MTP-DR1, 5'-TGT GCT TTC CCC TAT AGA TAA ACA CTG TTG-3'; L-FABP-DR1, 5'-TGA CCT ATG GCC TAT ATT TGA GGA GGA AGA-3'; mutant L-FABP-DR1, 5'-TGT GCT ATG GCC TAT ATT TGA GGA GGA AGA-3'. The probes were prepared by annealing the complementary oligonucleotides and by end labeling with (γ-32P)ATP (3000mCi/mmol), PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs), followed by purification on a G50 column. For binding reactions, 15µg of nuclear extracts were incubated with 3x104 cpm probe on ice for 20min in a total volume of 15µl of solution (20mM HEPES, pH7.9 at 4°C, 10% glycerol, 100mM KCl, 1 mM EDTA, and 2µg poly(dI-dC). For supershift experiments, 1µl of specific antibodies were added to preincubated DNA-protein complexes for an additional 20min on ice. Antibodies against COUP-TFII (sc-6576X), RXRα (sc-553X), and PPARα (sc-9000X) were obtained from Santa Cruz Biotechnology, Inc. DNA-protein complexes were resolved on 4% native polyacrylamide gel electrophoresis containing 0.5x TBE buffer.

6. RNA isolation, cDNA synthesis and Real-time PCR

Total RNA was isolated from either frozen liver using the Versagene RNA Tissue Kit (Gentra Systems, Inc.) or from cells using the Versagene RNA Cell Culture Kit (Gentra Systems, Inc.) with on-column DNA removal per manufacturer's instructions. The RNA concentrations were determined by spectrophotometer at 260nm. First strand cDNA was synthesized from 0.5µg of total RNA using the BioRad iScript for reverse transcription (BioRad).
Specific primers for each gene were designed using gene sequences from GenBank™. To avoid amplification of genomic DNA, the primers were positioned to span exon junctions. All primers were synthesized by IDT.

Real-time PCR analysis was performed with the BioRad iCycler using BioRad SyBr Green supermix according to manufacturers instruction. The reactions were analyzed in triplicate with specific product monitored using melt-curve analysis. The expression data were normalized to an endogenous control, either 18S ribosomal RNA or acidic ribosomal phosphoprotein P0 (36B4). The level of both 18S RNA and 36B4 was invariable among samples of all experiments. The relative expression levels were calculated according to the formula 2^{-\Delta Ct}, where \Delta Ct is the difference in threshold cycle (Ct) values between the target and either the 18S or 36B4 endogenous control.

7. Chromatin Immunoprecipitation Assay and Relative Quantitation

Cells were cultured in complete medium in 150-mm dishes until ≈70-80% confluent. Where indicated the agonists WY (10µM) and cRA (1µM) were added to cell culture medium for 48h prior to harvesting. The cells were then fixed by the addition of 280µl of 37% formaldehyde (Sigma) to 10ml of culture medium for 10min at 37°C, harvested, and processed for immunoprecipitation using the ChIP-IT Shearing Kit (Active Motif) and ChIP-IT Chromatin Immunoprecipitation Kit (Active Motif) for chromatin immunoprecipitation according to manufacturer’s protocol with minor modifications. Briefly, formaldehyde treated cells were rinsed with ice-cold PBS and scraped in
100 mM Tris-HCL (pH 9.4), 10 mM DTT and incubated for 15 min at 30°C. Cells pellets were collected and washed with 1 ml ice cold PBS, followed by sequential washes with Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5) and Buffer II (200 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5). Washed pellets were resuspended in 400 μl of Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing 1x protease inhibitor cocktail and sonicated with 8 sets of 10-second pulses using a Sonifier Cell Disruptor (Branson Sonic Power Inc., Danburg, CT). Sheared chromatin samples were centrifuged for 10 min at 12,000 rpm at 4°C, and the supernatants were collected and diluted in Dilution Buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1). Samples were pre-cleared with 2 μg of sheared salmon sperm DNA, 20 μl pre-immune serum and 45 μl of protein A-sepharose (50% slurry) for 2 hours at 4°C. Immunoprecipitation with pre-immune serum, COUP-TFII antisera, or PPARα antisera was performed overnight at 4°C. Immune complexes were eluted, reverse cross-linked using 5 M NaCl at 65°C, treated with proteinase K, and purified using mini-columns provided with ChIP-IT kit.

Specific genomic DNA fragments from immunoprecipitated samples and inputs were quantitated by Real-time PCR with BioRad SyBr Green Supermix as indicated above. For PCR 5 μl of immunoprecipitated DNA and 1 μl of input DNA was utilized as template amounts. As a control for region selectivity of immunoprecipitation-specific enrichment differences, amounts of non-coding distal untranslated regions were determined for each sample. The
antibodies used were against COUP-TFII (a gift from Dr. S. Karathanasis), PPARα (sc-9000X, Santa Cruz Biotechnology) and control IgG (Active Motif). Primer sets were designed to amplify the following rat genomic DNA regions: MTP-DR1 forward-5'-TAG TGA GCC CTT CCA TGA AC-3', MTP-DR1 reverse-5'-CAG AAT CTG CGA CAA CAG TG-3'), L-FABP-DR1 forward-5'-GAG TTA ATG TTT GAT CCT GGC C-3', L-FABP-DR1 reverse-5'-CCA CCC ACT GTT GGC TAT TTT-3'), L-FABP-3'-untranslated region forward-5'-GTC TTC CGC TAC CTA AGA GG-3', L-FABP-3'-untranslated region reverse-5'-CTG TCA TCT GAC CAG CTC TC-3'). All values were normalized to values from both input DNA and immunoprecipitation with IgG using the ΔΔCt method. Briefly, for every promoter studied a ΔCt value was calculated for each sample by subtracting the Ct value for the input DNA from the Ct value obtained for the immunoprecipitated sample. A ΔΔCt value was then calculated by subtracting the ΔCt value for the sample immunoprecipitated with the specific antibody (PPARα or COUP-TFII) from the ΔCt value for the corresponding sample immunoprecipitated with normal rabbit serum (IgG). Fold differences (factor-specific ChIP relative to control IgG ChIP) were then determined by raising 2 to the ΔΔCt power.

8. RNA interference

Knockdown of PPARα in FAO cells was achieved by using the Smartpool siRNA (Dharmacon) specific for rat PPARα. The sequence for
siRNA directed against rat PGC-1β was selected as described (212). The rat PGC-1β siRNA (sense sequence; 5'-GAT ATC CTC TGT GAT GTT A-3') was synthesized by Dharmacon as a 21-nucleotide duplex, using option A4, with 3' dinucleotide (TT) overhangs. The siCONTROL non-targeting siRNA #1 (Dharmacon) was utilized as a negative control sequence to monitor non-specific targeting. Cells were plated in 12-well plates at a concentration of 5x10^4/well 24 hours prior to experiment. Prior to transfection, siRNAs were resuspended in 1X siRNA Buffer (Dharmacon) to a concentration of 20µM. All siRNAs were transfected into FAO cells using the DharmaFECT™ 4 transfection reagent (Dharmacon) according to manufacturer's instructions. FAO cells were transfected with the indicated siRNAs for 48-72h at working concentrations of 100nM as indicated in figure legends. For the knockdown of PGC-1β, cells were transfected with the indicated siRNAs for 72 hours; cells were exposed to the agonists WY-14,643 (10µM) and 9-cisRA (1µM) 24 hours post-transfection and treated for 48 hours prior to harvesting. RNA isolation, cDNA synthesis and Real Time PCR expression analyses were performed as described above.

9. Animal Studies

Male PPARα-/- mice and age matched WT littermates (on SV/129 background) were fed a standard chow diet, supplemented with either the PPARα agonist GW-7647 (2.5mg/kg/d) or equivalent amount of solvent vehicle (DMSO), for 7 weeks. All animals had ad libitum access to water.
Mice were weighed every 2 weeks and drug intake was adjusted according to mean weight. Upon end of treatment animals were sacrificed, liver was isolated, and total RNA was extracted using Versagene RNA Tissue Kit (Gentra Systems, Inc.). First-Strand cDNA synthesis and Real Time PCR expression analyses were performed as described above.

For MTP inhibitor (8aR) studies male L-FABP -/- mice and age matched WT controls (C57BL/6) were fed a standard chow diet, then administered the 8aR compound orally each day for 7 days at a dose of 50mg/kg body weight. Upon end of treatment animals were sacrificed, plasma and livers were isolated, and the various hepatic and plasma lipid levels were determined using commercially available kits as described previously (7).

10. Adenovirus Infections

Adenoviral vectors expressing either PGC-1β (Ad-PGC-1β) or GFP (Ad-GFP) were generous gifts from Dr. B. Spiegelman. L35 cells were infected with either the Ad-PGC-1β or Ad-GFP for 2 hours in serum-free media, then treated for 48 hours with the complete media with the agonists WY-14,643 (10µM) and 9-cisRA (1µM). Cells were infected ~75-85% as determined by GFP expression. RNA isolation, cDNA synthesis and Real Time PCR expression analyses were performed as described above.
11. Western Blot Analysis

Nuclear Protein isolated from L35 and FAO cells were separated on a 4-12% gradient SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. After the blots were blocked with 10% dry milk in water for 1 hour at room temperature, they were incubated overnight at 4°C with an antibody specific for COUP-TFII (a gift from Dr. S. Karathanasis), RXRa (sc-553), HNF-1 (sc-6547), or PPARα (sc-9000) (Santa Cruz Biotechnology). After rinsing with TBS-T, the blots were incubated with secondary antibodies diluted in TBS-T for 1 hour at room temperature. The hybridized bands were visualized by chemiluminescence as in the protocol of the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

12. Apo B Immunoprecipitation and SDS-PAGE

Secreted apoB was immunoprecipitated as previously described (206). Briefly, L35 cells were cultured in 60mm dishes in the absence or presence of WY-14,643 (10µM) and 9-cisRA (1µM) for 72 hrs. Cells were then switched to methionine-free DMEM for 2 hrs and then labeled with 3ml (35S)-methionine (100 µCi/ml) in DMEM for 24 hrs. Media were collected and incubated with polyclonal anti-apoB antibody overnight at 4°C. Protein A-sepharose was then added and the mixture was further incubated for 2 hrs at 4°C. The immunoprecipitate complex was washed 3 times with TETN buffer (25 mM Tris at pH 7.5, 5 mM EDTA, 250 mM NaCl and 1% Triton X-100) and once with PBS. The pellet was resuspended in SDS-PAGE loading buffer, boiled
for 5 min and resolved on a 4-12% Tris-glycine gel by electrophoresis. Radioactive proteins were detected by autoradiography.

13. Lipid Extraction and Analysis

Livers were homogenized in PBS and protein concentration determined. 300µl of homogenate was extracted with 5 ml of chloroform methanol (2:1) and 0.5 ml 0.1% sulfuric acid. An aliquot of the organic phase was collected, dried under nitrogen, and resuspended in 2% Triton X-100. Hepatic FFA, TG, and cholesterol content were determined using commercially available kits. Data were normalized for differences in protein concentration.
III. RESULTS

1. Similar DR1 elements in proximal regions of the L-FABP and MTP promoters confer coordinate cell type-specific transcriptional activity and mRNA expression

A DR1 element located within the proximal MTP promoter region was previously shown to be responsible for the lack of expression in L35 cells and the high level expression exhibited by FAO cells (207). Occupation of this DR1 element by COUP-TFII was shown responsible for repressed MTP gene transcription exhibited by L35 cells (207). In this study, we examined whether the L-FABP gene, whose product is involved in the regulation of VLDL assembly and secretion (7), would be regulated by a similar mechanism. Sequence analysis of the proximal region of the L-FABP promoter (Figure 1) indicated that it contains a DR1 element which is similar to the DR1 element responsible for transcriptional regulation of the MTP gene (207). Moreover, expression of L-FABP mRNA by L35 and FAO cells paralleled the expression of MTP mRNA (Figure 2) suggesting that the transcription of both genes may be coordinately regulated.

Luciferase reporter constructs driven by either the L-FABP- or MTP-proximal promoter regions displayed similar cell-type specific differences; promoter activities were approximately 8-fold higher in FAO cells relative to levels in L35 cells (Figure 3). Mutational inactivation of the DR1 element, in either the L-FABP or MTP promoter constructs, decreased the promoter
activities in FAO cells to levels similar to those exhibited by L35 cells (Figure 3). Thus, the proximal DR1 elements residing within the promoter regions of the L-FABP and MTP genes are sufficient to confer relative promoter activities which correlate with the endogenous mRNA levels of both genes displayed by the L35 and FAO cell lines.

**Figure 1. Comparison of proximal promoter sequences of the MTP and L-FABP genes.** 5' flanking sequences of rat MTP and L-FABP genes are given with *cis*-elements common to each promoter indicated. Proximal DR1 elements of both promoters have 5' and 3' hexameric half-sites underlined. The position of each base is marked in reference to the transcription start site.
Figure 2. **MTP and L-FABP demonstrate similar cell-type specific mRNA levels**. 4µg of total RNA isolated from L35 and FAO cells was used to synthesize cDNA, then subject to quantitative real-time PCR analysis using SYBR green. All reactions were performed in triplicate. The relative mRNA levels of MTP and L-FABP were calculated using the comparative Ct method, with 36B4 mRNA as the invariant control. L35 indicated as open bars and FAO indicated as filled bars. All values shown as mean ± SD (n=3).
Figure 3. **MTP and L-FABP demonstrate similar cell-type specific differences in promoter activity levels.** Luciferase constructs driven by either the MTP (-135/+66) or L-FABP (-141/+66) promoters were transiently transfected into L35 and FAO cells. Constructs containing mutant DR1 elements consist of base pair changes in the 5' hexameric half sites of each promoter from AC to TG as indicated. Activities of mutant constructs are indicated as MTP-mutDR1 and L-FABP-mutDR1. Luciferase activities are represented by filled bars (FAO cells) and empty bars (L35 cells). All luciferase values were normalized to a Renilla control and given as mean ± SD (n=3).
2. Binding of COUP-TFII to the proximal DR1 site mediates transcriptional repression of the L-FABP gene.

2.1 The transcriptional inactivation of L-FABP in L35 cells is associated with COUP-TFII binding to the L-FABP-DR1 element.

Nuclear extracts obtained from the L35 and FAO hepatoma cell lines formed distinct DNA-protein complexes with the oligonucleotide probe containing the DR1 element of the L-FABP promoter (Figure 4). The DNA-protein complex formed using nuclear extracts from L35 cells exhibited a supershift with an antibody specific for COUP-TFII (Figure 4). In contrast, the DNA-protein complex formed using nuclear extracts from FAO cells did not display a supershift with the COUP-TFII antibody (Figure 4). Since mutation of the DR1 site blocked the formation of the cell-type specific DNA-protein complexes, they required an intact DR1 site (data not shown).

To determine whether COUP-TFII was associated with the L-FABP-DR1 region in the context of chromatin, chromatin immunoprecipitation (ChIP) analyses were developed. Amplified promoter fragments included the promoter region containing the L-FABP-DR1 and a control distal untranslated region from the L-FABP gene over 1kb away from the proximal DR1 element. The distal untranslated region does not contain a DR1 element, thus it will serve as a background control for the immunoprecipitation. ChIP assays using a COUP-TFII-specific antiserum showed that chromatin immunoprecipitated from L35 cells exhibited a 4-fold enrichment of the L-FABP-DR1 region,
compared to chromatin from FAO cells (Figure 5). The level of L-FABP-DR1 obtained in FAO cells was similar to that of the distal untranslated region, thus is likely representative of background associated with the immunoprecipitation. This suggests that COUP-TFII is not associated with the L-FABP-DR1 in FAO cells. Since the levels of the distal untranslated region immunoprecipitated from both cell lines were similar (Figure 5), the ~4-fold enrichment of the L-FABP-DR1 region reflects cell-type specific differences in binding of COUP-TFII to the L-FABP promoter. Thus, the data obtained from the ChIP analyses (Figure 5) were concordant with the data obtained from the EMSA-supershift analysis (Figure 4), indicating that in L35 cells COUP-TFII is associated with the DR1 element in the proximal promoter of the L-FABP gene.
Figure 4. The L35-specific complex formed with the L-FABP-DR1 site contains COUP-TFII. Utilizing EMSA with a radiolabeled L-FABP-DR1 probe, differential complexes were attained comparing nuclear extracts from L35 and FAO cells. To assess the presence of COUP-TFII in the L35-/FAO-specific complexes, antibodies specific for COUP-TFII were added to the nuclear extract during incubation with the L-FABP-DR1 probe. The L35-specific COUP-TFII complexes with and without antibody addition, are indicated by a filled arrow and open arrow, respectively.
Figure 5. **In L35 cells COUP-TFII associates with the L-FABP-DR1 region in the context of chromatin.** The association of COUP-TFII with the L-FABP promoter in L35 and FAO cells was measured by ChIP analysis. Sheared chromatin from both cell lines was immunoprecipitated with COUP-TFII antisera or pre immune serum as a normalization control. Subsequently, relative amounts of the L-FABP-DR1 promoter region (filled bars) and a control distal untranslated region (open bars) were determined by real-time PCR. Relative level of the distal untranslated region demonstrates region specificity and IP background. Values were normalized to input DNA and immunoprecipitation with pre immune serum using the ΔΔCt method. All values were given as mean ± SD (n=3).
2.2 **COUP-TFII expression demonstrates an inverse relationship with L-FABP in various hepatoma cell lines.**

To determine whether differential expression levels of COUP-TFII in L35 and FAO cells contributes to the L35-specific recruitment of COUP-TFII to the L-FABP-DR1 element, the relative mRNA and protein levels were determined comparing the two cell lines. Results from real-time PCR (Figure 6A) and western blot (Figure 6B) analyses revealed that L35 cells express nearly 2-3 fold higher levels of both COUP-TFII mRNA and protein when compared to levels detected in FAO cells. The increased expression of COUP-TFII in L35 cells correlates with its occupation of the L-FABP-DR1 (Figures 4 and 5).

To further validate the finding that COUP-TFII expression levels are inversely correlated with L-FABP expression in hepatoma cells, relative mRNA levels of COUP-TFII and L-FABP were determined comparing four different hepatoma cell lines (L35, FAO, H35 and BNL). Quantitation of COUP-TFII and L-FABP mRNA levels in the various hepatoma cell lines revealed an inverse correlation (Figure 7). These results further support the proposed role of COUP-TFII in mediating L-FABP gene repression.
Figure 6. **Relative COUP-TFII mRNA and protein levels in L35 and FAO cells.** A. Quantitative Real-Time PCR. 4µg of total RNA isolated from L35 and FAO cells was used to synthesize cDNA, then subject to real-time PCR analysis using SYBR green. All reactions were performed in triplicate. The relative mRNA levels of COUP-TFII in L35 (open bars) and FAO (filled bars) was determined using the comparative Ct method, with 36B4 mRNA as the invariant control. All values shown as mean ± SD (n=3). B. Western blot analysis. Western analysis was performed using nuclear extracts prepared from L35 and FAO cells. 6µg of protein from each cell line was separated on a 4-12% gradient SDS-polyacrylamide gel, and the transferred blot was probed with specific antibodies recognizing COUP-TFII or HNF-1 (as loading control) as indicated.
Figure 7. **COUP-TFII expression demonstrates an inverse correlation with L-FABP in various hepatoma cell lines.** 4µg of total RNA isolated from L35, FAO, H35 and BNL hepatoma cells was used to synthesize cDNA, then subjected to real-time PCR analysis using SYBR green. All reactions were performed in triplicate. The relative mRNA levels of COUP-TFII (open bars) and L-FABP (filled bars) in the various cell lines were determined using the comparative Ct method, with 36B4 mRNA as the invariant control. All values shown as mean ± SD (n=3).
2.3 Increased COUP-TFII expression decreases L-FABP promoter activity in FAO cells.

Since increased cellular content of COUP-TFII was associated with decreased L-FABP expression, the effect of COUP-TFII expression on L-FABP promoter activity was determined in FAO cells (high basal L-FABP levels). Ectopic expression of COUP-TFII in FAO cells transfected with a COUP-TFII expression plasmid resulted in a dose dependent decrease in L-FABP promoter activity (Figure 8). Since increased COUP-TFII expression did not alter the activity of an L-FABP promoter construct harboring a mutational inactivation of the DR1 sequence (Figure 8), the repression of the L-FABP promoter by COUP-TFII is dependent on a functional DR1 element. The maximal reduction of transcription exhibited by FAO cells transfected with the plasmid expressing COUP-TFII only partially (50-70%) recapitulated the low level exhibited by L35 cells suggesting that additional factors are likely to contribute to the cell-type specific differences in expression. These findings, similar to those obtained using the MTP-DR1 element (207), support the conclusion that in L35 cells occupancy of these DR1 elements by COUP-TFII is responsible for transcriptional inactivation of both genes (Figure 3).
Figure 8. **Ectopic expression of COUP-TFII in FAO cells decreases L-FABP promoter activity in a dose dependent manner.** A luciferase reporter plasmid containing sequences –141/+66 of the rat L-FABP promoter was cotransfected with the indicated amounts of COUP-TFII expression plasmid into FAO cells. The construct containing the mutant DR1 element is as described in Figure 3. All luciferase values were normalized to a Renilla control and given as mean ± SD (n=3).
3. **PPARα-RXRα** heterodimers compete with COUP-TFII for binding to the DR1 promoter elements of both the MTP and L-FABP genes

EMSA-supershift analyses of the complexes formed with the MTP-DR1 site revealed a FAO cell-specific complex containing RXRα (207). PPARα and RXRα agonists have been shown to stimulate the promoter activity of the L-FABP gene, an effect that was dependent on the presence of an intact DR1 site in the proximal promoter region (213, 214). However, no detailed analysis of promoter binding by these factors has been established for the L-FABP promoter. It is likely that MTP gene transcription may be activated via a similar DR1-dependent mechanism, and that the activation of both genes by PPARα-RXRα could be responsible for the phenotype exhibited by FAO cells. The possibility of PPARα-RXRα association with the proximal DR1 elements of both L-FABP and MTP was explored using EMSA-supershift and ChIP analyses. EMSA-supershift analyses using nuclear extracts from FAO cells demonstrated that DNA probes containing either the L-FABP- or MTP-DR1 sites formed a similar FAO-specific complex (indicated by arrow in Figure 9). The FAO-specific complex for both DR1 elements was identical to the RXR-containing complex reported in our previous findings with MTP (207). The two slower migrating bands were contained in both cell types and thus are likely functionally insignificant, in regards to the cell-type specific phenotypes (207). The FAO-specific complexes formed with probes containing either the L-FABP-DR1 and MTP-DR1 sites did not supershift with a COUP-TFII specific antiserum (Figure 9, lane 2), but did supershift with an antiserum recognizing
either RXRα (Figure 9, lane 3) or PPARα (Figure 9; lane 4). In contrast, PPARα-RXRα supershifts were not detected with the nuclear extracts obtained from L35 cells ((207) and data not shown). These findings indicate that in FAO cells, the DR1 sites of both the L-FABP and MTP promoters are occupied by PPARα-RXRα heterodimers while these same sites are occupied by a COUP-TFII complex in L35 cells.

ChIP analyses using a COUP-TFII-specific antiserum showed that chromatin immunoprecipitated from L35 cells exhibited a 4-fold enrichment of L-FABP-DR1 (Figure 10) and MTP-DR1 (Figure 11) regions, compared to chromatin immunoprecipitated from FAO cells (Figures 10 and 11 respectively). In contrast, using a PPARα-specific antiserum, chromatin obtained from FAO cells, exhibited a ~5-fold enrichment of the proximal L-FABP-DR1 (Figure 10) and MTP-DR1 (Figure 11) regions compared to chromatin from L35 cells. The levels of control distal untranslated regions (regions devoid of DR1 elements) were similar to those of the COUP-TFII-specific immunoprecipitation in FAO cells and levels of PPARα-specific immunoprecipitation in L35 cells. This indicates that COUP-TFII is not associated with the L-FABP-DR1 and MTP-DR1 regions in FAO cells, while PPARα is not associated with these regions in L35 cells. Furthermore, since the levels of untranslated regions immunoprecipitated from both cell lines were similar (Figures 10 and 11), the enrichment of DNA sequences containing the proximal L-FABP-DR1 (Figure 10) and MTP-DR1 (Figure 11) regions reflect cell-type specific differences in binding of PPARα-RXRα (FAO cells) or
COUP-TFII (L35 cells) to both the L-FABP and MTP promoters. Thus, the data obtained from the ChIP analyses (Figures 10 and 11) were concordant with the data obtained from the EMSA-supershift analyses for the proximal DR1 elements of both genes (207 and Figures 4 and 9).

Figure 9. PPARα/RXRα bind to the L-FABP-DR1 and MTP-DR1 probes in FAO cells. Utilizing EMSA with a radiolabeled L-FABP-DR1 or MTP-DR1 probes, similar FAO-specific complexes (filled arrows) were attained. To assess the presence of PPARα/RXRα in the FAO-specific complexes, antibodies specific for COUP-TFII (C, lane2), RXRα (R, lane3), or PPARα (P, lane4) were added to the nuclear extract during incubation with either the L-FABP-DR1 or MTP-DR1 probe as indicated.
Figure 10. **Cell-type specific complex formation with the L-FABP-DR1 element in L35 (COUP-TFII) and FAO (PPARα/RXRα) cells.** The association of COUP-TFII and PPARα with the L-FABP promoter in L35 and FAO cells was measured by ChIP analysis. Sheared chromatin from both cell lines was immunoprecipitated with COUP-TFII antisera, PPARα antisera or pre immune serum as a normalization control. Subsequently, relative amounts of the L-FABP-DR1 promoter region (filled bars) and a control distal untranslated region (open bars) were determined by real-time PCR. Relative level of the distal untranslated region demonstrates region specificity and IP background. Values were normalized to input DNA and immunoprecipitation with pre immune serum using the ΔΔCt method. All values were given as mean ± SD (n=3).
Figure 11. **Cell-type specific complex formation with the MTP-DR1 element in L35 (COUP-TFII) and FAO (PPARα/RXRα) cells.** The association of COUP-TFII and PPARα with the L-FABP promoter in L35 and FAO cells was measured by ChIP analysis. Sheared chromatin from both cell lines was immunoprecipitated with COUP-TFII antiserum, PPARα antiserum or pre immune serum as a normalization control. Subsequently, relative amounts of the MTP-DR1 promoter region (filled bars) and a control distal untranslated region (open bars) were determined by real-time PCR. Relative level of the distal untranslated region demonstrates region specificity and IP background. Values were normalized to input DNA and immunoprecipitation with pre immune serum using the \( \Delta \Delta Ct \) method. All values were given as mean ± SD (n=3).
4. Relative expression levels of COUP-TFII, PPAR\(\alpha\), and RXR\(\alpha\) in L35 and FAO cells correlate with complex occupancy at the L-FABP-DR1 and MTP-DR1 elements.

In an attempt to explain the differences in DR1-specific complex assembly seen in the L35 and FAO cell lines, the relative cellular concentrations of mRNA and protein COUP-TFII, PPAR\(\alpha\), and RXR\(\alpha\) were measured. Both cell lines contained relative levels of COUP-TFII mRNA that were inversely related to the amounts of both PPAR\(\alpha\) and RXR\(\alpha\) (Figure 12A). L35 cells are characterized by high levels of COUP-TFII coinciding with drastically lower levels of PPAR\(\alpha\) and RXR\(\alpha\) (Figure 12A). The markedly reduced content of PPAR\(\alpha\) (which is associated with activation of L-FABP and MTP in FAO cells) in L35 cells likely explains the finding that RNAi mediated decrease in COUP-TFII in these cells was unable to result in stimulation of MTP (Kang, unpublished data). In contrast, FAO cells demonstrate high levels of PPAR\(\alpha\) and RXR\(\alpha\), and a relatively decreased level of COUP-TFII (Figure 12A). The relative differences in protein level for each of the nuclear receptors correlate with those observed for their respective mRNAs (Figure 12B). This suggests that the observed L35/FAO cell type specific differences in both the L-FABP and MTP promoter occupancy (Figures 10 and 11) and expression levels (Figure 2) are likely due to the differential cellular contents of COUP-TFII, PPAR\(\alpha\), and RXR\(\alpha\) (Figure 12).
Figure 12. **Relative mRNA and protein levels of COUP-TFII, PPARα, and RXRα in L35 and FAO cells.** A. Quantitative Real-Time PCR. 4µg of total RNA isolated from L35 and FAO cells was used to synthesize cDNA, then subject to real-time PCR analysis using SYBR green. All reactions were performed in triplicate. The relative mRNA levels of COUP-TFII, PPARα, and RXRα in L35 (open bars) and FAO (filled bars) were determined using the comparative Ct method, with 36B4 mRNA as the invariant control. All values were relative to PPARα in L35, which was set to 1. All values shown as mean ± SD (n=3). B. Western blot analysis. Western analysis was performed using nuclear extracts prepared from L35 and FAO cells. 6µg of protein from each cell line was separated on a 4-12% gradient SDS-polyacrylamide gel, and the transferred blot was probed with specific antibodies recognizing COUP-TFII, PPARα, and RXRα as indicated.
5. PPARα and RXRα agonist treatment of L35 cells results in a conversion to the FAO phenotype.

5.1 Agonist-mediated stimulation of mRNA and transcriptional activity of both L-FABP and MTP in L35 cells.

Treatment of L35 cells with either a PPARα or RXRα agonist markedly increased the levels of both L-FABP (~60-75 fold) and MTP (~55-65 fold) mRNAs (Figure 13). Treatment with both agonists synergistically increased L-FABP and MTP mRNA levels by nearly 300-fold (Figure 13). L-FABP and MTP promoter luciferase reporter constructs exhibited similar responses to the PPARα and RXRα agonists (Figure 14). Treatment of L35 cells with PPARα and RXRα agonists (both separately and combined) enhanced the transcriptional activities of both the MTP- and L-FABP-DR1 promoter reporter constructs by up to 10-fold (Figure 14). No significant changes in the activities of either promoter were detected using reporter constructs harboring mutant DR1 sites (Figure 14). These findings indicate that the PPARα-RXRα agonist-mediated stimulation of the L-FABP and MTP genes are mediated through the proximal DR1 elements.

It should be noted that treatment with the vehicle DMSO alone caused a relatively modest but significant increase in both the expression of L-FABP and MTP mRNA and the activities of their promoter-reporters (Figure 13 and 14, respectively). Clearly, PPARα and RXRα agonists added to cells using DMSO as a vehicle exhibited a far greater induction of L-FABP and MTP than
did DMSO vehicle alone (Figure 13 and 14). The induction of PPARα activated genes by DMSO has been described (215).

Figure 13. PPARα/RXRα agonist treatment of L35 cells results in the coordinate induction of L-FABP and MTP mRNAs. 4μg of total RNA isolated from untreated L35 cells (-) and those treated for 48 hours with either the PPARα agonist 10 μM WY-14,643 (WY), the 1 μM RXRa 9-cis retinoic acid (RA) agonist, or the vehicle (DMSO) as indicated, were used to synthesize cDNA, then subject to real-time PCR analysis using SYBR green. WY/RA indicates L35 cells treated with both agonists simultaneously. All reactions were performed in triplicate. The relative mRNA levels of L-FABP AND MTP were determined using the comparative Ct method, with 36B4 mRNA as the invariant control. All values shown as mean ± SD (n=3).
Figure 14. **PPARα/RXRα** agonist treatment of L35 cells results in the coordinate induction of L-FABP and MTP promoter activities. Utilizing both the wild type and mutant-DR1 luciferase reporter constructs (described in figure 3), relative promoter activity levels, for both L-FABP and MTP, were determined comparing untreated L35 cells (-) to those treated for 48 hours with either the PPARa agonist 10 µM WY-14,643 (WY), the 1 µM RXRa 9-cis retinoic acid (RA) agonist, or the vehicle (DMSO) as indicated. WY/RA indicates L35 cells treated with both agonists simultaneously. Promoter activities are indicated for wild type as open bars and for the mutant-DR1 constructs as filled bars. All luciferase values were normalized to a Renilla control and given as mean ± SD (n=3).
5.2 Agonist treatment of L35 cells restores their ability to secrete apoB.

L35 cells lack the ability to assemble and secrete apo B-containing lipoproteins due to transcriptional inactivation of the MTP gene (206, 207). Treating L35 cells with PPARα-RXRα agonists markedly enhanced the secretion of de novo synthesized 35S-labeled apo B (Figure 15). Thus, PPARα-RXRα agonists restored expression of L-FABP and MTP lead to an activation of apoB-containing lipoprotein assembly and secretion in L35 cells.

![Figure 15](image-url)

**Figure 15.** PPARα/RXRα agonist treatment of L35 cells results in rescue of their ability to secrete apo B. L35 cells were cultured in the absence (lanes 1-3) or presence (lanes 4-6) of 1 µM 9-cis retinoic acid and 10 µM WY-14,643 for 72 hrs before being labeled with [35S]-methionine. Media was collected 24 hrs after the addition of radioactivity. Secreted apo B was immunoprecipitated with a polyclonal anti-apoB antibody and resolved by SDS-PAGE (4-12%). Labeled proteins were detected by autoradiography. The locations of apo B48 and apo B100 was determined by molecular weight markers and human LDL standards.
5.3 Agonist induction of L-FABP and MTP in L35 cells is associated with changes in the relative cellular content of COUP-TFII, PPARα, and RXRα.

The data to this point indicates that the relative expression levels of the DR1-associated factors contributes to the phenotypes of L35 and FAO cells, in regards to the differential proximal DR1 complex occupancy and subsequent differential expression of both L-FABP and MTP. Since treatment of L35 cells with PPARα-RXRα agonists restored the expression of L-FABP and MTP, mRNA and protein levels of COUP-TFII, PPARα, and RXRα were measured comparing control L35 cells (untreated and DMSO-treated) to those treated simultaneously with PPARα and RXRα agonists (context of maximal L-FABP/MTP induction). Real-time PCR analysis of mRNA levels revealed that treating L35 cells with PPARα-RXRα agonists altered the levels of nuclear receptors so that they resembled the levels displayed by FAO cells (Figures 12 and 16). Thus, treatment of L35 cells with PPARα-RXRα agonists induced the expression of both PPARα (5-fold) and RXRα (2.3-fold) mRNAs, whereas the level of COUP-TFII was decreased (~60%) (Figure 16A). L35 cells treated with DMSO alone also exhibited a drastic reduction in COUP-TFII levels, but no change in the levels of either PPARα or RXRα (Figure 16A). The DMSO-mediated reduction in COUP-TFII may explain why DMSO alone was associated with increased transcription and expression of L-FABP and MTP (Figures 13-14). Western blot analysis revealed that the agonist-mediated
changes in protein levels of COUP-TFII, PPAR\(\alpha\), and RXR\(\alpha\) (Figure 16B) were concordant with the changes in mRNA levels of these factors.
Figure 16. **PPARα/RXRα agonist treatment of L35 cells changes the relative levels of mRNA and protein of COUP-TFII, PPARα, and RXRα so that they resemble the profile seen in FAO cells.**

A. Quantitative Real-Time PCR. Relative mRNA levels of COUP-TFII, RXRα, and PPARα were determined comparing untreated L35 cells (open bars) to those treated for 48 hours with WY-14,643 and 9-cis retinoic acid simultaneously (filled black bars) or DMSO (filled grey bars) as indicated. All values were normalized to levels of 36B4 mRNA as an invariant control and shown as mean ± SD (n=3).

B. Western blot analysis. Western analysis was performed using nuclear extracts prepared from L35 either untreated or treated with WY-14,643 and 9-cis retinoic acid simultaneously. 6µg of protein from indicated L35 cells was separated on a 4-12% gradient SDS-polyacrylamide gel, and the transferred blot was probed with specific antibodies recognizing COUP-TFII, PPARα, and RXRα as indicated.
5.4 Agonist-mediated changes in the cellular levels of the DR1-associated factors result in altered complex occupation of the proximal elements of both the L-FABP and MTP genes.

To assess whether the agonist-mediated changes in expression of the transcription factors (Figure 16) coincided with an alteration in complex occupancy of the DR1 sites in the endogenous promoters, ChIP analyses of agonist-treated L35 cells were compared to that of untreated L35 and FAO cells. PPARα-RXRα agonist treatment of L35 cells decreased (~3-fold) the amount of L-FABP-DR1 (Figure 17) and MTP-DR1 (Figure 18) region containing chromatin immunoprecipitated with the COUP-TFII-specific antiserum, while it led to enriched levels (~3-fold) immunoprecipitated by a PPARα-specific antiserum (Figure 17, L-FABP-DR1 and Figure 18, MTP-DR1). The levels of control distal untranslated region immunoprecipitated from both untreated and treated L35 cells were similar (Figures 17 and 18). This indicates that the changes in levels of DNA sequences containing the proximal L-FABP-DR1 (Figure 10) and MTP-DR1 (Figure 11) regions reflect cell-type specific differences in binding of COUP-TFII (untreated L35 cells) and PPARα-RXRα (treated L35 cells) to both the L-FABP and MTP promoters. Thus, the results from the ChIP analyses were concordant with the changes in expression levels of the nuclear receptors (Figure 16). The combined data indicate that PPARα-RXRα ligand activation converts the L35 cell type phenotype into the FAO cell type phenotype by increasing the cellular content of activator complex PPARα-RXRα relative to the repressor COUP-TFII.
These changes result in parallel alterations in the complex associating with the DR1 elements of both the L-FABP and MTP genes, favoring DR1 occupation by the activating PPARα-RXRα complex.

Figure 17. PPARα/RXRα agonist treatment of L35 cells results in altered occupancy of the L-FABP-DR1 region from the repressive COUP-TFII complex to an activating PPARα/RXRα complex. ChIP assays, comparing untreated L35 and FAO cells to L35 cells treated with both WY-14,643 and 9-cis retinoic acid (L35W/R), were performed utilizing antibodies specific for COUP-TFII and PPARα as indicated. Sheared chromatin from both cell lines was immunoprecipitated with COUP-TFII antisera, PPARα antisera or pre immune serum as a normalization control. Subsequently, relative amounts of the L-FABP-DR1 promoter region (filled bars) and a control distal untranslated region (open bars) were determined by real-time PCR. The relative level of distal untranslated region demonstrates region specificity and IP background. Values were normalized to input DNA and immunoprecipitation with pre immune serum using the ΔΔCt method. All values were given as mean ± SD (n=3).
Figure 18. **PPARα/RXRα agonist treatment of L35 cells results in altered occupancy of the MTP-DR1 region from the repressive COUP-TFII complex to an activating PPARα/RXRα complex.** ChIP assays, comparing untreated L35 and FAO cells to L35 cells treated with both WY-14,643 and 9-cis retinoic acid (L35W/R), were performed utilizing antibodies specific for COUP-TFII and PPARα as indicated. Sheared chromatin from both cell lines was immunoprecipitated with COUP-TFII antisera, PPARα antisera or preimmune serum as a normalization control. Subsequently, relative amounts of the MTP-DR1 promoter region (filled bars) and a control distal untranslated region (open bars) were determined by real-time PCR. The relative level of distal untranslated region demonstrates region specificity and IP background. Values were normalized to input DNA and immunoprecipitation with preimmune serum using the ΔΔCt method. All values were given as mean ± SD (n=3).
6. **PPARα is necessary for high expression levels of L-FABP and MTP in hepatoma cells and *in vivo*%

The data thus far suggests that the PPARα-RXRα activation complex is essential for the coordinated expression of L-FABP and MTP. To test this hypothesis, RNA interference was employed to decrease the expression of PPARα in the FAO cell line. Real-time PCR was used to determine if any changes in L-FABP or MTP mRNA would be associated with the decreased PPARα expression. FAO cells transfected with PPARα specific siRNAs demonstrated a 75% decrease in PPARα mRNA, when compared to cells transfected with control siRNA (Figure 19). This decrease in PPARα mRNA was associated with a reduced cellular content of both L-FABP and MTP mRNAs to nearly 50% of the control (Figure 19). The PPARα specific RNAi did not alter the mRNA levels of PPARα independent apoB, suggesting that the decreases in L-FABP and MTP were PPARα reduction specific (Figure 19). These findings indicate that PPARα is necessary for the relatively high expression levels of L-FABP and MTP exhibited by FAO cells.

To more directly address the necessity of PPARα in liver gene expression, the ability of PPARα agonist to coordinately induce the hepatic expression of L-FABP and MTP mRNAs *in vivo* was measured. Control SV/129 and PPARα -/- mice were treated with the PPARα agonist GW-7647 (216). While control mice displayed increased levels of both L-FABP and MTP mRNAs following treatment with the PPARα agonist (Figure 20), mice lacking
functional PPARα displayed no significant changes in neither L-FABP nor MTP expression levels (Figure 20). These in vivo data support the findings obtained using L35 and FAO hepatoma cells. Together these findings demonstrate the important role PPARα plays in regulating the transcription of both L-FABP and MTP genes.
Figure 19. **PPARα is necessary for maintenance of L-FABP and MTP expression in FAO cells** A, RNA interference knockdown of PPARα was achieved by transfecting FAO cells for 72 hours with either PPARα-specific siRNAs or non-targeting control siRNAs as a negative control. Using real time PCR, relative mRNA levels of PPARα, MTP, L-FABP, and ApoB were determined comparing FAO cells treated with PPARα-specific siRNA (filled bars) to those treated with the negative control siRNA (open bars). The mRNA levels in FAO cells treated with PPARα-specific siRNAs are expressed as percentages of the negative control set to 100%. All values were normalized to levels of 18S RNA as an invariant control and shown as mean ± SD (n=3).
Figure 20. **PPARα is necessary for the GW-7647 mediated induction of L-FABP and MTP expression in vivo.** Control SV/129 and PPARα -/- mice (5 mice/group) were treated with the PPARα agonist GW-7647 for 7 weeks. Using real time PCR, relative mRNA levels of L-FABP and MTP were determined. Relative mRNA levels are represented as filled (GW-7647 treated) and open (vehicle treated) bars. All values were normalized to levels of 18S mRNA as an invariant control and shown as mean ± SD (n=5).
7. **PGC-1β Acts in Concert with PPARα to Cooperatively Induce the L-FABP and MTP Genes.**

7.1 **PGC-1β expression directly correlates with cellular levels of both the L-FABP and MTP in hepatoma cells and in vivo.**

The transcriptional coactivators PGC-1α and PGC-1β (217) exhibit distinct gene targets (212, 218). While PGC-1α activates genes involved in gluconeogenesis and mitochondrial biogenesis/fatty acid oxidation (218), PGC-1β activates genes involved in mitochondrial biogenesis/fatty acid oxidation and hepatic lipid transport (e.g. MTP (212)). The observations that L35 cells express relatively high levels of PGC-1α and nearly undetectable levels of PGC-1β, whereas FAO cells express high levels of PGC-1β and nearly undetectable levels of PGC-1α (Figure 21) are consistent with the proposal that PGC-1β activates MTP gene expression (212). This proposal is further supported by the finding that both L35 cells and mice treated with PPARα agonists resulted in a nearly 3-fold induction of PGC-1β mRNA (Figure 22); both are contexts in which L-FABP and MTP were coordinately induced (Figures 13 and 20 respectively).
Figure 21. **PGC-1β and PGC-1α mRNA levels in L35 and FAO.** 4μg of total RNA isolated from L35 and FAO cells was used to synthesize cDNA, then subject to quantitative real-time PCR analysis using SYBR green. All reactions were performed in triplicate. The relative mRNA levels of PGC-1α and PGC-1β were calculated using the comparative Ct method, with 36B4 mRNA as the invariant control. L35 indicated as open bars and FAO indicated as filled bars. All values shown as mean ± SD (n=3).
Figure 22. **PGC-1β expression correlates with L-FABP and MTP in hepatoma cells and in vivo.** Real-time PCR analysis of PGC-1β mRNA levels comparing untreated L35 (open bars) and L35 cells treated with WY-14,643 and 9-cis retinoic acid simultaneously (filled bars) for 48 hours. Additionally, wild type C57BL/6 (5 mice/group) were treated with the PPARα agonist GW-7647 for 7 weeks. Using real time PCR hepatic PGC-1β mRNA levels were determined comparing untreated wild mice (Wt, open bars) and wild type mice treated with the PPARα agonist GW-7647 for 7 weeks (Wt GW-7647, filled bars). All values were normalized to levels of 36B4 mRNA as an invariant control and shown as mean ± SD (n=5).
7.2 PGC-1β is necessary for the maximal induction of both the L-FABP and MTP by PPARα-RXRα agonists in hepatoma cells.

To examine the role of PGC-1β in the induction of L-FABP and MTP, FAO cells were treated with PPARα-RXRα agonists and the effect of siRNA-mediated knockdown of PGC-1β was determined. PPARα-RXRα agonist treated FAO cells given the siRNA specific for PGC-1β demonstrated a 65% reduction in PGC-1β mRNA levels, which was associated with coordinate decreases in both L-FABP (-38%) and MTP (-48%) mRNA levels, whereas PGC-1α mRNA levels remained unchanged (Figure 23). PPARα-RXRα agonist treated FAO cells given a negative control siRNA exhibited no change in any of these mRNA levels (Figure 23). Thus, the siRNA demonstrated target-specificity and the associated reductions in L-FABP and MTP mRNA expressions were due to the reduction in PGC-1β content.
Figure 23. **PGC-1β is necessary for the PPARα-RXRα mediated induction of L-FABP and MTP in hepatoma cells.** RNA interference knockdown of PGC-1β was achieved by transfection of FAO cells for 72 hours with either PGC-1β specific siRNAs or non-targeting siRNAs as a negative control. 48 hours prior to harvesting, cells were treated simultaneously with the agonists WY (10 μM) and RA (1 μM). Using real time PCR, relative mRNA levels of PGC-1β, MTP, L-FABP, and PGC-1α were determined comparing FAO cells treated with PGC-1β-specific siRNA (filled bars) to those treated with the negative control siRNA (open bars). The mRNA levels in FAO cells treated with PGC-1β-specific siRNAs are expressed as percentages of the negative control set to 100%. All values were normalized to levels of 18S RNA as an invariant control and shown as mean ± SD (n=3).
7.3 PPAR\(\alpha\)-RXR\(\alpha\) activity is necessary for the PGC-1\(\beta\) mediated induction L-FABP and MTP mRNA in L35 cells.

To further examine the role of PGC-1\(\beta\) in L-FABP and MTP gene expression, PGC-1\(\beta\) was introduced ectopically into untreated and PPAR\(\alpha\)-RXR\(\alpha\) agonist treated L35 cells via transduction with an adenovirus expressing PGC-1\(\beta\) (212, 219). Cellular mRNA levels of L-FABP and MTP were measured via real-time PCR. The PGC-1\(\beta\) adenovirus did not affect L-FABP (Figure 24) or MTP (Figure 25) mRNA levels in untreated L35 cells. In contrast, treating PPAR\(\alpha\)-RXR\(\alpha\) agonist-stimulated L35 cells with the PGC-1\(\beta\) adenovirus significantly increased both L-FABP (Figure 24) and MTP (Figure 25) mRNAs (~3fold), relative to levels in both the agonist-treated uninfected and GFP-infected controls. Treating PPAR\(\alpha\)-RXR\(\alpha\) agonist-stimulated L35 cells with an adenovirus that expressed GFP had no effect on the expression of L-FABP (Figure 24) and MTP (Figure 25) mRNAs. Thus, PGC-1\(\beta\) mediated induction of L-FABP and MTP expression is dependent on the presence of active PPAR\(\alpha\)-RXR\(\alpha\) at the proximal DR1 elements of both genes. These data provide additional support for the proposal that both PPAR\(\alpha\)-RXR\(\alpha\) and PGC-1\(\beta\) are necessary for enhancing the coordinated transcription of L-FABP and MTP.
Figure 24. **PGC-1β mediated increase of L-FABP in L35 cells is PPARα/RXRα dependent.** Real time PCR analysis of L-FABP mRNA levels in L35 cells treated with adenoviral constructs and agonists as indicated for 48 hours. L35 cells were infected with either Ad-PGC-1β, Ad-GFP or uninfected as indicated. Infection coincided with simultaneous agonist treatment WY and RA for 48 hours where indicated. All values were normalized to levels of 36B4 mRNA as an invariant control and shown as mean ± SD (n=3).
Figure 25. **PGC-1β mediated increase of MTP in L35 cells is PPARα/RXRα dependent.** Real time PCR analysis of MTP mRNA levels in L35 cells treated with adenoviral constructs and agonists as indicated for 48 hours. L35 cells were infected with either Ad-PGC-1β, Ad-GFP or uninfected as indicated. Infection coincided with simultaneous agonist treatment WY and RA for 48 hours where indicated. All values were normalized to levels of 36B4 mRNA as an invariant control and shown as mean ± SD (n=3).
8. Coordinate inactivation of L-FABP and MTP prevents hepatic steatosis \textit{in vivo}.

Inactivation of MTP through the use of chemical inhibitors has been a strategy employed to therapeutically ameliorate hyperlipidemia (149, 150, 220, 221). However, the use of MTP inhibitors is associated with the development of hepatic steatosis (222). While L35 cells do not express MTP or secrete apo B-containing lipoproteins, they do not accumulate triglycerides even when challenged with fatty acids (e.g. oleic acid) (206). Genetic deletion of L-FABP in mice resulted in a marked reduction in both hepatic fatty acid uptake and formation of triglycerides (7). Thus the lack of fat accumulation associated with the complete absence of MTP expression in L35 cells may be explained by the coordinate decrease in L-FABP.

To test this hypothesis the responses of both C57BL/6 and L-FABP null mice (L-FABP-/-), to treatment with an MTP inhibitor (8aR) (223) for 7 days, were measured. As expected, plasma levels of triglyceride and cholesterol were decreased to similar levels in both the C57BL/6 and L-FABP-/- mice (Figure 26), indicating that the 8aR compound was active and lipoprotein secretion was inhibited in both strains. Treatment with the MTP inhibitor caused a nearly 4-fold increase in hepatic triglyceride levels in the control C57BL/6 mice (Figure 27). In contrast, L-FABP -/- mice demonstrated no significant change hepatic triglyceride levels upon treatment with 8aR (Figure 27). Thus in the absence of L-FABP, which facilitates fatty acid uptake and delivery into triglyceride biosynthesis, inactivation of MTP was not sufficient to
cause deleterious triglyceride accumulation. These data show that coordinate transcription regulation of L-FABP and MTP genes allows variation in VLDL assembly and secretion in the absence of hepatic steatosis.

Figure 26. Treatment of C57BL/6 and L-FABP/- mice with an MTP inhibitor decreases plasma levels of VLDL-associated lipids. Male C57BL/6 and L-FABP/- mice (5 mice/group) were given the MTP inhibitor 8αR (50mg/day/kg) for 7 days. Mice were sacrificed, plasma was obtained and lipid levels determined. Plasma triglyceride and cholesterol levels were determined for both C57BL/6 and L-FABP/- mice comparing vehicle treated (open bars) to those treated with 8αR (filled bars). All values represent the mean ± SD (n=5).
Figure 27. **Prevention of the MTP inhibitor induced hepatic steatosis by ablation of the L-FABP gene.** Male C57BL/6 and L-FABP-/- mice (5 mice/group) were given the MTP inhibitor 8aR (50mg/day/kg) for 7 days. Mice were sacrificed, livers were obtained and lipid levels determined. Liver triglyceride levels were measured for both strains comparing untreated (open bars) to those treated with 8aR (filled bars). All values represent the mean ± SD (n=5).

The text and figures presented in the RESULTS section (Chapter III) are, in part, reprints of the material as it appears in:


The dissertation author and the co-authors listed in these publications directed and supervised the research that forms the basis for Chapter III.
IV. DISCUSSION

1. Proximal DR1 elements mediate coordinate transcription and expression of the L-FABP and MTP genes.

The concerted efforts of the lipid-transfer enzymes, L-FABP and MTP, allow for the production and transfer of the various VLDL-associated lipids to the nascent apo B, resulting in a secretion-competent lipoprotein particle. Thus it is plausible that the two genes would be coordinately regulated to ensure that neither would become rate-limiting when the cellular context dictates a necessity for lipid to be partitioned into the secretory pathway. Several lines of evidence support this hypothesis. Structurally, the lipid binding domains of L-FABP and MTP are quite similar and it has been suggested that they evolved from a common gene which coded for an ancestral lipid-binding protein (26, 170, 224, 225). In addition, parallel to the similarity in protein structure can be seen when comparing the promoters of the L-FABP and MTP genes. The proximal promoter regions of the both genes demonstrate a strikingly similar architecture with various trans factor-specific response elements spatially conserved (Figure 1) (207, 226-228). Furthermore, the proximal promoter sequences of L-FABP (-132/+21) (229) and MTP (-123/+66) (207, 226) have been shown to be sufficient to confer hepatocyte-specific expression for both genes.

Previous studies indicated that L35 and FAO hepatoma cells displayed reciprocal expression of MTP caused by differential, cell-type specific
transcripitional regulation mediated through a DR1 element residing within the proximal promoter region of the MTP gene (207). Since sequence analysis revealed that L-FABP promoter contained a DR1 element similarly located in the proximal region of the promoter (Figure 1), it was determined whether L-FABP would demonstrate the cell-type specific regulation exhibited by MTP. Both L-FABP and MTP demonstrated identical cell-type specific expression patterns with relatively high mRNA levels in FAO cells, while the mRNA concentrations in L35 cells were barely above background (Figure 2), suggesting that the transcription of both genes may be coordinately regulated. Accordingly, the proximal promoter regions of the L-FABP (-141/+66) and MTP (-135/+66) displayed similar cell-type specific activities (Figure 3) that paralleled the mRNA levels (Figure 2). Mutation of the 5' half-site of the DR1 element, in either the L-FABP or MTP promoter constructs, resulted in an abrogation of the cell-type specific activity differential (Figure 3). Thus, the proximal DR1 elements, residing within the promoter regions of the L-FABP and MTP genes, are sufficient to confer relative promoter activities which correlate with the endogenous mRNA levels of both genes displayed by the hepatoma cell lines.

In the context of hepatoma cells it has been shown that the proximal HNF-1 site (Figure 1) is necessary for maintenance of basal transcriptional activity of both the L-FABP (227) and MTP (226) promoters. In fact, HNF-1 null mice demonstrate a drastic reduction in basal L-FABP expression levels (227). While MTP levels have yet to be determined in these mice, they exhibit fatty
livers, which are a common characteristic associated with abrogation of MTP expression/function. The findings here reveal that the proximal DR1 element is yet another cis-element important in the regulation of both L-FABP and MTP. While the HNF-1 response element may confer appropriate basal expression in the liver, it is likely that the DR1 sites were retained in the promoters of these two genes allowing for appropriate coordination of two elements (L-FABP and MTP) key for lipid utilization through the VLDL biosynthetic pathway.

2. Molecular mechanisms facilitating both the coordinate transcriptional repression and activation of the L-FABP and MTP genes via their DR1 elements.

2.1 Transcriptional repression of the L-FABP and MTP genes by COUP-TFII

Numerous reports have implicated the "orphan" nuclear receptor COUP-TFII as a negative transcriptional regulator of various enzymes involved in the metabolism of fatty acids, via occupation by a COUP-TFII homodimer of DR1 elements in the promoters of these genes (230-233). Previously, it was determined that COUP-TFII binding to a well-conserved DR1 element in the proximal promoter region of the MTP gene facilitated transcriptional repression in L35 cells (207), which led to an inability to divert fatty acids into the secretory pathway as lipid components of VLDL (206). The following
observations reveal that L-FABP, an intracellular fatty acid binding protein necessary for the delivery of fatty acids into the VLDL-glycerolipids biosynthetic pathways (7), can be negatively regulated by COUP-TFII in a manner similar to that seen with MTP.

The lack of L-FABP transcription (Figure 3) and expression (Figure 2) exhibited by L35 cells, correlated with an L35-specific complex associating with the L-FABP-DR1 element as demonstrated by EMSA (Figure 4). It was determined by EMSA-supershift analysis that this L35-specific complex consisted of COUP-TFII (Figure 4). Similar to that observed for MTP, in L35 cells, a COUP-TFII complex occupied the proximal DR1 element of the L-FABP gene, as demonstrated by EMSA-supershift (Figure 4) and ChIP (Figure 5) analyses. The presence of COUP-TFII at the DR1 site was due to increased cellular content of COUP-TFII in L35 cells, relative to the level in FAO cells (Figures 6A-mRNA and 6B-protein). This is supported by the finding that COUP-TFII causes a dose dependent repression of L-FABP transcriptional activity when overexpressed in FAO cells, and this effect is DR1 site dependent (Figure 8). Furthermore, an inverse correlation between L-FABP and COUP-TFII expression was observed when comparing relative levels of the two mRNAs in various hepatoma cell lines (Figure 7). Thus, L-FABP and MTP expressions can be coordinately repressed by binding of COUP-TFII to similar DR1 elements residing within the proximal promoter regions of each gene (Figure 28), modulating the directionality of hepatic fatty acid flux away from the secretory pathway.
Figure 28. The role of COUP-TFII in the coordinate repression the L-FABP and MTP genes. COUP-TFII association with the DR1 elements in the proximal promoters of the L-FABP and MTP genes results in coordinate repression and subsequent inactivation of apo B lipoprotein synthesis.
2.2 Transcriptional activation of both the L-FABP and MTP genes by PPARα-RXRα

2.2.1 Occupancy of the DR1 element by a PPARα-RXRα heterodimer is associated with high transcription and expression of L-FABP and MTP in FAO cells.

Mutational inactivation of the DR1 element lead to marked reductions in the promoter activities of both L-FABP and MTP in FAO cells (Figure 3), suggesting that the proximal DR1 site serves as a positive regulatory cis-element in this cell line. Thus, the proximal DR1 elements with each promoter exhibit a dual functionality, allowing for either transcriptional repression (as in L35 cells) or activation (in FAO cells) depending on the cellular context. This is consistent with the dual regulatory role of DR1 elements in the regulation of many other genes involved in hepatic lipid metabolism. More specifically, a number of DR1 regulated genes are key components of various fatty acid metabolic pathways, including fatty acid uptake, transport, esterification, and oxidation (207, 230-233). In the context of these genes COUP-TFII and PPARα-RXRα compete with each other for binding to DR1 promoter elements. Occupation of these elements by PPARα-RXRα is associated with activation of transcription, whereas occupation by COUP-TFII is associated with transcriptional repression.

PPARα and RXRα agonists have been shown to activate L-FABP promoter activity in hepatoma cells, an effect dependent on the DR1 site in the
proximal promoter region (213, 214). However, detailed binding studies demonstrating a direct effect of PPARα-RXRα on L-FABP via their binding to the DR1 element are lacking. We have demonstrated previously that EMSA-supershift analyses of the complexes formed with the MTP-DR1 site revealed a FAO cell-specific complex containing RXRα (207). Given these findings, it is likely that the high expression levels of L-FABP and MTP exhibited by FAO cells is due to transcriptional activation by PPARα-RXRα heterodimer via association with the proximal DR1 elements of both genes. Indeed EMSA-supershift analyses revealed that the FAO-specific complex, formed with either the L-FABP-DR1 or MTP-DR1, consisted of PPARα-RXRα heterodimers (Figure 9). Assessment of the complexes bound to the proximal DR1 regions in the in vivo context of chromatin were in accordance with the EMSA-supershift analyses (Figures 10 and 11). In FAO cells PPARα-RXRα heterodimers were associated with the proximal regions of both promoters, while in L35 cells COUP-TFII was bound to these regions (Figure 10-L-FABP and Figure 11-MTP). This provides the immediate molecular mechanism allowing the dual functionality of the proximal DR1 elements in the regulation of both the L-FABP and MTP genes. When COUP-TFII is present the DR1 sequences act as negative regulatory elements, while PPARα-RXRα occupation of the DR1 sites confers their role as positive cis-elements (Figure 29).
Figure 29. **The dual role of the proximal DR1 elements in the coordinate regulation the L-FABP and MTP genes.** COUP-TFII and PPARα-RXRα complexes compete for binding to the DR1 elements in the proximal promoters of the L-FABP and MTP genes. When COUP-TFII is present the DR1 sequences act as negative regulatory elements resulting in coordinate repression of L-FABP and MTP, while PPARα-RXRα occupation of the DR1 sites confers their role as positive *cis*-elements resulting in coordinate activation of both genes. The coordinate regulation of L-FABP an MTP results in a subsequent parallel modulation of the lipoprotein secretory pathway.
The role of PPAR\(\alpha\) serving as an activator of MTP is supported by the recent finding that treatment of wild type, but not PPAR\(\alpha\) knockout mice, with a PPAR\(\alpha\) agonist increased hepatic expression of MTP (234). The stimulation of MTP coincided with an increase in apo B secretion (234). PPAR\(\alpha\) is well documented as a nuclear receptor controlling the transcription of genes involved in several lipid metabolism pathways, such as \(\beta\)-oxidation and fatty acid uptake and transport, as well as lipoprotein clearance (235). The results here now reveal that it plays a prominent role in the regulation of lipoprotein assembly and secretion, in the context of hepatoma cells, through its coordinate activation of L-FABP and MTP. Since the endogenous ligands for PPAR\(\alpha\) include fatty acids (236-238), and given that hepatic expression of both L-FABP (198) and MTP (159) can be induced by fatty acids, this mechanism likely persists in vivo.

During the fasting state, fatty acids supplied by adipose tissue can provide sufficient substrate for the glycerolipid synthesis and VLDL assembly/secretion (12). Fasted PPAR\(\alpha\) null mice demonstrated reduced levels of both L-FABP and MTP when compared to fasted wild type mice (239, 240). Furthermore, the decreased hepatic concentrations of L-FABP and MTP are associated with the development of fatty liver in these mice (240). Thus, physiological states resulting in increased hepatic influx of fatty acids, such as fasting and high-fat consumption, will result in the coordinate induction of L-FABP and MTP via PPAR\(\alpha\), driving the flux of excess fatty acids into the lipoprotein biosynthetic pathway.
2.2.2 Relative expression levels of the DR1-associated factors

The finding that repression of L-FABP and MTP in L35 cells was due to increased COUP-TFII levels (Figures 6) leading to its subsequent occupation of the proximal DR1 elements of both genes (Figures 4, 5, 10 and 11), was further supported by the relative expression level data from the various hepatoma cell lines (Figure 7). The efficiency of COUP-TFII competition for DR1 elements with activator complexes has been shown to be dependent on relative cellular concentrations of the competing DR1-associated factors (241).

The relative expression levels of COUP-TFII, PPARα, and RXRα indicate a similar mechanism for the determination the L35- and FAO-specific binding patterns for the L-FABP-DR1 and MTP-DR1 elements. In FAO cells, in which the DR1 elements are occupied by PPARα-RXRα, the expression profile favors high cellular contents of PPARα and RXRα, with relatively a low level of COUP-TFII (Figure 12). In contrast, L35 cells are characterized by a high cellular content of COUP-TFII, and relatively low levels of both PPARα and RXRα (Figure 12), coinciding with COUP-TFII occupation of the proximal DR1 elements of the L-FABP and MTP genes. Thus, the relative expression levels of the competing DR1-associated factors reflect the nature of the complex associated with the proximal promoters of both genes. Suggesting that the plasticity in the cellular phenotypes of a given hepatocyte, in regards to VLDL assembly and secretion, may be dependent upon the relative cellular contents of COUP-TFII (repressor) and PPARα-RXRα (activator).
2.2.3 PPAR\(\alpha\) and RXR\(\alpha\) agonist treatment of L35 cells results in a conversion to the FAO phenotype.

Several lines of evidence support the hypothesis that the proximal DR1 elements of L-FABP and MTP serve as "switches" for determining hepatocyte phenotypes, in regards to lipoprotein metabolism. L35 cells, which are normally characterized by a near complete lack of L-FABP and MTP expressions, and an inability to assemble and secrete lipoprotein particles, can be converted to a cell-type with characteristics resembling an FAO cell by treatment with agonists for PPAR\(\alpha\) and RXR\(\alpha\). Exposure to the PPAR\(\alpha\) (WY-14,643) and RXR\(\alpha\) (9cisRA) agonists caused a 250-300 fold induction of both L-FABP and MTP mRNAs (Figure 13). This was associated with coordinate DR1-dependent increases in the transcriptional activities of both promoters (Figure 14).

The relative cellular concentrations of the competing complexes correlated with the induction of L-FABP and MTP transcription and expression. PPAR\(\alpha\)-RXR\(\alpha\) agonist induction of both genes in L35 cells was associated with changes in the relative contents of the DR1-associated factors COUP-TFII, PPAR\(\alpha\) and RXR\(\alpha\) (Figure 16), with the expression profile resembling that seen in the FAO cell line (Figure 12). Agonist treatment resulted in a marked reduction in COUP-TFII, with coordinate increases in both PPAR\(\alpha\) and RXR\(\alpha\) (Figure 16), thus reversing the relative levels of the complexes competing for the DR1 element. Changing the nuclear milieu from a repressor (COUP-TFII) dominant context to an activator (PPAR\(\alpha\)-RXR\(\alpha\)) dominant
context resulted in coordinate alterations in complex-occupancy of the DR1 elements in the L-FABP and MTP promoters. Agonist treatment of L35 cells lead to the presence of a PPARα-RXRα complex associated with the proximal DR1 regions of both promoters, in comparison to untreated L35 cells in which COUP-TFII remained bound to this region (Figures 17-L-FABP and 18-MTP).

The sum of agonist-mediated events that resulted in restored expression of L-FABP and MTP led to a restored ability of L35 cells to secrete apo B (Figure 15). This provides detailed mechanistic insight regarding the findings demonstrating PPARα agonist stimulation of apo B secretion in primary mouse hepatocytes (184) and in vivo (234). However, in these studies the induction of apo B levels in either the media or plasma was exclusively attributed to the increased secretion of apo B100, as changes in apo B48 levels were insignificant. In contrast, the PPARα agonist treatment of L35 cells resulted in secretion of both apo B48 and apo B100, yet the majority of secreted apolipoprotein was of the apo B48 variety (Figure 15). The reason for this differential effect could be ascribed to the differential context in the hepatoma cells versus the in vivo context of the other studies. It has been shown that, in comparison to apo B48, apo B100 is more susceptible to pre-secretory degradation due to insufficient lipidation. Making it possible that the lipid availability was decreased, in L35 cells, relative to the other studies, resulting in the preference for secretion of apo B48 over apo B100. Interestingly, treatment of rat hepatocytes with oleate for 3 days lead to a selective recruitment of apo B48 in the formation of VLDL, with no significant
change in secreted apo B100 levels (242). Given the findings of this study, it is possible that the difference in PPARα agonist effects on specific apo B recruitment and secretion could be due to factors that are species-specific.

The combined results from the agonist treatment of L35 cells supports the findings that the proximal promoter sequences of L-FABP (229) and MTP (207, 226) have been shown to be sufficient to confer hepatocyte-specific expression patterns. The data further suggest that events occurring at proximal DR1 elements within each of these genes may be quintessential determinants for hepatocyte plasticity in regards to appropriate accommodation of changing lipid flux to and from the liver (Figure 30).
**Figure 30.** **The proximal DR1 elements as "switches" for maintenance of hepatocyte plasticity.** PPARα-RXRα stimulation of L35 cells promotes a change in DR1 element occupation from the repressive COUP-TFII, to an activation-competent PPARα-RXRα complex. This causes coordinate induction of L-FABP and MTP expressions, and subsequent restoration of apo B-containing lipoprotein assembly and secretion pathway.
2.2.4 PPARα is necessary for L-FABP and MTP expression in hepatoma cells and in vivo.

The agonist studies in L35 cells confirmed the role of PPARα-RXRα as an essential component in the coordinate transcriptional activation of the L-FABP and MTP genes. The L35 cell line was an ideal model system due to their lack of both L-FABP and MTP expression. The necessity of PPARα was further validated in the FAO cell line, which normally express L-FABP and MTP at relatively high levels. RNAi-mediated knockdown of cellular content of PPARα by 75%, in FAO cells, caused coordinate reductions in both L-FABP and MTP expression levels by nearly 50% (Figure 19). This result supports the previous data indicating that the relative cellular levels of trans-acting factors is the key determinant dictating transcriptional activity levels of these two genes in the context of the hepatoma cell model system. Further, this provided an alternative context to validate the role of PPARα in the coordinate regulation of L-FABP and MTP. The necessity of PPARα was also demonstrated in vivo as PPARα agonist treatment of both wild type and PPARα -/- mice demonstrated that the presence of PPARα is necessary for the induction of both L-FABP and MTP in the liver (Figure 20). This in vivo data support the findings obtained using L35 and FAO hepatoma cells, combined these findings provide extensive confirmation of the role of PPARα in the coordinate regulation of L-FABP and MTP genes.
2.3 Transcriptional activation of the L-FABP and MTP genes by the coactivator PGC-1β

2.3.1 Direct correlation between expression of PGC-1β and both L-FABP and MTP in hepatoma cells and in vivo

Overexpression of PGC-1β led to increased hepatic expression of MTP in mice (212). The findings here support the role of PGC-1β in the regulation of MTP, and reveal L-FABP as a putative target for this coactivator. The relative levels PGC-1β in the L35 and FAO cell lines (Figure 21) were directly proportional to their abilities to express both MTP and L-FABP (Figure 2). L35 cells (lacking L-FABP and MTP) display a nearly undetectable level of PGC-1β, while FAO cells (expressing L-FABP and MTP) exhibit a relatively high cellular content of PGC-1β mRNA (Figure 21).

Fatty acids, which have been shown induce the expression of both L-FABP and MTP, also result in a marked induction of PGC-1β expression levels in primary hepatocytes and in vivo (212). Similarly, the PPARα agonist stimulation of both the L35 cells and in vivo, contexts leading to increased L-FABP and MTP expressions (Figures 14 and 20), were associated with coordinate induction of PGC-1β (Figure 22). Thus, cellular content of L-FABP and MTP directly correlated with the level of PGC-1β in hepatoma cells and in
vivo, suggesting a role for this coactivator in the transcriptional regulation of both genes.

2.3.2 PGC-1β acts through PPARα-RXRα complex to induce expression of both L-FABP and MTP.

PGC-1β preferentially associates with PPARα leading to specific induction of PPARα-target genes (212), making it likely that PGC-1β might be recruited by the DR1-associated PPARα-RXRα complex facilitating transcriptional activation of the MTP and L-FABP genes. Several lines of evidence support this hypothesis. RNAi-knockdown studies indicate that PGC-1β is required for PPARα agonist-mediated induction of L-FABP and MTP expressions (Figure 22), suggesting that PGC-1β is required for PPARα agonist induction of L-FABP and MTP.

Interestingly, while PPARα agonist treatment of PPARα -/- mice failed to induce L-FABP and MTP mRNA expression (Figure 20), hepatic expression of PGC-1β mRNA was increased 2-fold (data not shown). Given that PGC-1β induces hepatic expression of MTP in mice (212), this suggests that in the absence of PPARα, PGC-1β is not sufficient to increase the expression of L-FABP and MTP. This is supported by the finding that adenovirus-mediated expression of PGC-1β requires the presence of an active PPARα-RXRα at the DR1 element for this coactivator to enhance the transcription of the L-FABP (Figure 24) and MTP (Figure 25) genes. Our combined findings support the
proposal that PGC-1β participates in the transcriptional activation of MTP and subsequent induction of apoB-dependent VLDL secretion (212, 243).

In ob/ob, diabetic mice, adenovirus mediated expression of PGC-1β and Foxa2 induced MTP expression, suggesting a mechanism through which insulin blocks VLDL assembly/secretion (243). It has been demonstrated that Foxa2 is completely excluded from the nucleus of ob/ob mice (244), while both MTP expression and VLDL assembly/secretion are increased (245). Furthermore, a PGC-1β mediated increase in MTP expression was retained in the ob/ob mice (lacking nuclear Foxa2), indicating that induction of MTP transcription can occur via Foxa2 independent mechanisms (243). In fat-fed hyperlipidemic mice, PGC-1β activation of SREBP and LXRα is associated with enhanced MTP expression and VLDL assembly/secretion (212). PGC-1β has been demonstrated to interact with PPARα (219) and activate the transcription of PPARα target genes (218). Our findings show that in the context of rat hepatoma cells, PGC-1β activates PPARα-RXRα (Figure 8).

It has been reported that RNAi-mediated knockdown of PGC-1β in high-fat fed mice causes a decrease in plasma levels of triglycerides, while resulting in a concomitant increase in hepatic accumulation (212). Our data would suggest that the reduction in hepatic content of PGC-1β results in a decreased expression of downstream targets L-FABP and MTP resulting in reduced secretion of lipoprotein associated-triglyceride into the plasma (120). Our combined data show that PPARα-RXRα with the coactivator PGC-1β
activate the transcription of L-FABP and MTP, allowing the hepatocyte to
direct fatty acids into the VLDL assembly/secrection pathway and subsequent
delivery of fat to the peripheral tissues. Thus, the availability of fatty acids
serves both as agonists of PPARα-RXRα-PGC-1β facilitated transcriptional
activation and as substrates for biosynthesis of the glycerolipids essential for
VLDL particle assembly, allowing a coordinate multilevel regulation of hepatic
lipoprotein secretion (Figure 32)
Figure 31. **Detailed molecular mechanism for coordinate activation of L-FABP and MTP genes via fatty acids.** Increased flux of fatty acids to the liver causes increased cellular content and activity of a PPARα-RXRαPGC-1β complex, resulting in coordinate transcription of both L-FABP and MTP genes via the proximal DR1 elements, and subsequent stimulation VLDL secretion.
3. **Coordinate downregulation of L-FABP and MTP prevents hepatic steatosis.**

Plasma levels of apoB-containing lipoproteins, such as VLDL and LDL, directly correlate with the susceptibility to coronary artery atherosclerosis, which is the leading cause of death in industrialized nations. Several studies have demonstrated a direct correlation between MTP expression/activity and the ability to secrete apoB-containing lipoproteins (141, 246, 247). Mice with a liver-specific inactivation of MTP, via the Cre-lox system, demonstrated a >95% reduction in plasma apoB-100 levels (9). In addition, liver-specific MTP ablation prevented the development of atherosclerosis in "apo-B100-only" LDL receptor-deficient mice, a recently developed animal model of atherosclerosis (248). In the human condition abetalipoproteinemia, the lack of a functional MTP results in an inability to assemble and secrete apoB-containing lipoproteins (249). As a result, abetalipoproteinemics demonstrate a near-complete absence of apo-B containing lipoproteins in the plasma and are markedly resistant to development of cardiovascular disease (250).

In light of these studies several chemical inhibitors targeting MTP lipid transfer activity have been developed that are effective in lowering plasma lipid levels in both animal models (149, 150, 221) and humans (150). In fact, treatment with the MTP inhibitor Implitapioide caused a marked reduction in the progression of atherosclerosis in ApoE knockout mice fed a high-fat diet (148). However, the decrease in plasma lipid profiles brought upon by the use of these MTP inhibitors is concomitant with many adverse effects due to
hepatic fat accumulation (149, 150, 222). The finding that the lack of MTP expression and activity exhibited by L35 cells is not associated with accumulation of intracellular triglycerides (206), suggested that decreasing the expression and/or activity of L-FABP provides the necessary context for the prevention of the hepatic steatosis that is associated with inhibition of MTP activity. In support of this hypothesis, primary hepatocytes from L-FABP -/- mice demonstrate a marked reduction in both hepatic fatty acid uptake and their subsequent incorporation into triglyceride (7). Furthermore, this decrease in liver triglyceride accumulation occurs in the face of decreased VLDL assembly and secretion (7). As a result of the absence of MTP, L35 cells exhibited a complete block in VLDL assembly/secretion (206). Unlike other situations in loss of MTP expression or function leads to cellular triglyceride accumulation (223), the inability of L35 cells to express MTP was not associated with the accumulation of triglycerides (206). The discovery that L35 cells also exhibit a coordinate transcriptional inactivation of L-FABP as MTP (Figures 2 and 3) can explain why L35 cells lack MTP yet do not accumulate fat. This is made clear by the finding that L-FABP plays an essential role in the uptake and delivery of fatty acid to the liver (7). Thus, the coordinate inactivation of both MTP and L-FABP allowed L35 cells to maintain cellular lipid homeostasis in the absence of the VLDL assembly/secretion pathway.

The hypothesis drawn from the L35 cell phenotype was validated in vivo by treating L-FABP -/- mice with an MTP inhibitor. This revealed that while
wild type C57BL/6 mice treated with the MTP inhibitor 8aR accumulate 4-fold more hepatic triglyceride relative to levels in untreated animals, L-FABP -/- mice are resistant to the accumulation of fat within the liver (Figure 27). These studies demonstrate that ablation of L-FABP completely blocks the hepatic accumulation of triglycerides even in the face of MTP inactivation and the subsequent decreased VLDL-triglyceride secretion (Figure 27). Thus, the development of agents that block either the function and/or expression of both L-FABP and MTP would reduce hyperlipidemia without causing the development of fatty liver; providing an efficient therapy for various pathologic states associated with increased plasma lipid profiles.

4. Future Studies

4.1 Coordinate regulation of L-FABP and MTP: The intestine and liver as a unit for efficient utilization of dietary energy

The bulk of lipids found in the intestinal lumen are derived from dietary fat. These lipids consist mainly of triglycerides, with substantially lesser proportions of free cholesterol, cholesterol esters and phospholipids (251). Dietary fat is hydrolyzed by pancreatic-derived lipases, the liberated long-chain fatty acids are then taken up by the intestine (252). Intestinally, absorbed long-chain fatty acids undergo esterification into triglyceride and phospholipid, allowing for subsequent MTP-mediated assembly and secretion of triglyceride-rich lipoproteins called chylomicrons (253). These intestinally
derived apo B-containing lipoproteins provide direct peripheral transport of both dietary lipids and fat-soluble vitamins (253).

Several lines of evidence suggest that the regulatory mechanism that we have demonstrated for the coordinate transcriptional control of hepatic L-FABP and MTP by fatty acids, via proximal DR1 mediated PPARα-RXRα activation, also occurs in the intestine to allow for efficient utilization of dietary fat. Both L-FABP (214, 254) and MTP (159, 255) are co-spatially expressed in the proximal third of the small intestine, primarily in the jejunum. In mammals, this proximal region of the small intestine is the primary region where absorption of lipids and other nutrients occurs (256, 257).

Treatment of intestinal cell lines with fatty acids causes the stimulation of mRNA levels of both L-FABP (214) and MTP (162, 255). Similarly, high-fat feeding leads to acute increases in intestinal mRNA and protein levels of L-FABP (214) and MTP (162) in various animals in vivo. One study revealed that intestinal expressions of both L-FABP and MTP demonstrated acute coordinate induction (159). The small intestine also expresses I-FABP; however, its expression appears to be constitutive and is not modulated by dietary fat (214). Furthermore, I-FABP null mice exhibit no phenotype as they lack changes in the ability of the intestine to absorb and secrete dietary lipid as apo B-containing lipoproteins (258).

While studies have shown that the dietary fat driven increases in intestinal expression of both LFABP (259) and MTP (159) are associated with increased transcription of both genes, molecular mechanisms for this process
have not been determined. Intestinal expression of PPARs includes both PPARα and PPARδ (260). Both PPARα and PPARδ are activated by fatty acids and have been shown to bind as heterodimeric complexes with RXRα to the L-FABP DR1 element (261). Furthermore, treatment of mice with specific agonists for either subtype causes induction of L-FABP expression in the intestine (198). In the absence of PPARα, dietary fat stimulation of L-FABP expression in the intestine can occur through PPARδ (198). Thus, intestinal fatty acid regulation of L-FABP at the level of transcription can occur via the proximal DR1 element through the actions of either PPARα or PPARδ. While the molecular mechanism of fatty acid mediated induction of intestinal MTP has yet to be addressed, given the fact that both intestinal L-FABP and MTP are upregulated by dietary fat, it is likely that the proximal DR1 elements of these promoters confer coordinate regulation in the intestine similar to the findings of this report in the liver.

Carbohydrate-enriched diets cause increased flux of glucose into liver via portal circulation stimulating the liver to store excess glucose as glycogen (262). Once hepatic glycogen stores are restored the excess glucose is catabolized via the glycolytic pathway which results in stimulation of lipogenesis via substrate generation (263). Lipogenic gene expression has been shown to be induced in this context, by insulin via the transcription factor SREBP-1C (264) and by glucose metabolites via ChREBP (265). The carbohydrate driven de novo synthesis of lipid provides fatty acids for incorporation into glycerolipids (triglycerides and phospholipids) and ultimately
leads to increased secretion of VLDL from the liver for storage of this excess energy in adipose tissue (266). The coordinate regulation of hepatic lipogenic and VLDL secretory pathways has been well documented (128, 164).

The induction of VLDL secretion by dietary carbohydrate has been associated with increased MTP expression (128, 159, 164). Interestingly, a recent report has revealed that fatty acids synthesized from carbohydrate catabolism can act as ligands for the stimulation of PPARα activated genes in the liver (267). This suggests the hypothesis that the dietary carbohydrate induction of hepatic MTP is a result of DR1-dependent transcriptional activation by PPARα. Effects of dietary carbohydrate on L-FABP expression have not been explored; however, given that L-FABP is necessary for intracellular trafficking of fatty acids to sites of glycerolipid synthesis, it is likely to be coordinately upregulated with MTP in this context. Thus, the lipogenesis-driven increase in hepatic fatty acid levels would cause a coordinate PPARα-mediated increase in the expressions of both L-FABP and MTP. This would ensure that there is sufficient machinery for VLDL assembly in the face of increased substrate levels to allow for efficient utilization of dietary energy and as a measure protecting against hepatic steatosis as a result of increased lipogenesis.

As the effects of carbohydrate feeding, in regards to lipoprotein assembly and secretion, are liver-specific and given that the intestine is the primary tissue responding to dietary fat consumption, the two organs function synergistically in the name of efficient dietary energy utilization. The liver acts
as an energy transducer to flux dietary carbohydrate-derived carbon units into fatty acids and then into VLDL-triglycerides for adipose storage. The intestine processes dietary fat into chylomicrons for direct transport to the periphery for utilization and storage. The existence of this context-specific coordinate fatty acid regulation of L-FABP and MTP, resulting in substrate driven transcriptional modulation of lipoprotein synthesis, would allow both the liver and intestine to function as a unit efficiently maximizing dietary energy intake, storage and utilization.

4.2 Regulation of PGC-1β by nuclear receptors via L-FABP

The competence for transcriptional activation of many DNA-binding transcription factors is achieved through the docking of specific coactivator proteins, usually regulated in a ligand-dependent manner. While most studies concerning transcriptional control have focused on changes in cellular levels and activities of the immediate trans-acting factors, expressional regulation of coactivators remains relatively uncharted territory. The regulation of the PGC-1 family of coactivators has recently been explored in the most detail, relative to other coactivator proteins (212). Even though various mechanisms have been elucidated concerning the regulation of various PGC-1 family members (mainly PGC-1α) (212), very little is known about the regulation of the more recently discovered coactivator PGC-1β.

PGC-1β is expressed in brown fat, heart and liver (219) and hepatic expression is activated in response to fasting (218, 268). The induction by
fasting is likely due to influx of fatty acids from adipose stores. It has been reported that hepatic expression of PGC-1β is stimulated by intake of dietary fat in vivo and by fatty acids in primary hepatocytes (212). The molecular mechanism leading to the fatty acid stimulation of PGC-1β in the liver is currently unknown. It is within reason to suggest that either of the fatty acid responsive nuclear receptors PPARα or HNF4α might allow for transcriptional upregulation of PGC-1β. The data from this study detailing the PPARα activation of L-FABP and MTP, and other reports demonstrating HNF4α regulation of L-FABP and MTP (269), support the possibility that these factors might also regulate PGC-1β to ensure that it does not become rate-limiting. While PPARα null mice do not exhibit reduced hepatic levels of PGC-1β mRNA, relative to control wild type mice (data not shown), this assessment was not from fasted livers so this possibility cannot be ruled out. The expression of PGC-1β has not been determined in HNF4α null mice, yet both L-FABP and MTP are markedly reduced in these mice, which may be attributed to decreased availability of PGC-1β (269).

The L35 and FAO cell lines provide an ideal model system to study the regulation of PGC-1β as they demonstrate reciprocal expression levels (Figure 21). Further support of PPARα regulation of PGC-1β is the finding that in L35 and FAO cells the expressions of both factors are directly correlated (Figures 12 and 21 respectively). L35 cells exhibit low levels of both PPARα and PGC-1β, while FAO cells express high levels of both factors. Since both cell lines
expressed equivalent levels of HNF4α protein (207), thus it is unlikely that this factor contributes to the cell-type specific expressions of PGC-1β in these hepatoma cell lines. However, it cannot be ruled out that HNF4α activity could demonstrate cell-type specific differences via post-translational mechanisms.

Several lines of evidence suggest that L-FABP may play a role in the regulation of PGC-1β via nuclear transport of fatty acids. Various findings have suggested that L-FABP can regulate PPARα activity by transport of fatty acid into the nucleus and transfer of these ligands directly to PPARα. An initial report revealed that L-FABP localizes within the nuclear membrane in a ligand-dependent manner (270). Subsequent studies localized L-FABP within the nucleoplasm (271) and demonstrated its ability to shuttle long chain fatty acids from the cytoplasm to nucleus (271, 272). Additional studies have shown colocalization (271) and direct interaction (273) between L-FABP and PPARα within the nucleus. Finally, the degree of ligand transport into the nucleus demonstrated L-FABP dose dependence (271); accordingly, the cellular concentration of L-FABP directly correlated with the ability of PPARα to activate its target genes (273).

L-FABP (Figure 2) and PGC-1β (Figure 21) expression exhibited a direct correlation in both L35 (L-FABP and PGC-1β are markedly reduced) and FAO cells (L-FABP and PGC-1β are expressed at high levels), and fatty acids stimulate the expression of PGC-1β. This raises the hypothesis that L-FABP may be necessary for the fatty acid induction of PGC-1β by transporting fatty
acids into the nucleus as ligands for nuclear receptor mediated activation of the PGC-1 gene. Thus, the lack of L-FABP expression in L35 cells causes the reduced cellular content of PGC-1β, and the relatively high levels of PGC-1β in FAO cells may be allowed by the increased cellular concentration of L-FABP. The combined findings from this report and others predicts that L-FABP may play a role in the regulation of PGC-1β via transport of fatty acids into the nucleus for activation by a fatty acid activated nuclear receptor such as PPARα. This could result in a complex multilevel regulation of L-FABP and MTP, and further fine-tune the modulation of the VLDL assembly and secretion pathway.
V. REFERENCES


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