Coordinate Transcriptional Repression of Liver Fatty Acid-binding Protein and Microsomal Triglyceride Transfer Protein Blocks Hepatic Very Low Density Lipoprotein Secretion without Hepatosteatosis

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Unlike the livers of humans and mice, and most hepatoma cells, which accumulate triglycerides when treated with microsomal triglyceride transfer protein (MTP) inhibitors, L35 rat hepatoma cells do not express MTP and cannot secrete very low density lipoprotein (VLDL), yet they do not accumulate triglyceride. In these studies we show that transcriptional co-repression of the two lipid transfer proteins, liver fatty acid-binding protein (L-FABP) and MTP, which cooperatively shunt fatty acids into de novo synthesized glycerolipids and the transfer of lipids into VLDL, respectively, act together to maintain hepatic lipid homeostasis. FAO rat hepatoma cells express L-FABP and MTP and demonstrate the ability to assemble and secrete VLDL. In contrast, L35 cells, derived as a single cell clone from FAO cells, do not express L-FABP or MTP nor do they assemble and secrete VLDL. We used these hepatoma cells to elucidate how a conserved DR1 promoter element present in the promoters of L-FABP and MTP affects transcription, expression, and VLDL production. In FAO cells, the DR1 elements of both L-FABP and MTP promoters are occupied by peroxisome proliferator-activated receptor α-retinoid X receptor α (RXRα), with which PGC-1β activates transcription. In contrast, in L35 cells the DR1 elements of both L-FABP and MTP promoters are occupied by chicken ovalbumin upstream promoter transcription factor II, and transcription is diminished. The combined findings indicate that peroxisome proliferator-activated receptor α-RXRα and PGC-1β coordinate up-regulate L-FABP and MTP expression, by competing with chicken ovalbumin upstream promoter transcription factor II for the DR1 sites in the proximal promoters of each gene. Additional studies show that ablation of L-FABP prevents hepatic steatosis caused by treating mice with an MTP inhibitor. Our findings show that reducing both L-FABP and MTP is an effective means to reduce VLDL secretion without causing hepatic steatosis.

Hepatic production of apolipoprotein B-containing lipoproteins is the major pathway by which essential lipids and fat-soluble nutrients are transported to peripheral tissues for anabolic and energy requirements (reviewed in Refs. 1–3). Three distinct gene products, apoB2 (4–6), MTP (7, 8), and L-FABP (9), share “lipid binding” structural domains (10–14) and act in concert to package fatty acids and apolipoprotein B (apoB) into triglyceride-rich VLDL.

apoB is a uniquely large (>500 kDa) amphipathic protein essential for the assembly and secretion of triglyceride-rich VLDL (3, 15–17). The inability to produce apoB of sufficient size (~35 kDa) is associated with an inability of both the liver and intestine to assemble and secrete VLDL (18). Normally, hepatic expression of apoB is constitutive; changes in hepatic secretion of apoB-containing lipoproteins are the result of variation in the amount of de novo synthesized apoB that is either secreted or degraded within hepatocytes (1, 2).

MTP acts as both a lipid transfer protein (19) and as a facilitator of apoB folding and translocation (3, 17). MTP facilitates the transfer of four major lipid classes (free cholesterol, phospholipids, triglycerides, and cholesterol esters) to the nascent apoB-containing lipoprotein particle (20) via a two-step process (21, 22). Abrogation of one or more of these concerted MTP-dependent processes leads to co-translational degradation of nascent apoB by the proteasome (23–27).

L-FABP, a highly abundant lipid-binding protein in the cytosol of liver parenchymal cells, facilitates fatty acid transport and utilization (28, 29). Genetic disruption of L-FABP expres-

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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sion impairs the ability of the liver to efficiently import and transfer fatty acids into glycerolipid biosynthesis (9).

Hepatic VLDL assembly and secretion are highly variable among individuals and sensitive to changes in nutritional state (17). It is induced by carbohydrate feeding (30) and repressed by fasting (31). These nutritional changes in VLDL secretion are linked to sterol regulatory element-binding protein-mediated changes in the expression levels of key lipogenic enzymes (32, 33). 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 synthesis and VLDL assembly/secretion (34). Variations in hepatic expression levels of both L-FABP (9) and MTP (35) control the flux of fatty acids into glycerolipid biosynthesis and VLDL assembly/secretion.

In this study we use two distinct lines of hepatoma cells, each displaying distinct abilities to assemble and secrete VLDL (36, 37) in order to uncover how the transcriptions of L-FABP and MTP are coordinately regulated. FAO cells, a rat hepatoma cell line used to study hepatic lipoprotein synthesis and secretion (38, 39), express both L-FABP and MTP and exhibit the ability to assemble and secrete apoB-containing lipoproteins. In contrast, L35 cells derived as a single cell clone from FAO cells express neither L-FABP nor MTP and lack the ability to assemble and secrete apoB-containing lipoproteins. Our combined data support the proposal that L-FABP and MTP were derived from a common ancestral lipid-binding protein (40). Retention of the DR1 site in the promoter allowed the distinct lipid transfer functions of L-FABP and MTP to evolve while retaining a common mechanism to ensure that their expression varied concomitantly, and their concerted function is readily adaptable to fatty acid substrate supply.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were cultured and transfected as described (37). FAO cells were obtained as a gift from Franz Simon (University of Colorado). L35 cells were obtained as described (31).

Cells were transfected using Lipofectamine reagent (Invitrogen) according to manufacturer’s protocol, with minor modifications (37). One day prior to transfection, L35 and FAO cells (2 × 10^5) were seeded on 12-well plates. On the day of transfection, cells were transfected with 0.8 µg of promoter/luciferase reporter construct and with 6 ng of pRL-CMV plasmid as an internal control for normalization of L-FABP and MTP promoter activities. The normalized pRL-CMV activities are reported relative to activity of the empty vector from parallel transfections. Varying doses of COUP-TFII expression vector was added as indicated in figure legends. The total DNA concentration for each assay was maintained constant by the addition of empty expression vector pCR 3.1 (Invitrogen). Upon transfection, cells were incubated for 48 h and harvested using passive lysis buffer (Promega). Luciferase activities were measured using the dual-luciferase reporter assay system (Promega).

The PPARα and RXR agonists, WY-14,643 (WY) and 9-cis-retinoic acid (RA), respectively (A.G. Scientific, Inc.), were dissolved in dimethyl sulfoxide (Me_2SO, 0.15% v/v) and used at working concentrations of 10 µM (WY) and 1 µM (9-cis-RA). Briefly, upon transfection cells were treated for 48 h with agonists or Me_2SO alone as indicated. Cells were harvested and promoter activity assayed as described above.

Reporter Gene Constructs and Expression Vectors—The wild type and mutant rat MTP reporter vectors (−135/+66) were as described previously (37). To generate the wild type rat L-FABP reporter vector (−141/+66), genomic DNA was isolated and purified from FAO cells using the DNeasy tissue kit (Qiagen). The promoter fragment was generated by PCR using the primers with indicated restriction enzyme sites as follows: forward, 5′(KpnI)-GAACAAATCTGTCCGCCGATCATTCTGTATTTTA-3′; and reverse, 5′(BglII)-TTCATGGATCTGCATATGCCCTATTT-3′. The promoter fragment was then cloned into KpnI and BglII sites of the empty luciferase reporter vector PGL3Basic (Promega).

To generate the mutant L-FABP reporter vector, a specific mutation in the proximal DR1 sequence was generated using the QuickChange site-directed mutagenesis kit (Stratagene). For the in vitro mutagenesis, the wild type rat L-FABP (−141/+66)-luciferase reporter vector was used as the template along with two oligonucleotide primers (mutated bases underlined), each complementary to opposite strands of the vector as follows: forward, 5′-AAC GCA TCA TCG TG CAT GGC CTA TAT TT-3′; reverse, 5′-AAA TAT AGC CCA TAG CAC AGT GAT GTG 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 College of Medicine).

Preparation of Nuclear Extracts—Nuclear extracts from L35 and FAO cells were prepared as described previously (37). Briefly, cells were trypsinized and harvested by centrifugation, washed with 1× phosphate-buffered saline, and resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). After a 10-min incubation on ice, cells were lysed with the use of a Dounce homogenizer. The nuclei were pelleted by centrifugation and resuspended in low salt buffer (20 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 1.5 mM MgCl₂, 0.02 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonil fluoride, 0.5 mM dithiothreitol). Subsequently, the high salt buffer (20 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 1.5 mM MgCl₂, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonil fluoride, 0.5 mM dithiothreitol) was added dropwise with stirring. The resulting suspension was rocked gently for 30 min to allow extraction of nuclear proteins. The nuclei were centrifuged again for 30 min, and the resulting supernatant was dialyzed for 1 h against dialysis buffer (20 mM HEPES, pH 7.9, at 4 °C, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonil fluoride, 0.5 mM dithiothreitol).

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.
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FIGURE 1. MTP and L-FABP demonstrate similar cell type-specific differences in both mRNA and promoter activity levels. A, the conserved DR1 elements (5'- and 3'-hexameric half-sites are underlined) within the proximal regions of both MTP and L-FABP promoters (rat) are provided. B, real-time PCR analysis of MTP and L-FABP mRNA levels in L35 and FAO cells. All values are normalized to levels of 36B4 RNA and shown as mean ± S.D. (n = 3). C, 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 detailed under “Experimental Procedures.” Luciferase activities are represented by filled bars (FAO cells) and empty bars (L35 cells). All luciferase values were normalized to a Renilla control and represent the mean ± S.D. (n = 3).

with [γ-32P]ATP (3000 mCi/mmol) (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs), followed by purification on a G-50 column. For binding reactions, 15 μg of nuclear extracts were incubated with 3 × 10^4 cpm probe on ice for 20 min in a total volume of 15 μl of solution (20 mM HEPES, pH 7.9, 4 °C, 10% glycerol, 100 mM KCl, 1 mM EDTA, and 2 μg of poly(dI-dC)). For supershift experiments, 1 μl of specific antibodies were added to preincubated DNA-protein complexes for an additional 20 min on ice. Antibodies against COUP-TFII (sc-6576X), RXRα (sc-553X), and PPARα (sc-9000X) were obtained from Santa Cruz Biotechnology. DNA-protein complexes were resolved on 6% native PAGE containing 0.5× TBE buffer.

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.) on-column DNA removal per the manufacturer's instructions. The RNA concentrations were determined by spectrophotometer at 260 nm. First strand cDNA was synthesized from 0.5 μg of total RNA using the Bio-Rad iScript for reverse transcription (Bio-Rad). Specific primers for each gene (supplemental Table 1) 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 iCycler using SyBr Green Supermix according to manufacturer's instructions (Bio-Rad). The reactions were analyzed in triplicate with specific product monitored using melt-curve analysis. The expression data were normalized to an endogenous control, either 18 S ribosomal RNA or acidic ribosomal phosphoprotein P0 (36B4). The level of both 18 S RNA and 36B4 was invariant among samples of all experiments. The relative expression levels were calculated according to the formula 2^−ΔΔCt, where ΔΔCt is the difference in threshold cycle (Ct) values between the target and either the 18 S or 36B4 endogenous control.

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 (10 μM) and 9-cis-RA (1 μM) were added to cell culture medium for 48 h prior to harvesting. The cells were then fixed by the addition of 280 μl of 37% formaldehyde (Sigma) to 10 ml of culture medium for 10 min 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 the manufacturer's protocol. 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 SyBr Green Supermix (Bio-Rad) as indicated above. As a control for region selectivity of immunoprecipitation-specific enrichment differences, amounts of noncoding 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'-ATG TGA GCC CTT CCA TGA AC-3'; reverse, 5'-CAT AAT CTG CGA CAA CAG TG-3'), L-FABP-DR1 (forward, 5'-GAG TTA ATG TTT GAT CCT GCC C-3'; reverse, 5'-CCA CCC ACT GTT GGC TAT TTT-3'); and L-FABP 3'-untranslated region (forward, 5'-GTC TCC CGC TAC CTA AGA GG-3'; reverse, 5'-CTG TCA TCT GAC CTC TC-3'). All values were normalized to values from both input DNA and immunoprecipitations 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

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Immunoprecipitation of Secreted ApoB—Secreted apoB was immunoprecipitated as described previously (23, 36). Briefly, L35 cells were cultured in 60-mm dishes in the absence or presence of WY-14,643 (10 μM) and 9-cis-RA (1 μM) for 72 h. Cells were then switched to methionine-free Dulbecco’s modified Eagle’s medium for 2 h and then labeled with 3 ml of [35S]methionine (100 μCi/ml) in Dulbecco’s modified Eagle’s medium for 24 h. 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 h at 4 °C. The immunoprecipitate complex was washed three times with TETN buffer (25 mM Tris, pH 7.5, 5 mM EDTA, 250 mM NaCl, and 1% Triton X-100) and once with phosphate-buffered saline. 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

**FIGURE 2. COUP-TFII binds to the proximal DR1 site acting as a repressor of L-FABP promoter activity.** A, EMSA analysis with a radiolabeled L-FABP-DR1 probe. Antibodies (Ab) specific for COUP-TFII were added to the nuclear extract (either L35 or FAO) during incubation with the L-FABP-DR1 probe as indicated. The L35-specific COUP-TFII complexes without and with antibody addition are indicated by a filled arrow and empty arrow, respectively. B, luciferase reporter plasmid containing sequences −141/−66 of the rat L-FABP promoter was co-transfected with the indicated amounts of COUP-TFII expression plasmid into FAO cells. The construct containing the mutant DR1 element is as described in Fig. 1. Wild type and mutant constructs are indicated by filled and empty diamonds, respectively. All luciferase values were normalized to a Renilla control and represent the mean ± S.D. (n = 3).
autoradiography. The locations of apoB48 and apoB100 was determined by molecular weight markers and human low density lipoprotein standards.

RESULTS

Transcriptional Activities of L-FABP and MTP Promoter-Reporter Constructs Reflect Cell Type-specific Differences in mRNA Expression—A DR1 element located within the proximal MTP promoter region was shown to be responsible for the lack of expression in L35 cells and the high level expression exhibited by FAO cells (37). Occupation of this DR1 element by COUP-TFII caused transcription repression (37). Because the L-FABP promoter contains a similar DR1 element (Fig. 1A), we examined if its expression would vary in parallel with expression of MTP. Indeed, the cell type-specific differences in L-FABP mRNA displayed by L35 and FAO cells were similar to those of MTP mRNA (Fig. 1B), 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 higher (8-fold) in FAO cells compared with L35 cells (Fig. 1C). Mutation of the DR1 sites decreased L-FABP and MTP promoter activities in FAO cells to levels similar to those of L35 cells (Fig. 1B). These findings show that the proximal DR1 elements of both L-FABP and MTP genes are sufficient to confer the cell type-specific differences in mRNA expression displayed by the L35 and FAO cells.

Binding of COUP-TFII to the Proximal DR1 Site Mediates Transcriptional Repression of the L-FABP Gene—Nuclear extracts from L35 and FAO hepatoma cells formed distinct DNA-protein complexes with the oligonucleotide probe containing the DR1 element of the L-FABP promoter (Fig. 2A). The DNA-protein complex formed using nuclear extracts from L35 cells exhibited a supershift with an antibody specific for COUP-TFII (Fig. 2A). In contrast, the DNA-protein complex formed using nuclear extracts from FAO cells did not display a supershift with the COUP-TFII antibody (Fig. 2A). Because mutation of the DR1 site blocked the formation of the cell type-specific DNA-protein complexes (37), they require an intact DR1 site.

Ectopic expression of COUP-TFII in FAO cells resulted in a dose-dependent decrease in L-FABP promoter activity (Fig. 2B). Because increased COUP-TFII expression did not alter the activity of the mutated L-FABP promoter (Fig. 2B), the repression of the L-FABP promoter by COUP-TFII is dependent on a functional DR1 element. The maximal reduction of L-FABP promoter activity exhibited by FAO cells transfected with the
COUP-TFII plasmid was only ~50–70% of 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 (37), support the conclusion that in L35 cells occupancy of the DR1 element by COUP-TFII is responsible for transcriptional inactivation of both genes (Fig. 2A).

PPARα-RXRα Heterodimers Compete with COUP-TFII for Binding to the DR1 Promoter Elements of Both the L-FABP and MTP Genes—EMSA supershift analysis of the MTP-DR1 site revealed an FAO cell-specific complex containing RXRα (37). Because PPARα and RXRα agonists have been shown to activate the transcription of L-FABP via the DR1 site in the proximal promoter region (43), we assessed whether the MTP gene occupied by COUP-TFII is responsible for transcriptional inactivation of both genes (Fig. 2A).

ChIP analyses of both cell types showed that chromatin from L35 cells immunoprecipitated by a COUP-TFII-specific antiserum was enriched with the DR1 elements of both L-FABP and MTP genes by 4-fold compared with chromatin from FAO cells (Fig. 3B). In contrast, using a PPARα-specific antiserum, DR1 element-specific chromatin immunoprecipitated from FAO cells was enriched 4-fold compared with chromatin from L35 cells (Fig. 3B). Because immunoprecipitated chromatin obtained using both antisera contained similar enrichment of DNA sequences corresponding to regions distal to the DR1 regions from both cell lines (Fig. 3B), the ~4-fold enrichment of DR1 elements from both the L-FABP and MTP genes reflects cell type-specific differences in binding of PPARα-RXRα (FAO

PPARα-RXRα agonist treatment of L35 cells allows for the coordinate induction of L-FABP and MTP mRNAs, DR1 site-dependent increased promoter activity levels, and a restored ability for apoB secretion. A, real time PCR analysis of relative L-FABP and MTP mRNA levels comparing untreated L35 cells (−) to those treated for 48 h with either the PPARα agonist WY-14,643 (WY), the RXRa 9-cis-retinoic acid (RA) agonist, or the vehicle (DMSO) as indicated. WY/RA indicates L35 cells treated with both agonists simultaneously. All values normalized to levels of 36B4 RNA and shown as mean ± S.D. (n = 3). B, upon transfection of either wild type or mutant DR1 luciferase reporter constructs, relative activity levels of L-FABP and MTP promoters were determined by comparing untreated L35 cells (−) to those treated for 48 h as described in Fig. 4A. Promoter activities are indicated as empty bars (wild type DR1) and filled bars (mutant DR1). All luciferase values were normalized to a Renilla control and represent the mean ± S.D. (n = 3). C, apoB secretion was measured using media from L35 cells cultured in the absence (lanes 1–3) or presence (lanes 4–6) of 1 μM 9-cis-retinoic acid (RA) and 10 μM WY-14,643 for 72 h then labeled with [35S]methionine. Secreted apoB was immunoprecipitated with a polyclonal anti-apoB antibody, resolved by SDS-PAGE (4–12%), and detected by autoradiography.
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Concordant Changes in Cellular Expression of PPARα-RXRα and COUP-TFII Orchestrate Agonist-mediated Changes in Transcription by Determining Which Complex Occupies the DR1 Element—PPARα-RXRα agonists altered the levels of nuclear receptors in L35 cells so that they resembled the levels in FAO cells (Fig. 5A). Upon treatment of L35 cells with PPARα-RXRα agonists mRNA expression of both PPARα (5-fold) and RXRα (2.3-fold) increased, whereas COUP-TFII expression decreased (~60%) (Fig. 5A). Me2SO alone also reduced COUP-TFII mRNA expression by L35 cells, without affecting expression of either PPARα or RXRα (Fig. 5A). The Me2SO-mediated reduction in COUP-TFII may explain why its addition to L35 cells increased transcription and expression of L-FABP and MTP (Fig. 4). The agonist-mediated changes in nuclear receptor protein levels parallel those seen for the mRNAs (data not shown).

The effect of agonist-mediated changes in the relative expression of the DR1-associated factors, on the occupancy of the proximal DR1 region of both L-FABP and MTP promoters from the repressive COUP-TFII complex to an activating PPARα-RXRα complex. A, real time PCR analysis of relative mRNA levels of COUP-TFII, RXRα, and PPARα comparing untreated L35 cells (empty bars) to those treated for 48 h with WY-14,643 and 9-cis-retinoic acid simultaneously (black bars) or Me2SO (gray bars) as indicated. All values normalized to levels of 36B4 RNA and shown as mean ± S.D. (n = 3). B, ChIP assays, comparing untreated L35 and FAO cells to L35 cells treated with both WY-14,643 and 9-cis-retinoic acid (L35/R), were performed utilizing antibodies specific for COUP-TFII and PPARα as indicated. Relative amounts of region-specific DNA were determined by real time PCR using primers specific for either L-FABP-DR1 or MTP-DR1 promoter regions (filled bars) as indicated. Relative level of the control distal untranslated regions (empty bars) demonstrates region specificity and immunoprecipitation background. Values were normalized to input DNA and immunoprecipitation with preimmune serum using the ΔΔCt method. All values were given as mean ± S.D. (n = 3).

The inability of L35 cells to assemble and secrete apoB-containing lipoproteins is strictly caused by a lack of MTP; transfection with a constitutively expression MTP transgene allows L35 cells to secrete apoB-containing lipoproteins (36, 37). We now show that treating L35 cells with PPARα-RXRα agonists markedly enhanced the secretion of de novo synthesized ^35S-labeled apoB (Fig. 4C). Thus, PPARα-RXRα agonists restored expression of L-FABP and MTP allowed L35 cells to assemble and secrete apoB-containing lipoproteins.

FIGURE 5. PPARα/RXRα agonist treatment of L35 cells reduces the nuclear receptor ratio of COUP-TFII/PPARα/RXRα resulting in altered occupancy of the proximal DR1 region of both L-FABP and MTP promoters from the repressive COUP-TFII complex to an activating PPARα/RXRα complex. A, real time PCR analysis of relative mRNA levels of COUP-TFII, RXRα, and PPARα comparing untreated L35 cells (empty bars) to those treated for 48 h with WY-14,643 and 9-cis-retinoic acid simultaneously (black bars) or Me2SO (gray bars) as indicated. All values normalized to levels of 36B4 RNA and shown as mean ± S.D. (n = 3). B, ChIP assays, comparing untreated L35 and FAO cells to L35 cells treated with both WY-14,643 and 9-cis-retinoic acid (L35/R), were performed utilizing antibodies specific for COUP-TFII and PPARα as indicated. Relative amounts of region-specific DNA were determined by real time PCR using primers specific for either L-FABP-DR1 or MTP-DR1 promoter regions (filled bars) as indicated. Relative level of the control distal untranslated regions (empty bars) demonstrates region specificity and immunoprecipitation background. Values were normalized to input DNA and immunoprecipitation with preimmune serum using the ΔΔCt method. All values were given as mean ± S.D. (n = 3).

cells) or COUP-TFII (L35 cells). The concordant findings obtained from both EMSA supershift analyses (Figs. 2A and 3A) and ChIP analyses (Fig. 3B) suggest that the cellular content of activator (PPARα and RXRα) relative to repressor (COUP-TFII) in each cell type would determine which complex occupies the DR1 site and controls transcriptional activity. This interpretation is supported by the findings showing that both cell lines contained relative levels of COUP-TFII mRNA that were inversely related to the amounts of both PPARα and RXRα (Fig. 3C). (The relative differences in protein levels for each of the nuclear receptors correlates with those observed for their respective mRNAs (see Ref. 37 and data not shown).

PPARα and RXRα Agonist Treatment of L35 Cells Results in the Transcriptional Induction of L-FABP and MTP Expression and a Restored Ability to Secret ApoB—Treatment of L35 cells with either a PPARα or RXRα agonist increased the expression of both L-FABP (~60–75-fold) and MTP (~55–65-fold) mRNAs (Fig. 4A). Treatment with both agonists synergistically increased L-FABP and MTP mRNA expression by ~300-fold (Fig. 4A). L-FABP and MTP promoter luciferase reporters exhibited similar responses to the PPARα and RXRα agonists (Fig. 4B). Mutation of the DR1 sites blocked the activation of both the L-FABP and MTP by PPARα and RXRα agonists (Fig. 4B). Thus, PPARα and RXRα agonists coordinately activate L-FABP and MTP gene transcription via their respective DR1 elements.

It should be noted that the vehicle Me2SO alone caused a modest but significant increase in both the expression of L-FABP and MTP mRNA and their respective promoter activities (Fig. 4, A and B, respectively). Clearly, PPARα and RXRα agonists added to cells using Me2SO as a vehicle exhibited a much greater induction of L-FABP and MTP compared with Me2SO alone (Fig. 4, A and B). The induction of PPARα-activated genes by Me2SO has been described (44).
sequences immunoprecipitated with a PPARα-specific antibody (Fig. 5B). Thus, the ChIP analyses demonstrated that changes in the relative expression of the DR1-associated factors (Fig. 5A) resulted in concordant changes in the occupancy of each DR1 site (Fig. 5B).

**PPARα Is Necessary for High Expression Levels of L-FABP and MTP in Hepatoma Cells and in Vivo**—The combined data suggest that cellular levels of transcriptional activators (PPARαRXRα) relative to the transcriptional repressor (COUP-TFII) dictate which complex occupies the DR1 site and controls the coordinate transcriptional activity of L-FABP and MTP genes. PPARαRXRα agonist treatment induces L-FABP and MTP transcription by both increasing the cellular content of PPARαRXRα/COUP-TFII and by ligand binding. To examine if changes in cellular expression of PPARα-RXRα/COUP-TFII might change transcription independent of PPARα-RXRα agonists, we utilized RNA interference to knock down the expression levels of PPARα in FAO cells. FAO cells transfected with PPARα-specific siRNAs demonstrated a 75% reduction in PPARα mRNA compared with the cells transfected with control siRNA (Fig. 6A). 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 (Fig. 6A). The PPARα-specific RNA interference did not alter the mRNA levels of PPARα-independent apob, suggesting that the decreases in L-FABP and MTP were PPARα-reduction specific (Fig. 6A).

We also examined whether a PPARα agonist would coordinately induce the hepatic expressions of L-FABP and MTP mRNAs in vivo and, if so, whether the agonist-mediated increases were PPARα-dependent. Control SV/129 and PPARα−/− mice were treated with the PPARα agonist GW-7647 (45). Although control mice displayed increased levels of both L-FABP and MTP mRNAs following treatment with the PPARα agonist (Fig. 6B), mice lacking functional PPARα displayed no significant changes in either L-FABP or MTP expression levels (Fig. 6B). These findings obtained in vivo corroborate those obtained using FAO and L35 hepatoma cells indicating that ligand activation of PPARα coordinately induces both L-FABP and MTP genes.

**PGC-1β Acts in Concert with PPARα to Coordinately Induce the L-FABP and MTP Genes**—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 (Fig. 7A), are consistent with the proposal that PGC-1β activates MTP gene expression (41). This proposal is further supported by our findings showing both L35 cells and mice treated with PPARα agonists caused an ~3-fold induction of PGC-1β mRNA (Fig. 7, B and C, respectively). To examine if PGC-1β is essential for PPARα-mediated induction of L-FABP and MTP, FAO cells were treated with PPARα-RXRα agonists, and the effect of siRNA knockdown of PGC-1β was determined. PPARα-RXRα agonists-treated FAO cells given the siRNA specific for PGC-1β demonstrated no change in any of these mRNA levels (Fig. 7A). The PPARα-RXRα agonists-treated FAO cells given a negative control siRNA exhibited no change in any of these mRNA levels (Fig. 7A). Thus, the siRNA demonstrated target specificity and the associated reductions in L-FABP and MTP mRNA expressions were because of the reduction in PGC-1β content.

We further examined the role of PGC-1β in L-FABP and MTP gene transcription by enhancing the expression of PGC-1β in untreated and PPARα-RXRα agonist-treated L35 cells via transduction with an adenovirus expressing PGC-1β (41, 46). Whereas PGC-1β adenovirus infection did not alter L-FABP or MTP mRNA levels in untreated L35 cells (Fig. 8, A), L35 cells treated with the PPARα-RXRα agonists demonstrated...
PGC-1\(\beta\) adeno-virus-mediated increases (3-fold) in both L-FABP and MTP mRNAs relative to agonist-treated uninfected and GFP-infected controls (Fig. 8). These data show that PGC-1\(\beta\) plays an essential role in the PPAR\(\alpha\)-mediated induction of L-FABP and MTP gene transcription.

Coordinate Inactivation of L-FABP and MTP Prevents Hepatic Steatosis in Vivo—Chemical inhibition of MTP, a strategy developed to ameliorate hyperlipidemia (47–50), is of limited use because of the development of hepatic steatosis (35, 51). Although L35 cells do not express MTP or secrete apoB-100, fatty acid ligand-activated transcription factors (PPAR\(\alpha\)-RXR\(\alpha\)) or COUP-TFII. Thus, expression of two lipid transfer proteins (L-FABP and MTP), which function in concert with each other, can be coordinately regulated in response to the availability of fatty acid substrate. 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. glycerolipids and cholesterol esters), \(\beta\)-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. Changes in fatty acid flux through these pathways must occur rapidly and selectively. Substrate-driven “feed-forward” transcriptional regulation is a common mechanism allowing changes in gene expression to occur concomitantly with variations in metabolic needs (52). Because fatty acids also can activate PPAR\(\alpha\)-dependent gene transcription of L-FABP (53) and MTP (54), fatty acid flux to the liver both induces the enzymes controlling VLDL assembly/secretion as well as providing lipogenic substrate.

Several lines of evidence indicate that similar DR1 elements present in the L-FABP and MTP promoters provide coordinate transcriptional regulation necessary for the interdependent role of these two lipid transfer proteins in delivering fatty acids to the VLDL assembly/secretion pathway. Mutational deletion of the DR1 element, in both the L-FABP and MTP promoter reporter constructs, caused the following: 1) a reduction of the relatively high activity levels exhibited by FAO cells to the lower levels exhibited by L35 cells (Fig. 1C), and 2) abrogation of both the ability of COUP-TFII (Fig. 2B) and PPAR\(\alpha\)-RXR\(\alpha\) agonist (Fig. 4A) to repress or activate transcription, respectively. Further analysis of PGC-1\(\alpha\) even when challenged with fatty acids (51). Although L35 cells do not express MTP or secrete apoB-100, fatty acid ligand-activated transcription factors (PPAR\(\alpha\)-RXR\(\alpha\)) or COUP-TFII. Thus, expression of two lipid transfer proteins (L-FABP and MTP), which function in concert with each other, can be coordinately regulated in response to the availability of fatty acid substrate. 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. glycerolipids and cholesterol esters), \(\beta\)-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. Changes in fatty acid flux through these pathways must occur rapidly and selectively. Substrate-driven “feed-forward” transcriptional regulation is a common mechanism allowing changes in gene expression to occur concomitantly with variations in metabolic needs (52). Because fatty acids also can activate PPAR\(\alpha\)-dependent gene transcription of L-FABP (53) and MTP (54), fatty acid flux to the liver both induces the enzymes controlling VLDL assembly/secretion as well as providing lipogenic substrate.

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further evidence showing that these DR1 elements are the functionally relevant cognate binding sites responsible for competitive occupation by COUP-TFII (repressor) or PPARα-RXRα (activator) are provided by EMSA supershift (Fig. 2, L-FABP), MTP (37), and ChIP (Fig. 3) analyses. The combined analyses obtained from the complementary EMSA supershift and ChIP experiments of the DR1 elements indicate that occupation by PPARα-RXRα is associated with transcriptional activation of both genes (FAO cells), whereas occupation by COUP-TFII is associated with repression (L35 cells).

The principal determinant responsible for the phenotypic difference in the expression of L-FABP and MTP exhibited by FAO and L35 cells is the relative cellular content of COUP-TFII (repressor) to PPARα-RXRα (activator) (Fig. 3C). Three independent experiments demonstrate that the plasticity in the cellular phenotype of FAO and L35 cells is dependent upon the cellular content of COUP-TFII (repressor) relative to PPARα-RXRα (activator) as follows. 1) Treating L35 cells with PPARα-RXRα agonists increases the expression of PPARα-RXRα (activator), while decreasing COUP-TFII (repressor) expression (Fig. 5A). 2) These changes in cellular content of PPARα-RXRα/COUP-TFII are reflected by similar changes in the occupancy of the DR1 elements present in both the L-FABP and MTP promoters (Fig. 5B). 3) These changes resulted in DR1 site-dependent increases in the transcriptional activities of both L-FABP and MTP promoter-luciferase reporter constructs (Fig. 4B). 4) They enhanced expression of L-FABP and MTP mRNAs (Fig. 4A). Furthermore, the agonist-mediated changes in L35 cells were associated with a restored ability to assemble and secrete apoB-containing lipoproteins (Fig. 4C).

PPARα and RXRα agonists have been shown to activate the transcription of L-FABP in hepatoma cells, an effect dependent on the DR1 site in the proximal promoter region (43). Treating wild type but not PPARα knock-out mice with a PPARα agonist increased hepatic expression of MTP (54). These and additional findings obtained from studies examining transcriptional regulation of genes involved in fatty acid metabolism (55–58) support the proposal that PPARα-RXRα and COUP-TFII compete with each other for binding to DR1 promoter elements. In the context of these genes, occupation of these elements by PPARα-RXRα is associated with activation of transcription, whereas occupation by COUP-TFII is associated with transcriptional repression. Thus, this is a common regulatory paradigm for substrate-driven modulation of various fatty acid utilization pathways at the level of transcription.
Our findings show PGC-1β is required for PPARα agonist-mediated induction of L-FABP, and MTP expression suggests that PGC-1β is required for PPARα agonist induction of L-FABP and MTP (Fig. 7D). We also found that although PPARα agonist treatment of PPARα−/− mice failed to induce L-FABP and MTP mRNA expression (Fig. 4), hepatic expression of PGC-1β mRNA was increased 2-fold (data not shown). These data suggest that in the absence of PPARα, PGC-1β is not sufficient to increase the expression of L-FABP and MTP. Additional experiments using adenovirus-mediated expression of PGC-1β indicate that PPARαRXRα is necessary in order to enhance the transcription of L-FABP and MTP (Fig. 8).

Our combined findings support the proposal that PGC-1β participates in the transcriptional activation of MTP and subsequent induction of apoB-dependent VLDL secretion (41, 59). 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/secre-
tion (59). It has been demonstrated that Foxa2 is completely excluded from the nucleus of ob/ob mice (60), whereas both MTP expression and VLDL assembly/secrition are increased (61). Furthermore, PGC-1β-mediated increase in MTP expres-
sion was retained in the ob/ob mice indicating that induction of MTP transcription can occur via Foxa2-independent mecha-
nisms (59). In fat-fed hyperlipidemic mice, PGC-1β activation of sterol regulatory element-binding protein and liver X recep-
tor α is associated with enhanced MTP expression and VLDL assembly/secrition (41). PGC-1β has been demonstrated to interact with PPARα (62) and activate the transcription of PPARα target genes (63). Our findings show that in the context of rat hepatoma cells, PGC-1β activates PPARαRXRα (Fig. 8).

Our combined data suggest that retention of similar DR1 elements in the promoters of L-FABP and MTP ensures that their expression will be sufficiently induced to provide an efficient delivery of fatty acids to the VLDL assembly/secrition pathway. The same coordinate transcriptional regulation can attenuate the expression of both genes. The importance of coordinate decreased expression of L-FABP and MTP was clearly shown by additional studies showing that ablation of L-FABP blocked the accumulation of triglycerides in the livers of mice treated with the MTP inhibitor (Fig. 9).

Our findings have important implications regarding the effi-
cacy of MTP inhibitors to ameliorate hyperlipidemia (47–50). In a recent study, treatment of hyperlipidemic apoE knock-out mice with an MTP inhibitor caused a marked reduction in both plasma lipids and the progression of atherosclerosis (64). In other studies, liver-specific MTP gene ablation prevented the development of hyperlipidemia and atherosclerosis in mice made susceptible due in part to hepatic overproduction of apoB (65). The utility of MTP inhibitors has been greatly diminished because of the associated development of fatty liver (35, 51).

Our studies show that ablation of L-FABP completely blocks the accumulation of triglycerides in the liver of mice treated with the MTP inhibitor 8aR (Fig. 9B). Thus, our findings indicate that blocking the function of both L-FABP and MTP would reduce hyperlipidemia without causing the development of fatty liver.
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