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UNIVERSITY OF OKLAHOMA
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AN ANALYSIS OF THE ACTIVATION OF THE ADIPOGENIC DIFFERENTIATION PATHWAY IN THE BONE MARROW STROMA BY MEMBERS OF THE NUCLEAR HORMONE RECEPTOR FAMILY

A Dissertation

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By

Claudius Emet Robinson
Norman, Oklahoma
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AN ANALYSIS OF THE ACTIVATION OF THE ADIPOGENIC DIFFERENTIATION PATHWAY IN THE BONE MARROW STROMA BY MEMBERS OF THE NUCLEAR HORMONE RECEPTOR FAMILY

A Dissertation APPROVED FOR THE
DEPARTMENT OF ZOOLOGY

BY

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ABSTRACT

The bone marrow stroma is composed of a diverse array of cell types that have unique functions. Of the different types, the adipocyte is the most abundant. It is not clear what purpose adipocytes serve in the bone marrow. They may simply occupy space in the marrow cavity or they may play active roles in systemic lipid metabolism and provide an energy reservoir in the bone marrow. Marrow adipocytes also appear to contribute growth factors and cytokines that directly promote hematopoiesis and influence osteogenesis. The enzyme lipoprotein lipase (LPL) is an early marker of adipogenesis and its regulation is complex. It is the enzyme responsible for the hydrolysis of triglycerides into free fatty acids and the clearance of chylomicrons from the blood. It is regulated by a variety of transcription factors. The nuclear hormone receptor superfamily consists of an immense number of genes and is accepted as the largest transcription factor family in eukaryotes. We determine that peroxisome proliferator activated receptors (PPARs) with their ligands induce adipogenesis in bone marrow stromal cells and that they bind and activate the LPL gene promoter *in vitro*. The chicken ovalbumin upstream promoter transcription factors (Coup-TFs) are orphan receptors that bind certain DNA direct repeats as homodimers and heterodimers with other steroid receptor molecules such as the retinoid X receptor (RXR) and are accepted as repressors of other nuclear hormone receptors. We determine that Coup-TFs bind the LPL promoter *in vitro* and demonstrate in cotransfection analysis that they act in concert with the PPARγ2 and RXRα proteins to multiplicatively activate its transcription. Recently, new regulatory proteins have been described that affect the interaction of nuclear hormone receptors with the transcriptional

apparatus. We determine that the co-regulators of nuclear hormone receptors SMRT (silencing mediator for retinoid and thyroid-hormone receptors) and steroid receptor coactivator (SRC-1) help regulate the LPL promoter in conjunction with PPARγ2, RXRα and Coup-TFII (ARP-1) in negative and positive fashion respectively. Improved understanding of the mechanisms regulating stromal adipocyte differentiation may lead to therapeutic interventions that can enhance osteogenesis and hematopoiesis in afflicted individuals.

INTRODUCTION

The bone marrow stroma is composed of a diverse array of cell types that have unique functions. It is hypothesized that a single mesenchymal-derived precursor gives rise to the adipocytes, osteoblasts and hematopoietic-supporting cells within the bone marrow stroma (9,13,42,46,52,143). Of the different types, the adipocyte is the most abundant. While it is not completely established what roles bone marrow adipocytes play in the stroma, a number of reviews have been written on the subject (42,46,132). Bone marrow adipocytes may simply occupy space in the bone marrow cavity. They may also play active roles in systemic lipid metabolism and provide a localized energy reservoir in the bone marrow (90). Finally, marrow adipocytes appear to contribute growth factors and cytokines that directly promote hematopoiesis and influence osteogenesis (67,68). It is gradually becoming accepted that the bone marrow adipocyte plays an important role in health and disease. With the new interest in bone marrow adipogenesis, molecular and biochemical analyses of marrow adipogenesis has begun using both primary bone marrow cultures and clonal cell lines (8,42,46) for study. A drawback of primary bone marrow cultures is their greater phenotypic heterogeneity. Stromal stem cell clones offer the advantage of relatively greater homogeneity, but can present unrecognized artifacts as a consequence of the original cloning procedure. The hematopoietic and osteogenic literature reports an impressive array of stromal cell lines with over 50% exhibiting preadipocyte properties (see Ref. (30) for a comprehensive review). The BMS-2 (bone

marrow support-2) cell has many of the characteristics of a multipotential stem cell in the bone marrow stroma. It has hematopoietic support capacity (110), can display osteoblast-specific gene markers (33,45), and undergoes adipogenesis in response to a cocktail of agonists (methylisobutyxanthine, hydrocortisone, indomethacin, (MHI)) (43) and to the thiazolidinedione compounds BRL 49653 and pioglitazone (47). Thus, the BMS-2 cell provides a useful model for monitoring adipogenic events in the bone marrow stroma.

The enzyme lipoprotein lipase (LPL: EC 3.1.1.34) is an early marker of adipogenesis and its regulation is complex. It is the enzyme responsible for the hydrolysis of triglycerides into free fatty acids and the clearance of chylomicrons from the blood. It is an essential gene product. In LPL knockout mice, death occurs during the neonatal period due to a massive hypertriglyceridemia (21). In man, hereditary deficiencies in LPL activity cause extreme postprandial hypertriglyceridemia (151) and increased morbidity due to cardiovascular disease (36). Excess LPL activity is also detrimental. Elevated levels of LPL expression are found in macrophages of inbred strains of mice susceptible to atherosclerosis (114). The LPL gene is regulated by a variety of transcription factors. LPL is expressed in numerous tissues (36-38,121). Analysis of transgenic mice with the -1824 to +187 bp region of the LPL promoter fused to a luciferase reporter construct (44) revealed high levels of LPL promoter activity in the murine brain as well as in adipose and liver with a low level of activation in the kidney. We believe that the LPL promoter offers a useful tool for monitoring adipogenic events in the bone marrow stroma.

The nuclear hormone receptor superfamily consists of an immense number of genes and is accepted as the largest transcription factor family in eukaryotes. The peroxisome proliferator activated receptors (PPARs) with their ligands induce adipogenesis in cells that can proceed along the adipogenic pathway (reviewed in (119) and (51)). They form heterodimers with the retinoic acid X receptors (RXR) proteins and bind to DNA elements know as DR-1s (6 nucleotide direct repeat with a 1 nucleotide spacer) (88,106,140). Ligands for the PPARs increase LPL mRNA levels and adipogenesis in pre-adipocyte cell models (reviewed in (119)). These ligands include natural compounds such as certain prostaglandin's and long chain fatty acids, as well as synthetic drugs such as the fibrates and thiazolidinediones (119). In preadipocyte cell models, adipogenesis has been associated with increased PPARγ2 (12,15,16,47,136,137,147), PPARα (12,16), and PPARδ (2,59) mRNA levels, suggesting that each of these proteins may help regulate the proteins activated during adipogenesis, including LPL. mPPARα is predominantly expressed in liver, heart, kidney and brown adipose tissue (119) while mPPARδ demonstrates a high level of expression in brain and fat, with a low level of expression in the liver (119). PPARγ2 is predominantly expressed in adipose tissue (15,136,137,154) and is a strong candidate as a regulator of LPL transcription.

The Chicken Ovalbumin Upstream Promoter Transcription Factors (Coup-TFs) are orphan nuclear hormone receptors that bind direct repeats of AGGTCA motifs with spacing from 0 to 12 nucleotides (DR-1 to DR-12) as homodimers and heterodimers with other steroid receptor molecules such as the retinoid X receptor (RXR). Coup-TF was originally cloned based on its ability to promote transcription of the chicken ovalbumin

gene (107,116,142) and has become accepted as a repressor of other nuclear hormone receptors (22,23). It was independently cloned via homology to *erb* A and called *erb* A related protein 3 (Ear3) (92). Somewhat later, Coup-TFII (ARP-1) was cloned from a HeLa cell cDNA library through its homology to hCoup-TFI (141) and from a placental library as apolipoprotein AI regulatory protein-1 (ARP-1) (78). Another family member, *erb*A related protein 2 (Ear2) was cloned in the same way as Ear3 (92). In humans, Coups are expressed in a variety of cell lines (92). Coup-TFs are highly expressed in organs such as lung, testis, prostate, skin, intestine, pancreas, stomach, and salivary gland (62,85,108) and have recently been shown to be present in adipocytes (11). Coup-TFs bind PPAR response elements on DNA and exert a repressive force on the respective promoter (3,11,93). With these facts in mind, we decided to test if members of the Coup-TF family could bind the LPL promoter and influence regulation of its expression.

It is becoming recognized that other proteins affect the interaction of nuclear hormone receptors with the transcriptional apparatus. Once a nuclear hormone receptor is bound to its DNA response element, it either activates or represses gene transcription depending on what the cell needs. Transactivation by nuclear hormone receptors can occur through direct interaction with components of the transcriptional apparatus such as TFIIB (4,10,60). It is also suspected that other factors can act as bridges between nuclear hormone receptors and the transcriptional apparatus. The coregulators of nuclear hormone receptors SMRT (silencing mediator for retinoid and thyroid-hormone receptors) (18)and SRC-1 (steroid receptor coactivator-1) (104) help regulate nuclear receptor action in a negative or positive fashion (for reviews, see (57)). SMRT interacts with

unliganded nuclear hormone receptors to function as adapters that convey a repressive signal to the transcriptional apparatus (57,152). Upon binding of ligand, a conformational change occurs in the receptor that allows the corepressor to dissociate (57,152). In nuclear hormone receptors, there is a carboxyl terminal amphipathic α-helix that is known as AF2 (activation function-2) (27). When ligand is bound, AF2 serves to trigger the release of corepressor (5,18) and helps recruit a coactivator (6,27,35) such as SRC-1 which has recently been demonstrated to be a coactivator of PPARγ on a PPAR response element (155).

This dissertation explores the activation of the adipogenic pathway in the bone marrow stroma through the actions of the nuclear hormone receptor family members PPAR, RXR and Coup-TFs on transcription directed by the LPL promoter, and the coregulation of these actions by SMRT and SRC-1. Improved understanding of the mechanisms regulating stromal adipocyte differentiation and regulation of the lipoprotein lipase gene may lead to therapeutic interventions that can enhance osteogenesis and hematopoiesis and limit atherogenesis.

Peroxisome Proliferator-Activated Receptor γ Activation by Thiazolidinediones Induces Adipogenesis in Bone Marrow Stromal Cells

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Running Title: Thiazolidinediones Induce Bone Marrow Stromal Adipogenesis

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Abbreviations:
BAT, brown adipose tissue; BRL, BRL49653 or 5-(4-[(N-methyl-N(2-pyridyl)amino)ethoxy] benzyl)thiazolidine-2-4-dione; FACS, fluorescence activated cell sorter; PIO, pioglitazone; MHI, Methylisobutylxanthine/hydrocortisone/indomethacin; PPAR, peroxisome proliferator-activated receptor; WAT, white adipose tissue.

Key Words:
Adipocytes, Adipose Tissue, Bone Marrow, Peroxisome Proliferator-Activated Receptors, Stroma, Thiazolidinediones

Summary:

The thiazolidinediones improve insulin sensitivity in animal models and have promise as potent oral antidiabetic agents. To date, their clinical use has been limited due to anemia and cardiac hypertrophy. Some compounds of this class have been reported to induce bone marrow fat accumulation in animals and this effect could account for the observed anemia. This work examines the biological mechanism contributing to this phenomenon. The thiazolidinediones, BRL49653 and pioglitazone, induced adipocyte differentiation in the BMS2 bone marrow stromal cell line in a dose- and time-dependent manner. These actions were further enhanced by the presence of glucocorticoids and other adipogenic agonists. The thiazolidinediones increased the mRNA levels of adipocyte-specific genes, including that of their receptor, the peroxisome proliferator-activated receptor γ (PPARγ). In contrast, mRNA levels of genes encoding other PPAR family members (PPARα, PPARδ or NUC-1) were unchanged or decreased. Thiazolidinedione treatment of primary bone marrow stromal cells elicited a comparable dose-dependent response. Using a polyclonal antibody, PPARγ was detected in protein lysates from adipose-rich bone marrow. Thus, thiazolidinediones directly regulate bone marrow stromal cell differentiation; induced PPARγ expression may play a key regulatory role in this process.

The thiazolidinediones are potent oral antidiabetic agents. However, the clinical application of thiazolidinediones has been limited by their potential effects on blood cell production and cardiac hypertrophy. In both rodent and canine models, administration of thiazolidinediones was accompanied by fat accumulation in the bone marrow cavity and impaired hematopoiesis, resulting in anemia (29,146).

In vitro, thiazolidinediones have been found to induce adipocyte differentiation in pre-adipocyte cell lines derived from murine fetal tissue (3T3-L1) or ob/ob mice (Ob17) (123). Recently, the thiazolidinediones have been identified as ligands for the peroxisome proliferator-activated receptor γ (PPARγ) (82). This protein was originally identified as an adipogenic transcription factor (136,137). The PPARγ gene is subject to alternative promoter usage and splicing, giving rise to the tissue-specific subtypes, PPARγ1 (liver) and PPARγ2 (adipose) (17,136,137,154,156). Like other PPAR family members (PPARα, PPARδ), the PPARγ isoforms are members of the steroid receptor family and are closely related to the retinoid, vitamin D_3, and thyroxine receptors (140). In its ligand-bound form, PPARγ regulates transcription from adipocyte-specific genes and will induce adipocyte differentiation in fibroblasts (53,72,136,137).

The mechanism underlying the bone marrow effects of the thiazolidinediones remains unknown. Normally, the bone marrow stroma consists of a heterogeneous population of hematopoietic supporting fibroblasts, adipocytes and bone-forming osteoblasts (42,69). It is hypothesized that this phenotypically diverse group of cells derives from a multipotent stromal progenitor cell (7,105). The murine BMS2 bone marrow stromal cell line provides a well characterized *in vitro* model for this progenitor cell. The BMS2 cells support lymphohematopoiesis (110), display osteoblast specific gene

markers (33,45), and undergo accelerated adipogenesis in response to a cocktail of agonists (glucocorticoids, methylisobutylxanthine, indomethacin) (48). The current work determines that the thiazolidinediones, pioglitazone and BRL49653, induce BMS2 adipogenesis in a dose- and time-dependent manner. This correlates with increased levels of PPARγ mRNA. Thiazolidinediones exert a similar effect on primary cultures of bone marrow stromal cells. Moreover, the PPARγ protein is detected in adipose-rich bone marrow. Together, these data indicate that bone marrow stromal cells are a direct target for thiazolidinedione actions *in vivo*.

Materials and Methods:

Cell Culture: Reagents were obtained from Sigma Chemical Co. (St. Louis MO) or Fisher Scientific (Dallas TX) unless otherwise noted. The BMS2 cell line was originally cloned by limiting dilution from the adherent population of murine bone marrow stromal cells (110). The cells were selected based on their ability to support the proliferation of stromal-dependent B lineage lymphoid cell lines in culture (110). In these experiments, the BMS2 cells (110) were plated at a density of 4×10^4 cells/35 mm dish and cultured in Dulbecco's Modified Eagle's Medium (Mediatech, Washington DC) supplemented with 10% fetal bovine serum (Hyclone, Logan UT), 1 mM Na pyruvate, 50 μM 2-mercaptoethanol, 100 mg/ml streptomycin and 100 units/ml penicillin; this is referred to as "standard medium." Confluence was achieved within 7 days. Pioglitazone and BRL49653 were dissolved in dimethylsulfoxide (DMSO) and added at the indicated concentrations with a constant final concentration of 0.5% DMSO. The DMSO carrier alone had no effect on cell differentiation. An adipogenic agonist cocktail, MHI (500 μM methylisobutylxanthine, 0.5 μM hydrocortisone [Elkin Sinn, Cherry Hill NJ], 60 μM indomethacin), was used as a control (33). Confluent cultures were maintained in the presence of thiazolidinediones or MHI for 3 days. The medium was removed and replaced with "standard medium." Individual 35 mm plates were harvested after an additional 3 days in culture (day 6). Cell densities at this time averaged 4.7×10^5 cells/35 mm plate, independent of culture conditions (range: $4.2 - 5.2 \times 10^5$). Primary bone marrow stromal cells were harvested from the femurs and tibia of 6 week old female Balb/c mice in "standard medium." The mice were euthanized by CO_2 asphyxiation in accordance with an institutionally-approved protocol and the long bones of the lower extremities removed

under sterile conditions. The marrow cavity was flushed with "standard medium" using a #25 gauge needle. 10^7 cells were cultured in a 25 cm^2 flask. After 2 hours in culture, the nonadherent (primarily hematopoietic) cells were removed and the medium replaced. This step enriches for the adherent stromal population which includes fibroblasts, adipocytes, osteoblasts, and macrophages. Two weeks after the cultures were established, cells were treated with thiazolidinediones for one week, photographed using a Zeiss IM35 microscope (magnified X 102 under phase contrast), and harvested for total RNA. Cell densities were not determined in these studies.

Fluorescence-Activated Cell Sorting: The BMS2 were plated at a density of 10^4 cells/well in 24-well plates. After seven days in culture, the cells were induced with agents for three days and the medium changed. After an additional three days of culture, the 24-well plates containing BMS2 cells were harvested by treatment with 0.25% trypsin/1 mM EDTA, washed in phosphate-buffered saline (PBS), and fixed with the addition of a final concentration of 0.5% paraformaldehyde (33,124). A stock solution of Nile red (1 mg/ml DMSO) was diluted 1:100 and added to the cells at a final concentration of 1 μg/ml. Cells were analyzed on a FACscan (Becton-Dickinson, San Jose CA) multiparameter flow cytometer. Gold fluorescence emission was detected between 564 nm and 604 nm with a bandpass filter 585/42. Sample sizes of 7.5-10 x 10^3 cells were analyzed from each well.

Northern Blot Analysis: RNA was harvested from BMS2 cells cultured in 35 mm plates as described under "Cell Culture" above and analyzed as previously described (20,64). Northern blots were hybridized with the following probes: aP2 (courtesy H. Green,

Harvard University) (127), actin and adipsin (courtesy W. Wilkison & B.M. Spiegelman, Dana Farber Cancer Center) (145), lipoprotein lipase (LPL) (ATCC 63117) (48), C/EBPα (courtesy S. Enerback & K. Xanthopoulos, University Gotesborg) and PPARγ2 (136). The PPARγ2 probe was cloned by reverse transcription polymerase chain reaction (PCR) using murine brown adipose total RNA and the following specific primers: N Terminal primer 5′ TTTGAGCTC GCTGTTATGGGTGAAACTCTG 3′(bp 34-54); C Terminal primer 5′ TTTGAGCTC CCTGCTAATACAAGTCCTTGTA 3′ (bp 1540-1561) (136). Intensity of mRNA signals on northern blots was quantitated using an Eagle Eye II Still Video System (Stratagene, La Jolla, CA).

Semi-quantitative PCR: Reactions were performed according to published methods (95). Aliquots containing 5 μg of total RNA in a 12.5 μl volume were heated for 5 min at 65°C. The cDNA was reverse transcribed in a 30 μl volume of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 0.5 mM spermidine, containing RNasin (1.25 units), random hexamer and oligo-dT primers (100 ng each), and AMV RT (2.5 units). The reaction was incubated for 60 min at 42°C, 30 min at 52°C, and 5 min at 95°C. Polymerase chain reactions were conducted in 100 μl volumes with oligonucleotide primers specific for PPARα, PPARδ, PPARγ1, PPARγ2, and β-actin **(Table 1)**; a reaction cycle consisted of 45 sec at 94°C, 45 sec at 62°C, and 2 min at 72°C using a Perkin-Elmer-Cetus DNA Thermal Cycler (Norwalk CT). Aliquots (12 μl) were removed at 3-cycle intervals between cycles 17 to 35 and examined on 3% agarose gels stained with ethidium bromide.

Polyclonal Antibody Preparation: A multiple antigenic peptide (111) was synthesized

based on the PPARγ2 amino acids 482-499 (136): HVIKKTETDMSLHPLLQE. Eight identical peptides were attached to a single poly lysine-resin matrix core, providing a potent antigen (111). After the collection of pre-immune serum, the goat was injected with 1 mg of the multiple antigenic peptide with incomplete Freund's adjuvant. Four weeks later, the animal was boosted with 1 mg of peptide alone. Immune serum was harvested weekly after the fifth week. Antibody was prepared by ammonium sulfate precipitation and affinity purified over a column prepared with the multiple antigenic peptide coupled to cyanogen bromide activated Sepharose 4B.

Transfections: The PPARγ2 cDNA was subcloned into the pSG5 and the PPARγ1 cDNA (provided courtesy of Drs. F. Chen and B. O'Malley, Baylor College of Medicine) (17) into the pEF-BOS eukaryotic expression vectors (provided by Dr. K. Oritani, Oklahoma Medical Research Foundation) (94). The plasmids were transiently transfected into the human kidney 293T cell line by calcium phosphate precipitation. Cell lysates from the transiently transfected 293T cells provided an enriched source of the PPAR γ2 protein. The non-transfected 293T cell lysates provided an appropriate negative control.

Western Blot Analysis: Cells or tissues were homogenized in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM iodoacetamide, 0.1% NaN_3, 5% aprotinin, 1 mM PMSF, .1% Triton X-100, soybean trypsin inhibitor [25 μg/ml], leupeptin [10 μg/ml]). Equal protein aliquots were loaded per lane and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (BioRad, Richmond CA), and blocked overnight in buffer (10 mM sodium phosphate, 150

mM NaCl, 0.5% gelatin, 0.05% Tween 20, 0.1% merthiolate) (79). Blots were sequentially incubated with goat primary antibody (21 μg/ml) and an anti-goat horseradish peroxidase coupled secondary antibody for 1 hr, followed by three washes (10 min) in phosphate-buffered saline 0.05% Tween 20, and visualized by chemiluminescent reagents according to the manufacturer's instructions (Amersham, Arlington Heights IL).

Results and Discussion:

Dose-dependent Effects of Thiazolidinediones on the BMS2 Bone Marrow Stromal Cell Line. The BMS2 cell line was used as an *in vitro* model to examine the response of bone marrow stromal cells to thiazolidinediones. Confluent cultures of BMS2 stromal cells were treated with varying concentrations of the thiazolidinediones BRL49653 and pioglitazone. Cellular accumulation of lipid vacuoles after 6 days in culture was quantified by staining with a lipophilic dye, Nile red, and fluorescence-activated cell sorting (FACS) analysis **(Figure 1, Table 2 A)**. In the absence of inducing factors, the cells did not contain lipid vacuoles after 6 days. As previously reported, a cocktail of adipogenic agonists (MHI: methylisobutylxanthine, hydrocortisone, indomethacin) induced lipid droplets in over 50% of the cells (33). Both pioglitazone and BRL49653 induced adipocyte differentiation in a dose-dependent manner; the cell response was greater to BRL49653 than pioglitazone at equal molar concentrations. At concentrations of 5 μM BRL49653 or 25 μM pioglitazone, up to 40% of the cells contained lipid vacuoles. The actions of the thiazolidinediones were partially additive with those of the MHI cocktail **(Table 2 B)**. While 50% of the cells contained lipid vacuoles following treatment with the MHI cocktail alone, treatment with either thiazolidinedione in combination with the MHI cocktail induced adipogenesis in up to 70% of the BMS2 cell population. Similar observations have been made in the ob/ob derived pre-adipocyte cell line, Ob1771, where glucocorticoids further enhanced the actions of BRL49653 on expression of adipocyte markers (59). This suggests that thiazolidinediones and glucocorticoids may activate distinct as well as common signaling pathways during adipogenesis. Evidence from

previous studies supports this hypothesis. For example, dexamethasone upregulated PPARα mRNA levels in hepatic cells (129).

Thiazolidinedione Induction of Adipocyte Gene Markers in BMS2 Cells. The temporal dependent expression of adipocyte mRNAs was examined in thiazolidinedione-treated BMS2 cells. Confluent BMS2 cultures were treated with "standard medium" alone (Control) or medium supplemented with either pioglitazone (25 μM) or BRL49653 (5 μM). Northern blot analyses were performed using total RNA harvested daily from the cells following treatment initiation **(Figure 2)**. The blots were hybridized with a β-actin probe to control for the relative RNA loading between lanes. The signal intensity of each mRNA on day 6 relative to β actin was quantitated **(Table 3A)**. Two relatively late adipocyte differentiation marker genes, the fatty acid binding protein aP2 and adipsin, were not detected in control cells. The thiazolidinediones increased both mRNAs which reached maximal levels after 3 to 5 days. Although the relatively early adipocyte differentiation marker gene, lipoprotein lipase (LPL), was present in control cells, its level was further induced and sustained by treatment with the thiazolidinediones; near maximal levels were reached after 3 days of treatment. These same genes exhibited a similar expression pattern in 3T3-derived and Ob1771 pre-adipocyte cell lines following induction with thiazolidinediones and other adipogenic agents (2,15,16,59,136).

The transcription factors C/EBPα and PPARγ are both known to regulate adipocyte differentiation (123). While control cells contained a detectable signal for each gene, thiazolidinediones accelerated the rate and extent of their accumulation by up to nine-fold **(Figure 2, Table 3A)**. In 3T3-derived pre-adipocytes, adipogenic agents were

found to induce PPARγ expression in a similar time-dependent manner (15,16,136). Hybridization of the Northern blots with probes for PPARα and PPARδ detected a weak mRNA signal at best.

Specific Induction of PPARγ mRNA Levels in BMS2 Cells: Two distinct murine PPARγ subtypes have been detected in adipose tissue (PPARγ2) and liver (PPARγ1), respectively. The PPARγ2 isoform utilizes an alternative promoter and 5′ non-coding region and contains an additional 30 amino acids in comparison to PPARγ1 (17,136,154,156). To determine if BMS2 cells expressed both PPARγ isoforms and to examine the PPARα and PPARδ genes more closely, specific oligonucleotides **(Table 1)** were synthesized for semi-quantitative polymerase chain reactions (PCR) (95). The β-actin gene was used as an internal standard to allow comparison between samples **(Figure 3)**. The signal intensity of each PCR product at 32 cycles under individual treatment conditions was quantitated and normalized relative to control levels based on densitometry **(Table 3 B)**; these values are intended only as an aid for comparative purposes and should not be viewed as quantitative. The control cells expressed detectable levels of mRNA for PPARα, PPARδ, and both PPARγ isoforms **(Figure 3)**. After induction with either MHI or thiazolidinediones, the signal intensities of both PPARα and PPARδ were reduced relative to control levels. In contrast, following 6 days of exposure to adipogenic agonists, PPARγ1 and PPARγ2 levels were equal to or greater than control signals; the only exception was the PPARγ1 level in response to pioglitazone **(Table 3B)**. These findings document that BMS2 adipocytes express both the PPARγ1 and PPARγ2 isoforms.

While the signals for PPARα and PPARδ are detected in BMS2 cells, adipogenesis reduces the levels of these rare mRNAs. This pattern of PPARδ expression differs from that reported in 3T3-derived and Ob1771 pre-adipocytes (2,16,136). In 3T3-L1 cells, PPARδ mRNA levels increased with adipocyte differentiation (16). Recently, Amri and colleagues cloned the murine PPARδ cDNA (also known as Fatty Acid-Activated Receptor, FAAR). Based on transfection experiments, they concluded that PPARδ mediated the transcriptional effects of fatty acids on Ob1771 adipocyte differentiation (2). Since their previous work indicated that fatty acids and thiazolidinediones share a common mechanism of action, this indicates that PPARδ mediates the effects of thiazolidinediones in epididymal-derived Ob1771 cells (59). The current results in bone marrow stromal cells are consistent with the original observations of Tontonoz and colleagues, indicating that the PPARγ isoforms are induced during adipogenesis (136). This suggests that PPARγ is partially responsible for the thiazolidinedione effects on bone marrow observed *in vivo*.

Thiazolidinedione Induction of Primary Murine Bone Marrow Stromal Cells. To more closely approximate the *in vivo* bone marrow microenvironment, the response of primary murine stromal cells to thiazolidinediones was examined. Treatment of confluent primary stromal cultures for one week with BRL49653 (0.005 μM to 5 μM) or pioglitazone (0.025 μM to 25 μM) increased the number of adipocytes relative to control cultures **(Figure 4 A)**. Based on visual examination, up to 15% of the stromal cells contained lipid vacuoles in the presence of 5 μM BRL49653 or 25 μM pioglitazone. This was accompanied by increased mRNA levels for the adipocyte gene markers aP2, adipsin, and lipoprotein lipase, as well as the transcription factor, PPARγ **(Figure 4 B, Table 4)**.

Levels of induction were dose-dependent. Based on quantitation relative to the β actin control, maximum mRNA induction was achieved with concentrations of 0.5 μM BRL49653 or 2.5 μM pioglitazone **(Table 4)**. Thus, these results demonstrate PPARγ expression in primary stromal cells and are consistent with those obtained using the BMS2 stromal cell clone. However, the possibility cannot be excluded that macrophages in the heterogeneous primary cultures may account for some of the PPARγ signal.

Antibody Detection of PPAR Protein in Bone Marrow. Additional experiments were undertaken to document the presence of the PPARγ in the bone marrow *in vivo*. To directly detect the PPARγ protein, a polyclonal antibody was prepared using a PPARγ C terminal peptide as antigen. Previously, a peptide from the comparable region of the retinoic acid receptor γ had been employed successfully for this same purpose (115). The final affinity-purified α-PPARγ antibody was tested on Western blots prepared with extracts from cells transfected with a PPARγ2 expression vector. The antibody specifically detected an approximately 63 kDa protein **(Figure 5)**. The protein was not detected in antisense oriented control expression constructs nor by the pre-immune serum.

Control studies determined that the α-PPARγ antibody detected an identically sized protein on Western blots of both murine and rat white adipose tissues (data not shown). Rats were used instead of mice to examine PPARγ protein expression in bone marrow due to their greater size and the relative prominence of adipocytes in their marrow cavity. Of course, the marrow is heterogeneous, containing macrophages and other blood cell lineages in addition to the stromal cells themselves. It cannot be ruled out that proteins derived from the hematopoietic cell population might account for some component of the

signal obtained from the bone marrow specimens. On Western blots prepared with bone marrow (BM), white (WAT) and brown (BAT) adipose tissue specimens, the antibody detected a major protein of 63 kDa **(Figure 6 A)**. This was identical in size to the transfected PPARγ2 control vector and was not detected by pre-immune antibody. Addition of the multi-antigenic peptide (MAP) antigen specifically competed away the protein signal in bone marrow **(Figure 6 B)**. Similar observations were made with WAT protein lysates (data not shown).

Conclusions:

The current study has examined the mechanism underlying the effects of the thiazolidinediones on bone marrow. Both clonal and primary bone marrow stromal cell cultures underwent adipocyte differentiation in a time- and dose-dependent manner following thiazolidinedione treatment **(Figure 1, Table 2)**. This induction was similar to that described in other murine pre-adipocyte cell lines (56,71,82,125). In the BMS2 cells, adipogenesis correlated with a specific increase in the mRNA levels of the thiazolidinedione receptor, PPARγ (82), while other PPAR mRNAs were unchanged or decreased. It is likely that the thiazolidinediones, acting as PPARγ ligands, directly induce transcription of adipocyte-specific genes **(Figures 2 and 4)**. In transient transfection assays, expression of PPARγ and its heterodimerization partner, RXRα, increases reporter gene expression under the regulation of the aP2 enhancer (136) or the LPL promoter **[unpublished observations, CER & JMG]**. Using an antibody reagent, the PPARγ protein was detected in bone marrow tissue extracts **(Figure 6)**. Together, these findings suggest that thiazolidinedione interactions with PPARγ as opposed to other receptor proteins underlies bone marrow stromal cell adipogenesis. In the future, it may be possible to decrease these bone marrow effects through the development of thiazolidinedione derivatives that do not activate PPARγ proteins in the marrow stromal cell lineages.

Acknowledgements:

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Table 1. Primers for Semi-Quantitative PCR Analyses.

Gene & (ref)	5′ Oligonucleotide	3′ Oligonucleotide
PPARα (61)	CGACAAGTGTGATCGGAGCT GCAAG bp 574-598	GTTGAAGTTCTTCAGGTAGGC TTC bp 800-777
PPARδ (2)	GGCCAACGGCAGTGGCTTCGTC bp 912-933	GGCTGCGGCCTTAGTACATGT CCT bp 1390-1367
PPARγ1(17,154)	TTCTGACAGGACTGTGTGACAG bp 391-412	ATAAGGTGGAGATGCAGGTTC bp 745-725
PPARγ2 (136)	GCTGTTATGGGTGAAACTCTG bp 34-54	ATAAGGTGGAGATGCAGGTTC bp 384-364
β-actin (134)	CCTAAGGCCAACCGTGAAAAG bp 414-434	TCTTCATGGTGCTAGGAGCCA bp 1059-1039

Table 2. FACS Analysis of Thiazolidinedione Induction of Adipocyte Differentiation.

Table 2 A. Dose-Dependent Adipogenic Response to Thiazolidinediones.

	0 μM	0.005 μM	0.05 μM	0.5 μM	5 μM	25 μM	MHI
PIO	0.00	N.D.	2.4 ± 3.1	4.2 ± 2.4	18.5 ± 5.7	34.5 ± 3.2	53.6 ± 3.2
BRL	0.00	0.00	13.6 ± 6.8	25.1 ± 9.9	42.9 ± .7	N.D.	53.4 ± 4.1

(In all studies, values are reported as percentage of all cells staining positive for Nile Red based on fluorescence intensity. P values are <0.0001 relative to the MHI point for all concentrations of thiazolidinediones. The results are the mean ± S.D. from 10 data points collected in four experiments. Abbr. BRL = BRL49563; MHI = methylisobutylxanthine/-hydrocortisone/indomethacin; N.D. = not done; PIO = pioglitazone.)

Table 2 B. Additive Effects of Thiazolidinediones and MHI as Adipogenic Agonists.

CONTROL	0
MHI	50.5 ± 12.2
MHI + BRL 5 μM	70.6 ± 2.2*
MHI + PIO 25 μM	68.9 ± 5.9*

(*indicates a P value <0.0001 relative to the MHI point. Values are expressed as the percentage of all cells positive for Nile red fluorescence. The results are the mean ± S.D. from 14 data points collected in four experiments.)

Table 3. Densitometric Quantitation of BMS2 mRNA Levels on Day 6 Relative to Actin.

A. Based on Northern Blot Analysis (Figure 2)

Gene:	aP2	Adipsin	LPL	C/EBP α	PPARγ	Actin
Agent						
Control	0	0	0	0	0.2	1.0
PIO	2.6	2.5	0.9	0.2	1.9	1.0
BRL	0.8	1.3	0.5	0.1	0.9	1.0

B. Based on PCR Analysis at 32 Cycles (Figure 3) and Normalized Relative to Control.

Gene:	PPARα	PPARδ	PPARγ1	PPARγ2	Actin
Agent					
Control	1.0	1.0	1.0	1.0	1.0
MHI	0.6	0.7	1.2	1.6	1.0
BRL	0.2	0.1	1.0	1.1	1.0
PIO	0.2	0.2	0.5	1.0	1.0

Abbreviations: BRL = 5 μM BRL49653; MHI = 0.5 mM Methylisobutylxanthine, 60 μM indomethacine, 5 x 10-7 M hydrocortisone; PIO = 25 μM pioglitazone. Values are based on the signal intensity of the positive image from northern blots (A) or the negative image from PCR gels (B), measured using a Stratagene Eagle Eye Video System as described in Materials and Methods. All signal intensities are normalized relative to actin at an equivalent day (A) and cycle number (B).

Table 4. Densitometric Quantitation of Primary Stromal Cell mRNA Levels Normalized Relative to Actin mRNA Signal Intensity.

	Agent		BRL (μM)				PIO (μM)			
Gene	C	D	.005	.05	.5	5	.025	.25	2.5	25
aP2	0	0	0	0.3	1.1	1.3	0	0	1.0	1.1
Adipsin	0	0	0	0.7	0.5	0.5	0	0.4	0.9	0.1
LPL	0	0	0	0.4	0.3	0.6	0.1	0.3	0.9	0.9
PPARγ	0	0	0	0.1	0.6	0.3	0	0	0.1	0.2
Actin	1	1	1	1	1	1	1	1	1	1

Abbreviations: C = Control, D = DMSO vehicle alone, BRL = BRL49653 treated cells at indicated μM concentrations, PIO = pioglitazone treated cells at indicated μM concentrations. Values are derived from data presented in Figure 4 as described in Materials and Methods.

FIGURE LEGENDS:

FIGURE 1. FACS ANALYSIS OF BMS2 STROMAL CELL ADIPOGENESIS IN RESPONSE TO THIAZOLIDINEDIONE COMPOUNDS.

Confluent, quiescent cultures of BMS2 stromal cells were untreated (CTRL) or induced with the thiazolidinedione compounds BRL49653 (BRL) or pioglitazone (PIO) at increasing concentrations for 3 days. The cells were returned to standard medium for an additional 3 days, at which time they were fixed, stained with the lipophilic fluorescent dye Nile red, and monitored by FACS for enhanced fluorescence in the gold wavelength (cells in the M1 region of the profile). This percentage value of the total cell population is reported in **TABLE 2 A.**

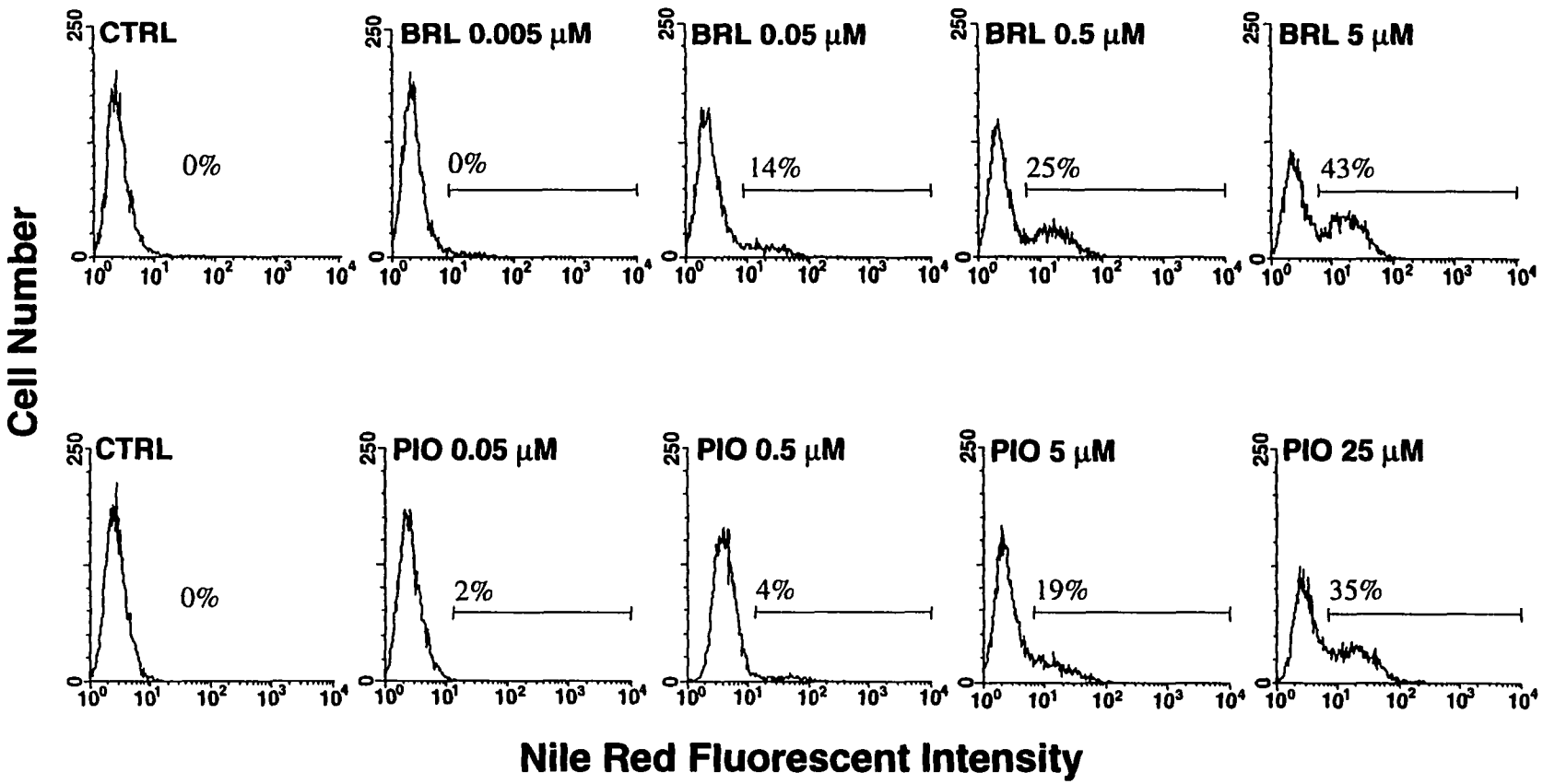

CTRL
BRL 0.005 μM
BRL 0.05 μM
BRL 0.5 μM
BRL 5 μM
CTRL
PIO 0.05 μM
PIO 0.5 μM
PIO 5 μM
PIO 25 μM
0%
0%
14%
25%
43%
0%
2%
4%
19%
35%
Cell Number
Nile Red Fluorescent Intensity

FIGURE 2. NORTHERN BLOT ANALYSIS OF ADIPOCYTE-SPECIFIC GENES INDUCED BY THIAZOLIDINEDIONES.

Confluent, quiescent BMS2 stromal cells were cultured without inducing factors in "standard medium" (Control) or in the presence of either 25 μM pioglitazone (PIO) or 5 μM BRL49653 (BRL) for 3 days; at this time, all cultures were converted to "standard medium" alone. Individual cultures were harvested daily from day 0 to day 6. Northern blots prepared with total RNA from these cells were hybridized with the following cDNA probes and the autoradiographs exposed for the number of days indicated within the parentheses: the fatty acid binding protein aP2 (1); adipsin (1); lipoprotein lipase, LPL (1); CAAT/enhancer binding protein α, C/EBPα (9); peroxisome proliferator-activated receptor γ, PPARγ (8); β-actin (1).

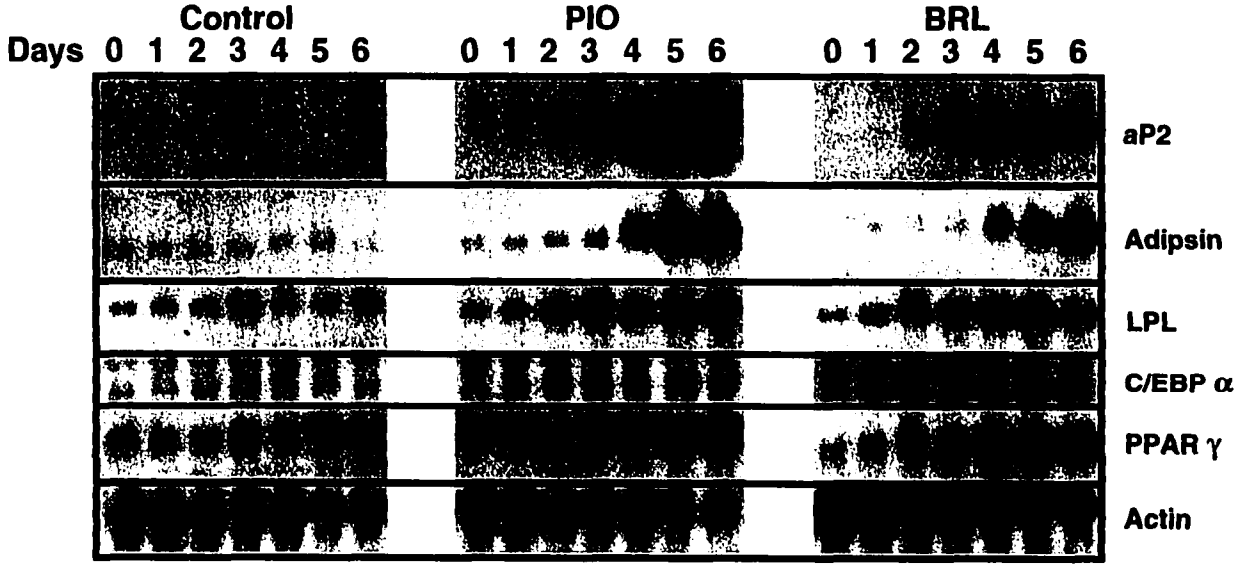

Control
PIO
BRL
Days 0 1 2 3 4 5 6
0 1 2 3 4 5 6
0 1 2 3 4 5 6
aP2
Adipsin
LPL
C/EBP α
PPAR γ
Actin

FIGURE 3. SEMI-QUANTITATIVE PCR DETECTION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR mRNA.

Total RNA was prepared from BMS2 cells 6 days after treatment initiation. Cells were cultured in standard medium alone (Control), with the classical adipogenic agonists (methylisobutyl-xanthine, hydrocortisone, indomethacin: MHI), with 5 μM BRL49653 (BRL), or with 25 μM pioglitazone (PIO). Equal aliquots of total RNA were reverse transcribed and amplified with oligonucleotide primers specific for PPARα, PPARδ, PPARγ1 and PPARγ2 (described in **TABLE 1**). The β-actin gene was used as a control. Aliquots were removed from each reaction volume at 3-cycle intervals and examined on 3% agarose gels to compare the relative signal intensity.

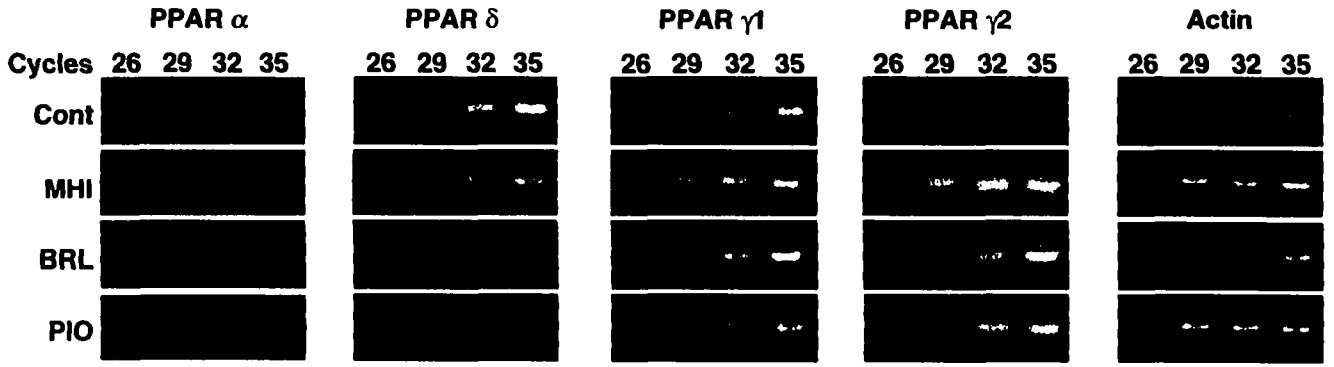
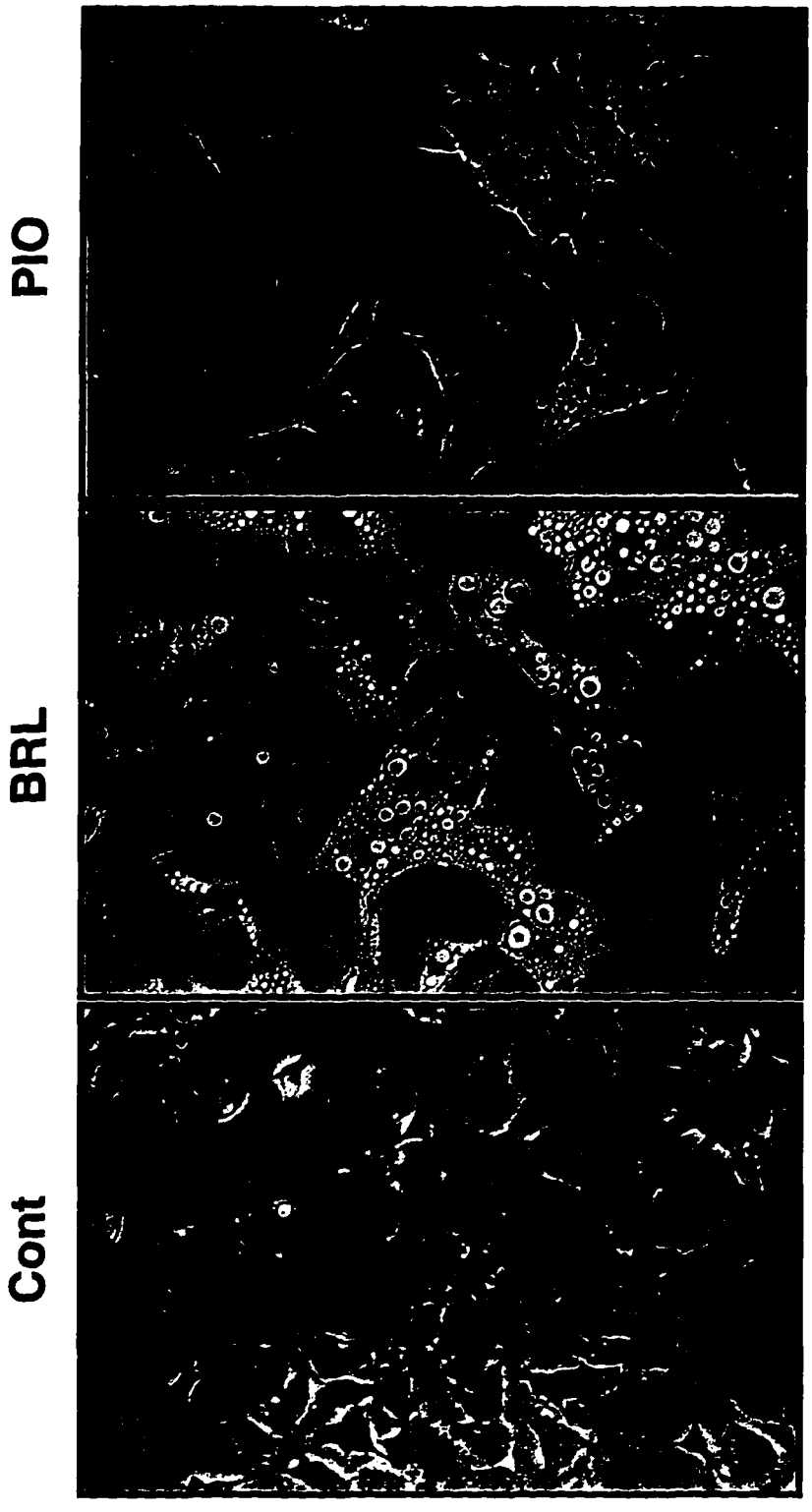

PPAR α
PPAR δ
PPAR γ1
PPAR γ2
Actin
Cycles
26 29 32 35
Cont
MHI
BRL
PIO

FIGURE 4. RESPONSE OF PRIMARY BONE MARROW STROMAL CELLS.

A. Confluent cultures of primary murine bone marrow stromal cells were cultured in the absence (Control) or presence of thiazolidinediones (0.5 μM BRL49653 or 2.5 μM pioglitazone) for one week. Cultures were photographed under phase contrast at X102 magnification.

B. Total RNA harvested from primary stromal cell cultures after one week in the presence of inducing agents (μM) was examined on Northern blots hybridized with the following cDNA probes, and the autoradiographs were exposed for the number of days indicated in parentheses: β-actin (1), aP2 (1), adipsin (1), lipoprotein lipase (LPL) (1) or peroxisome proliferator activated receptor γ (PPARγ) (5). Cells were cultured in standard medium (Control, C), in the presence of vehicle alone (0.25% DMSO, D), in the presence of BRL48653 (BRL 0.005 to 5 μM), or in the presence of pioglitazone (PIO 0.025 to 25 μM).


PIO
BRL
Cont

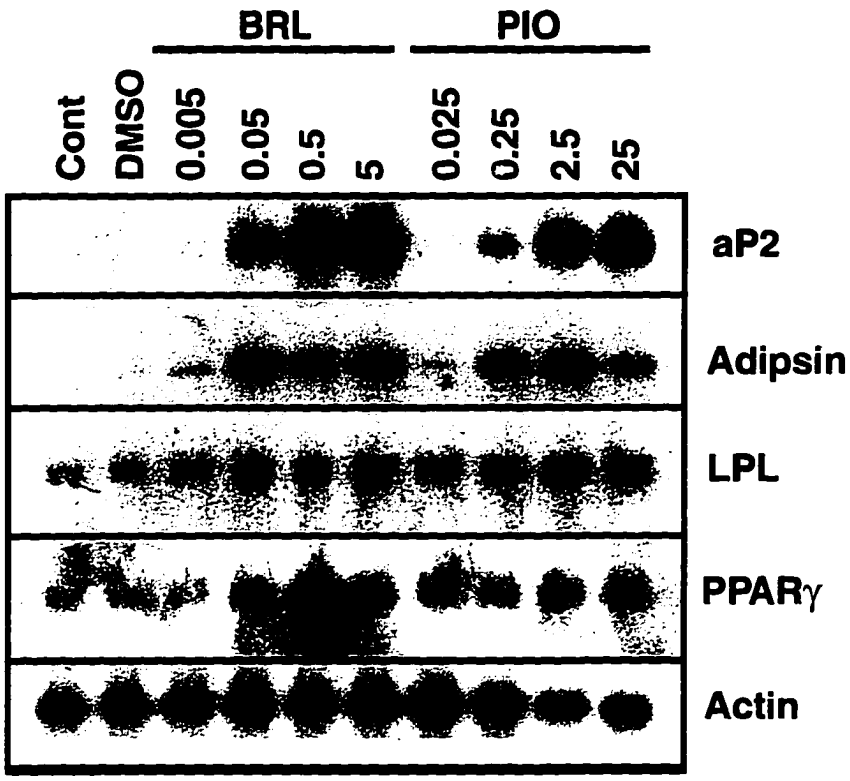

BRL
PIO
Cont
DMSO
0.005
0.05
0.5
5
0.025
0.25
2.5
25
aP2
Adipsin
LPL
PPARγ
Actin

FIGURE 5. ANTIBODY DETECTION OF THE RECOMBINANT PPARγ PROTEIN.

Equal aliquots of total cell lysates (51 μg) from transiently transfected 293T kidney cells were examined on Western blots probed with either the goat pre-immune antibody or the affinity- purified α-PPARγ antibody. Eukaryotic expression vectors contained the murine PPARγ2 full-length cDNA in the sense orientation or the murine PPARγ1 full-length cDNA in the antisense orientation. The empty pEF-BOS vector served as an additional control. Antibody complexes were detected using chemiluminescent reagents.

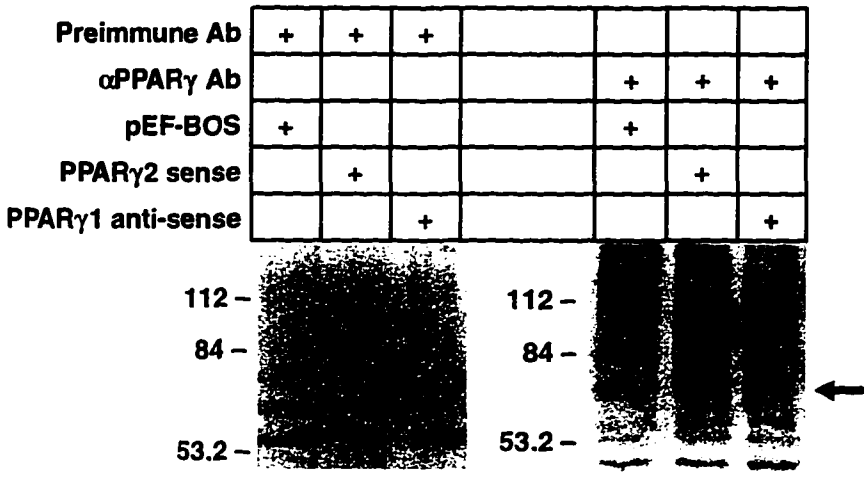

Preimmune Ab
αPPARγ Ab
pEF-BOS
PPARγ2 sense
PPARγ1 anti-sense
112 –
84 –
53.2 –
112 –
84 –
53.2 –

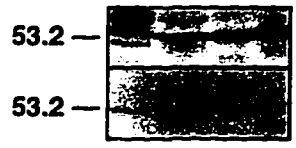
FIGURE 6. ANTIBODY DETECTION OF THE NATIVE PPARγ PROTEIN IN BONE MARROW AND ADIPOSE TISSUE.

A. Total tissue lysates were prepared from rat bone marrow (BM), brown adipose tissue (BAT) and white adipose tissue (WAT). Equal aliquots of tissue protein (124 μg) were examined on Western blots with the α-PPARγ antibody or pre-immune antibody and complexes detected using chemiluminescent reagents. Lysates (25 μg) from 293T cells transfected with the PPARγ2 expression construct served as a control. The arrows indicate the PPARγ specific protein of approximately 63 kDa.

B. Western blots prepared with rat bone marrow protein lysates were examined with the anti-PPARγ antibody in the absence or presence of increasing μg concentrations of the multiple antigenic peptide antigen (MAP). The arrow indicates the PPARγ specific protein complex.

6A

Rat Brown Adipose	+			
Rat White Adipose		+		
Rat Bone Marrow			+	



6B

Rat Bone Marrow	+	+	+
αPPARγ	+	+	+

Title: Peroxisome Proliferator Activated Receptors act in Concert with the Co-regulators SMRT (Silencing Mediator for Retinoid and Thyroid-hormone Receptors) and hSRC-1 (human Steroid Receptor Coactivator) to Regulate Transcription from the Murine Lipoprotein Lipase Promoter.

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Running Title: PPAR Regulation of LPL Transcription

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Key Words: Adipocytes, Bone Marrow Stroma, Lipoprotein Lipase, Peroxisome Proliferator Activated Receptors, Steroid Receptors, Thiazolidinediones, Transcription

Footnote: This work is submitted as partial fullfillment of the Ph.D. thesis requirement for C.E.R. in the Department of Zoology, University of Oklahoma.

ABSTRACT

Lipoprotein lipase (LPL) gene expression is complex and reflects the influence of multiple tissue-specific transcription factors. The novel steroid receptors, the peroxisome proliferator activated receptors (PPARs), and their ligands induce adipocyte differentiation accompanied by increased LPL mRNA levels. The coregulators of steroid receptor action SMRT (silencing mediator for retinoid and thyroid-hormone receptors) and SRC-1 (steroid receptor coactivator-1) are thought to help regulate nuclear receptor action in a positive or negative fashion. Based on this and the identification of a putative PPAR recognition element conserved within the murine and human LPL promoters, we examined the effect of each PPAR protein on LPL transcription in cotransfection experiments and the ability of SMRT and mSRC-1 to influence this effect. Using a full-length (-1824/+187 bp) murine LPL promoter/luciferase reporter construct, the PPARα/RXRα heterodimer increased transcription by 2-3 fold activation while PPARδ alone inhibited the reporter construct below baseline levels. Based on deletion analyses, the PPARα recognition element fell between -564 to -181 bp and was subject to negative regulation. The PPARγ2/RXRα heterodimer induced transcription by 13-fold from the full-length promoter in the absence of exogenous ligand; further increases were observed with thiazolidinedione compounds. Increasing addition of SMRT vector decreased the activation by the PPARγ2/RXRα heterodimer to baseline levels in a dose-dependent manner. With increasing amounts of SRC-1 vector, no increase in the level of activation by the PPARγ2/RXRα heterodimer was observed. However, addition of

SRC-1 to PPARγ2 in the presence of ligand activated the LPL promoter to the level observed with the PPARγ2/RXRα heterodimer. The addition of ligand to the PPARγ2/RXRα heterodimeric complex increased activation 2.5 fold over the PPARγ2/RXRα heterodimer alone. Addition of SRC-1 to this system did not further increase activation. Based on deletion and mutation analyses as well as electromobility shift assays, the PPARγ2 DNA recognition site was localized to a polypyrimidine tract between -171 bp and -156 bp. Upon binding, the PPARγ2/RXRα protein heterodimer causes distortion of the DNA. This DNA recognition element is conserved between the murine and human promoters and resembles the polypyrimidine sequences identified as single stranded DNA binding sites within the promoters of the adipsin and c-*myc* genes. These data support a role for PPAR proteins and the coregulators SMRT and SRC-1 in the regulation of LPL transcription.

INTRODUCTION

The enzyme lipoprotein lipase (LPL: EC 3.1.1.34) is responsible for the hydrolysis of triglycerides into free fatty acids and is necessary for the clearance of chylomicrons from the bloodstream (36,37). In mice where the LPL gene has been inactivated by homologous recombination, death occurs during the neonatal period due to a massive hypertriglyceridemia (21). Inborn errors of metabolism reveal similar defects in man; hereditary deficiencies in LPL activity cause increased morbidity due to cardiovascular disease (36). Lipid-lowering agents such as the fibrate compounds are used in the treatment of these disorders (36,119).

The LPL gene is subject to transcriptional regulation (37) and serves as one of the earliest markers of adipocyte differentiation, exhibiting increased mRNA levels less than 4 hours after cell exposure to adipogenic agonists (26). Both the human and murine LPL genomic genes have been cloned (28,38,58,70,153)and these sequences exhibit >65% identity within the initial 1.5 kb of their 5' flanking regions (58). This degree of evolutionary conservation suggests that important *cis*-acting DNA elements exist within the promoter region (58). *In vivo* and *in vitro* studies support this hypothesis. Analysis of transgenic mice has shown that the -1824 bp to +187 bp region of the murine LPL promoter is required to drive the correct tissue specific expression of a luciferase reporter gene; shorter regions of the LPL promoter were not sufficient (44). *In vitro* transfection studies have demonstrated the importance of a number of positive transcription factors in the expression of the LPL gene. These

include the octamer binding proteins, the CAAT box binding protein NF-Y, and the hepatic nuclear factor-3/forkhead family of proteins, all of which bind directly to the LPL promoter and increase transcriptional rates (25,38,101,113). In contrast, a silencer element has been identified between -169 bp and -152 bp of the human LPL promoter which is bound by unidentified nuclear proteins (131). This DNA sequence is evolutionarily conserved and consists of a polypyrimidine "coding" strand and its polypurine "non-coding" complement. Proteins responsible for the silencing activity and binding specifically to the "non-coding" polypurine sequence were detected in nuclear extracts from HeLa cells, a cell which does not express the LPL gene constitutively (131).

Other transcription factors have been implicated as direct or indirect regulators of LPL transcription. Of particular interest are the peroxisome proliferator activated receptors, novel members of the steroid receptor gene superfamily (reviewed in (50,120)) which include PPARα (61), PPARδ (2,34,73,118)and PPARγ (17,136,154). The PPARs act as transcriptional regulators, forming heterodimers with the retinoic acid X receptors (RXR) proteins and binding to DNA elements based on the direct-repeat 1 (DR-1) sequence "TGACCTnTGACCT" common to many steroid receptors (73,135,140). Ligands for the PPARs induce both LPL mRNA levels and adipogenesis in pre-adipocyte models (40,47,54,56,59,72,74,82,137,150). These ligands include natural compounds, such as prostaglandin J and long chain fatty acids, as well as synthetic lipid-lowering drugs, such as the fibrates and thiazolidinediones. Recently, indomethacin and other non-

steroidal anti-inflammatory drugs have been shown to activate PPAR γ2 and α (81). In representative cell models, adipogenesis has been associated with increased PPARγ2 (12,15,16,47,136,137,148), PPARα (15,148), and PPARδ (2,59) mRNAs levels, suggesting that each of these proteins may regulate LPL expression.

Evidence is accumulating that nuclear hormone receptors are further regulated in their actions by molecules that act as coactivators and corepressors of their function (see (57) for reviews). SMRT (silencing mediator for retinoid and thyroid hormone receptor) (18) was isolated by yeast two-hybrid screening using an unliganded hRXRα ligand binding domain fusion protein as bait. It is considered a corepressor protein because it interacts with unliganded nuclear hormone receptors to transmit a repressive signal to the transcriptional apparatus. It is thought that binding of ligand to the nuclear receptor results in dissociation of the corepressor. When this occurs, proteins known as coactivators of steroid receptor function such as SRC-1 (104) are able to bind to the nuclear receptor and enhance transcription. SRC-1 was isolated by a yeast two-hybrid screening of a human cDNA library using the ligand binding domain of the human progesterone receptor as bait (104). It is widely distributed among different cell types and enhances the activity of ligands that are bound by nuclear hormone receptors. Recently, murine SRC-1 (mSRC-1) was cloned (155). It was demonstrated to act as a coactivator to PPARγ activation of a PPAR response element (155) in the presence of ligand.

We noted that the polypyrimidine sequence between -169 bp to -152 bp of the murine

and human LPL promoters exhibited similarity to the consensus PPAR recognition element identified in the promoters of other adipocyte-inducible genes (131,135). Based on this observation and the fact that PPAR ligands induce LPL expression, we set out to determine whether the PPAR proteins and their ligands directly regulated transcription from the murine LPL promoter using a cotransfection approach. Upon confirmation of PPARγ2 regulation of the LPL promoter, we devised experiments to determine the role that the coregulators of nuclear hormone receptor function SMRT and SRC-1 play in the activation of the LPL promoter.

MATERIALS AND METHODS

Materials: All reagents were obtained from Sigma Chemical Co. (St. Louis MO) or Fisher Scientific (Dallas TX) unless noted otherwise. Oligonucleotides were synthesized by Dr. Ken Jackson, Oklahoma Center for Molecular Biology, Oklahoma City OK.

Plasmid Constructs: Constructs containing varying segments of the wild type murine LPL promoter linked to the luciferase reporter gene were prepared in the p19Luc vector (provided courtesy of D. R. Helinski, UCSD) (32) as previously described (44). Mutations in the promoter were introduced by PCR using the following specific primers anchored at the -181 bp relative to the transcription start site:

(MUT1) TTTGTCGACGCTTTCCTTAAAAAAATTTCCCCTTCTT

(MUT2) TTTGTCGACGCTTTCCTTCCTGAAAAAAACCCTTCTTCTCG

(MUT3) TTTGTCGACGCTTTCCTTCCTGCCCTAAAAAAATCTTCTCGCTGG

(MUT4)

TTTGTCGACGCTTTCCTTCCTGCCCTTTCCAAAAAAACTCGCTGGCACC

(MUT5)

TTTGTCGACGCTTTCCTTCCTGCCCTTTCCCCTTAAAAAAACTGGCACCGTT

G

Each PCR reaction was conducted with the following oligonucleotide anchored at +187 bp relative to the transcription start site:

TTTGGTACCCCCTTCTGCTTTGCTGCTGG.

The resulting PCR products were subcloned into pBluescript SKII, sequenced with Sequenase 2 (Amersham, Arlington Ht. IL) and subsequently subcloned into the SalI/KpnI site of the p19Luc vector.

Eukaryotic expression vectors were prepared by excising the murine PPARα, PPARγ2 and PPARδ (82)and RXRα cDNAs (kindly provided by Dr. Ron Evans, Salk Institute) (87) from their original vectors, ligating the fragments to BstXI linkers and subcloning these products into the BstXI site of the pEF-BOS vector (94).

Bacterial expression constructs were prepared using the M13mp18 derived pET-11cHRAP30 vector (kindly provided by Ron and Joan Conaway, OMRF) (130). This vector contains a $(histidine)_6$ tag followed by a SalI/BamHI cloning site. The following primers were designed to prepare in-frame inserts of the PPARγ2 and RXRα cDNAs by PCR amplification; the cDNA coding sequences are underlined:

(PPAR-1) GCAACGTCGACATGGGTGAAACTCTGGGAGA ;

(PPAR-2) GCAGCCCCGGGTCACTAATACAAGTCCTTGTAGAT;

(RXR-1) GCAACGTCGACATGGACACCAAACATTTCCT;

(RXR-2) GCAGCAGATCTTCACTAGGTGGCTTGATGTGG.

The PCR products were then digested with appropriate enzymes (PPARγ2, SalI/SmaI; RXRα, SalI/BglII) prior to subcloning into the SalI/BamHI site of the vector.

Transient Transfections: The human embryonic kidney cell line, 293T (49) (obtained courtesy Kenji Oritani, OMRF), was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Hyclone, Logan UT), penicillin 100 units/ml and streptomycin 100µg/ml. A total of 8 X 10^4 cells in a 2 ml volume were plated in 35 mm dishes 18 hours prior to transfection. Calcium phosphate/DNA co-precipitates (117) were prepared by mixing a total of (LPL reporter + receptor expression vector + empty pEF-BOS vector) up to 24 µg of DNA in 1/10 TE (1mM Tris-HCl (pH 8.0); 0.1 mM EDTA (pH 8.0)), adding 23 µl of 2M $CaCl_2$ with 183 µl of 2X HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2PO_4, 12 mM dextrose, 50 mM HEPES pH 7.05) 25 minutes prior to equal additions to two 35 mm plates. Following an overnight incubation, the cells were fed with fresh medium, incubated an additional 24 hours and harvested in a 100 µl volume of 25 mM glycylglycine, 15 mM $MgSO_4$, 1 mM dithiothreitol and 1% Triton X-100 (84). Protein concentrations were determined by the bicinchonic acid method (Pierce, Rockford IL) and adjusted to 3.5 µg/µl. Luciferase assays were performed over a 20 second period using a 25 µl (87.5 µg) aliquot of protein and 100 µl of reaction buffer (0.5 mM D-luciferin, 2.5mM ATP, 7.5 mM $MgSO_4$, 100 mM KH_2PO_4) in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego CA) as previously described (58).

Ligand treatment of transient transfections: Ligands were obtained from the following sources: BRL49653 and pioglitazone from Glaxo/Wellcome; Wy14653 (4-

chloro-6-(2,3-xylidino)-2pyrimidinylthio acetic acid), 9-*cis* retinoic acid (9-Cis RA) and ETYA (5,8,11,14-eicosatetraynoic acid from Biomol (Plymouth Meeting, PA); fenofibrate (FF) and docosahexanoic acid (DHA) from Sigma. Each ligand was prepared as a concentrated stock in dimethyl sulfoxide (DMSO). The 293T cells were transfected in bulk (4 X 10^5 cells/10 cm plate, in triplicate) with the full length LPL reporter construct and both the PPARγ2 and RXRα expression constructs. One day after transfection, the cells were released by trypsin digestion, pooled, and plated at equal cell numbers in 96 well plates. Control wells were treated with vehicle (DMSO) alone. Treatment of the -1824 to +187 LPL promoter/luciferase reporter construct resulted in no activation over baseline activation of the construct in the absence of added ligand or receptor constructs (data not shown). Duplicate experimental wells were treated with varying concentrations of each ligand; a minimum of n = 3 studies were performed.

His-tagged protein expression and purification: Recombinant PPARγ2 and RXRα his-tagged proteins were expressed and purified according to the method of Tan et al (130). The *E.coli* strain JM109(DE3) was cultured to a OD_{600}=0.6 in LB broth, inoculated with cells infected with the M13 constructs and incubated an additional 3 hours. The cultures were then induced with 0.4 mM isopropyl-1-thio-β-D-galactoside (IPTG) prior to an additional 7 hour incubation. Cells were collected by centrifugation at 1500 X g, 4°C, resuspended in sonication buffer (20% sucrose, 40 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM PMSF, 1.25 mg/ml lysozyme), and incubated 60 min on ice. After a 30 sec sonication, the cell supernatant was collected by

centrifugation for 20 min at 2000 X g, 4°C, loaded on a TALON (Clonetech, Palo Alto CA) column, washed with sonication buffer and eluted with single column volume step gradients containing 2, 15 and 80 mM imidazole. The protein purification was followed by western blot using goat polyclonal antibodies directed against the C terminal peptides of the PPARγ and RXRα proteins; the PPARγ antibody and the immunoblotting methods have been described previously (47). Routinely, the his-tagged proteins eluted in the 15 mM imidazole fractions.

Electromobility shift assays (EMSA): The DNA electromobility shift assays were performed using a 67 bp HindIII/SspI DNA fragment spanning bp -181 to -113 of the LPL promoter or complementary oligonucleotide primers based on the same sequence between bp -181 and -145. DNA labeling was performed using T4 polynucleotide kinase and γ^{32}P d-ATP (ICN, Irvine CA). Probes were labeled to a specific activity of 10^5-10^6 cpm/pmol. Reactions were conducted in a 30 µl volume containing 10 mM Tris pH8.0, 0.1 M KCl, 0.05 % NP40, 1 mM DTT, 6% glycerol, 1 ng PPARγ, 0.2 ng RXRα, and 2-10 X 10^5 cpm of probe for a 20 min period at room temperature. Volumes were immediately separated on a 5% acrylamide/bis-acrylamide (24:1) gel by electrophoresis at 100 v for 3 hours. The gels were dried at 80°C for 90 min and exposed on Kodak XAR film for 18 hours without an intensifying screen.

The following complementary oligonucleotides for the coding (c) and non-coding (nc) strands were used as competitors in these reactions:

IL6 C/EBP (c) TAAACGACGTCACATTGTGCAATCTTAA

IL6 C/EBP (nc) TTAAGATTGCACAATGTGACGTCGTTTA (1)

IL6 NFκB (c)AAATGTGGGATTTTCCCATGAG

IL6 NFκB (nc) CTCATGGGAAAATCCCACATTT (122)

LPL (c) GCTTTCCTTCCTGCCCTTTCCCCTTCTTCTCGCTGG

LPL (nc) CCAGCGAGAAGAAGGGGAAAGGGCAGGAAGGAAAGC (58)

PPRE (c) AGCTACCAGGTCAAAGGTCACGT

PPRE (nc) AGCTACGTGACCTTTGACCTGGT (40)

Circular permutation experiments: The 67 bp HindIII/SspI fragment of the LPL promoter spanning -181 bp to -113 bp was blunted by reaction with the Klenow fragment of DNA polymerase, ligated to XbaI linkers and subcloned into XbaI site of the pBend2 vector (provided courtesy Dr. C. Webb, OMRF) (65). Equal sized restriction fragments were prepared with the following enzymes: BglII, XhoI, EcoRV, SspI and BamHI. Electromobility shift assays using the recombinant PPARγ2/RXRα protein heterodimers were performed with each fragment and the mobility of the bound complex measured relative to that of the free probe. The degree of DNA distortion (α) was estimated based on the equation $\mu_M/\mu_E = \cos(\alpha/2)$ where μ_M and μ_E are the mobilities of the protein/DNA complex with the protein located at the middle ($_M$) or end ($_E$) of the DNA fragment (133).

RESULTS

Two members of the PPAR family activate the LPL promoter: Our initial experiments set out to determine if any member of the murine PPAR family (α,γ2, δ), either alone or in combination with RXRα, regulated transcription from the full length (-1824 bp to +187 bp) LPL promoter. The human embryonic kidney cell line, 293T, was chosen for its ease of transfection and the absence of LPL expression in its tissue of origin (44). Cotransfection with expression constructs for the different PPAR and/or RXRα expression vectors revealed the following pattern of LPL transcriptional regulation. The PPARγ2/RXRα heterodimer induced the highest transcriptional rate, inducing the luciferase reporter gene by 13-fold over baseline (**Figure 1**). Likewise, cotransfection with either PPARγ2 alone or PPARα/RXRα resulted in statistically significant induction's of approximately 2-fold over baseline. No other expression construct significantly altered LPL transcription, with the exception of PPARδ which reduced expression of the luciferase reporter by approximately 60% relative to baseline.

Addition of exogenous ligands increase activation of the full-length LPL promoter by the PPARγ2/RXRα heterodimer: The next experiments were designed to ask if the addition of exogenous PPAR ligands would further increase transcriptional activation by the transfected PPARγ2/RXRα heterodimer of the LPL promoter. To control for equivalent transfection efficiency, cells were transfected in bulk with the full-length promoter construct and the PPARγ2/RXRα expression

constructs prior to exposure to varying concentrations of exogenous ligands (**Figure 2**). In the absence of any added ligand, the luciferase expression in this system was already 13-fold greater than the baseline described in **Figure 1**. The addition of the thiazolidinedione compounds BRL49653 or pioglitazone resulted in a further 2.5-fold activation. The presence of ETYA, an arachidonic acid analog, caused a smaller (1.3-fold) but significant increase. However, fenofibrate, docosahexanoic acid and Wy 14643 did not change the level of luciferase expression. In contrast, the addition of 9-*cis* retinoic acid, the RXRα ligand, significantly decreased expression levels by over 50%. Preliminary experiments determined that there was only a slight activation of PPARα/RXRα by the compounds fenofibrate and docosahexanoic acid (data not shown) and no activation of PPARδ/RXRα by the compounds BRL 49653, fenofibrate, ETYA, Wy 14643, or carbacyclin (data not shown).

Deletion analysis of the LPL promoter localizes the sites of PPARα and PPARγ2 heterodimer binding: To localize the sites of PPAR recognition elements and to determine if negative regulatory elements might be present, we analyzed a series of luciferase reporter constructs with progressive deletions of the LPL promoter between -1824 bp and -101 bp 5' to the transcription start site (**Figure 3**). Each reporter construct was transfected alone or cotransfected with the appropriate eukaryotic expression constructs for the individual PPAR/RXRα heterodimers. Deletion of the LPL promoter between -1824 and -181 did not alter the high level of PPARγ2/RXRα activation. However, the deletion between -181 and -101 bp significantly reduced the level of PPARγ2/RXRα induction from 14-fold to 2-fold

relative to baseline (**Figure 3 A**). In contrast, activation by the PPARα/RXRα heterodimer increased with deletions between -1824 and -564 bp and decreased with further deletions to -181 bp (**Figure 3 B**). Independent of the length of the promoter construct, the PPARδ/RXRα heterodimer consistently had no significant effect on luciferase levels (**Figure 3 C**). These findings suggest that at least two members of the PPAR family recognize unique DNA elements within the LPL promoter and that both positive and negative *cis*-acting regulatory elements exist.

EMSA and transfection analyses identify the PPARγ2 DNA recognition element: Since the greatest activation of the LPL promoter was observed with the PPARγ2/RXRα heterodimeric complex, further experiments were designed to determine the site of PPARγ2/RXRα DNA binding and no further experiments were performed with PPARα or PPARδ. Electromobility shift assays were performed using a DNA probe spanning bp -181 to -113 of the LPL promoter and bacterially expressed, histidine tagged PPARγ2 and RXRα proteins (**Figure 4**). The presence of the RXRα protein alone did not shift the DNA probe while the PPARγ2 protein alone resulted in a weak signal of mobility shifted band "A". In contrast, both proteins together yielded a strong signal for two mobility shifted bands, "A" and "B". The binding activity demonstrated specificity. Double stranded oligonucleotide probes based on the IL6 NFκB (122)or IL6 C/EBP (1) binding sites failed to compete for protein binding. In contrast, double stranded oligonucleotide probes based on an optimal PPAR recognition element (40) or on bp -180 to -145 bp of the LPL promoter (58) competed for binding in a concentration dependent manner.

This region of the LPL promoter contains a polypyrimidine-rich sequence which shares features exhibited by the consensus PPAR recognition element. To define the PPARγ2/RXRα recognition site, we prepared a series of five overlapping mutations spanning -171 to -149 bp of the 5' flanking region (**Figure 5A**). In EMSA analyses, none of the mutations specifically competed for DNA binding except for that spanning bp -155 to -149 (Mutant E, **Figure 5 B**). In cotransfection analyses, the mutants decreased the level luciferase induction by PPARγ2/RXRα by 50-90% relative to the wild type control (**Figure 5C**). Together, these experiments localize the minimal PPARγ2/RXRα recognition element between -171 to -156 bp. However, additional flanking sequences remain important for transcriptional activity.

Circular permutation experiments reveal DNA distortion due to binding of the PPARγ2/RXRα heterodimer: Many DNA binding proteins regulate transcription by changing the DNA conformation. Experimentally, the presence of a DNA bend is reflected by altered electrophoretic mobility of the protein/DNA complex. Fragments migrate slower when the DNA recognition element is located at the center of the DNA fragment rather than the ends. We examined the mobility of the LPL promoter/heterodimer complex using circular permutation analysis using DNA fragments generated from pBend2 constructs (**Figure 6**). The extent of DNA distortion was estimated to be approximately 46° for the LPL promoter fragment (**Figure 6 A,B**). These results were comparable to circular permutation analyses of an optimal PPAR recognition sequence which displayed DNA distortion of

approximately 56° (**Figure 6 C,D**).

Coregulators of steroid receptor action modify the ability of PPARγ2/RXRα to activate the LPL promoter: To determine if coregulators of steroid receptor action interact with the PPARγ2/RXRα system, a corepressor protein SMRT (silencing mediator for retinoid and thyroid-hormone receptors) and a coactivator protein, steroid receptor coactivator (SRC-1) were expressed in 293T cells with PPARγ2 +/- RXRα and the -1824 LPL promoter/luciferase reporter construct with or without addition of ligand (**Figure 7**). To test the regulatory effect of the SMRT protein, cotransfections were performed with a constant concentration of the PPARγ2 and RXRα expression vectors as indicated, with increasing amounts of the SMRT (**Figure 7A**) expression vector with the -1824 LPL/luciferase reporter construct. The SMRT vector inhibited transcription from the LPL promoter to near baseline level of expression at the highest dose. This finding demonstrates that the SMRT protein disrupts the ability of PPARγ2/RXRα to activate the LPL promoter in the absence of added ligand. In experiments with hSRC-1, increasing concentration of hSRC-1 with a constant concentration of PPARγ2/RXRα in the absence of added ligand demonstrated no significant increase in the activation of the LPL promoter (data not shown). When PPARγ2 was transfected by itself in the presence of BRL 49653, a six-fold induction of the LPL promoter was observed (**Figure 7B**). This is approximately a 2.5-fold increase over the induction of the promoter by PPARγ2 by itself in the absence of added ligand (see fig. 1). When a DNA concentration of SRC-

1 twice that of PPARγ2 was added to the PPARγ2/BRL transfection the activation increased to 13-fold, the level of activation observed when PPARγ2 was transfected with RXRα alone in the absence of added ligand (**Figure 7B**). When ligand was added to the PPARγ2/RXRα transfection, a 30-fold induction was observed as in figure 2. Addition of SRC-1 to the BRL treated PPARγ2/RXRα transfection did not further increase activation. These experiments provide evidence of the ability of SRC-1 to enhance regulation of the LPL promoter by liganded PPARγ2.

DISCUSSION

The enzyme lipoprotein lipase plays a critical role in the clearance of plasma triglycerides. Adipocytes, macrophages, lactating mammary tissue and the central nervous system express the highest levels of this protein; other sites including the liver express the protein at lower levels. The complex tissue-specific regulation of the LPL promoter reflects the simultaneous input of multiple transcription factors (37). Several of these have been examined in some detail. The octamer binding proteins recognize the sequence ATTTGCAT which is perfectly conserved at bp -46 in the murine (58,153) and human (28,70) LPL promoters and bp-42 in the chicken (24) LPL promoter. This site and its immediate flanking sequences are critical for LPL transcription; when it is mutated, expression is reduced by 75% (25,113). Evidence suggests that the octamer protein regulates transcription initiation through protein/protein interactions with TFIIB, a component of the RNA polymerase II complex (101). The protein factor NF-Y recognizes the conserved CAAT box located at -66 bp in the murine and human LPL promoters and -68 bp in the chicken LPL promoters. Tumor necrosis factor, an inhibitor of LPL transcription, reduces binding of both NF-Y and the octamer binding proteins to their recognition elements in adipocytes (96). In contrast, exposure of macrophages to lipopolysaccharide, which inhibits LPL mRNA levels while inducing expression of tumor necrosis factor, increases binding of octamer binding proteins to the LPL recognition element (55). Additional proteins belonging to the hepatic nuclear factor 3/forkhead related activator family bind to DNA recognition elements located at approximately -669 bp

and -440 bp of the human LPL promoter (38,109). However, comparison across species reveals less conservation pressure at these sites and their role in the control of the murine LPL promoter remains unknown.

The current work extends these earlier studies on LPL transcription by demonstrating a role for both PPARγ2 and PPARα heterodimers in the regulation of the murine LPL promoter as well as examining the role of the coregulators SMRT and SRC-1 in modulating this effect. The actions of PPARγ2 may involve distortion or bending of the DNA. We also determine that the PPARα does not activate transcription as strongly as PPARγ2 and that PPARα may be subject to both positive and negative regulation. In contrast, we find that PPARδ does not activate the LPL promoter in the region from -1824 to +187 bp. Our findings confirm and extend those of Schoonjans et al. (120) who have recently reported that PPARγ binds to the highly conserved corresponding region of the human LPL promoter. Together, these findings provide a mechanistic explanation for the effect of thiazoldidinediones on LPL expression in bone marrow stromal cells and other pre-adipocyte models (47,120,136,137) and the ability of coregulator proteins to modify this effect.

Previous studies by Tanuma et al. (131) demonstrated that HeLa cell extracts bound the identical polypyrimidine region of the human LPL promoter. However, in their hands, the binding proteins acted as negative regulatory factors by binding to the single-stranded polypurine "non-coding" strand. Our preliminary experiments have failed to prove that the PPARγ2/RXRα heterodimer exhibits single-stranded DNA

binding activity (data not shown). However, single-stranded DNA binding activity has been attributed to at least one other steroid receptor, the estrogen receptor (80,97,98). Likewise, the sterol response element (GTGGGGTG), recognized by the transcription factor known as both "sterol response element binding factor 1" (SREBP-1) and "adipocyte determination- and differentiation-dependent factor 1" (ADD1) (66,126,138,149), is bound both as a single- and double-stranded DNA element by nuclear extract proteins (128). Other single-stranded DNA binding proteins include the heterogeneous nuclear ribonucleoprotein K (hnRNPK) (91), the nuclease-sensitive element protein-1 (NSEP-1)(75), and the zinc finger proteins Sp1 and ZF87/MAZ (31).

Transcription of genes by the RNA polymerase II complex requires assembly at the transcriptional start point of multiple factors that compose the initiation complex at the TATA box. While there is evidence that nuclear receptors themselves contact some of the factors of the initiation complex directly (4,19,60), it is thought that certain cofactors can form a bridge between a nuclear hormone receptor and the transcriptional apparatus. While the coactivator RIP 140 has recently been demonstrated not to bind TFIIB (14), the coactivator CBP/p300 does bind TFIIB (76). Evidence is accumulating that DNA bending by nuclear receptors is coupled to transcriptional induction (for review, see (102)). Thus, the stage is set for new findings involving nuclear hormone receptors and their coactivators activating the transcriptional apparatus through direct and indirect contact with members of the initiation complex through bending of the DNA to which they are bound.

Based on the current data, we can now add the PPARα and PPARγ2 proteins to the growing list of transcription factors which contribute to LPL regulation. A model outlining the location of known DNA/protein interaction sites within the LPL promoter is shown in **Figure 7**. The relative ratio of positive and negative regulatory proteins in a given cell may alter the DNA conformation and determine the level of LPL transcription and elongation. Clearly, the regulation of the LPL promoter is complex and is most likely defined by the tissue-specific expression of multiple transcription factors; it is unlikely that any single factor determines the level of LPL expression in all tissue sites.

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FIGURE LEGENDS

Figure 1. Activation of the lipoprotein lipase gene promoter by the peroxisome proliferator activated receptor family. Cultures of 293T cells (5 x 10^5/35 mm plate) were all transfected with 1 μg of the full-length (-1824/+187) LPL promoter/luciferase construct by calcium phosphate precipitation. Cells were additionally transfected with 4 μg each of pEF-BOS expression vectors containing either PPAR and/or RXRα and cultured in the presence of fetal bovine serum. Numbers at the ends of the bar graph indicate the fold-activation relative to the luciferase baseline activity in the absence of PPAR or RXRα expression constructs, defined as "1". Data is normalized relative to a constant protein concentration per assay (87.5μg) and represents the mean ± SE of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.

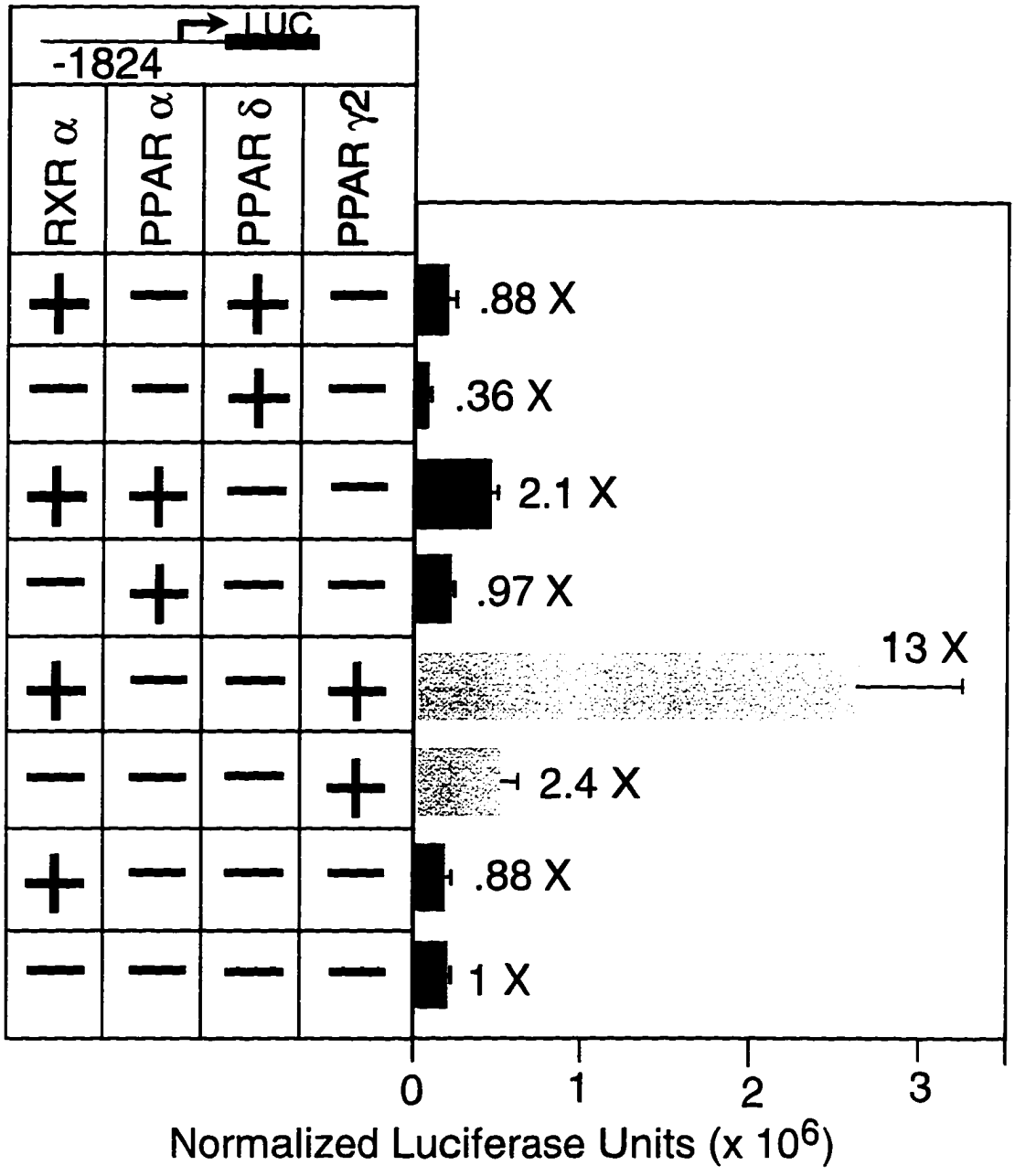

LUC
-1824
RXR α
PPAR α
PPAR δ
PPAR γ2
.88 X
.36 X
2.1 X
.97 X
13 X
2.4 X
.88 X
1 X
0
1
2
3
Normalized Luciferase Units (x 10^6)

Figure 2. Increased activation of the LPL promoter by treatment of PPARγ2/RXRα transfected cells with PPAR ligands. Cultures of 293T cells (2 x 10^6/100 mm plate) were transfected with 2.5 μg of the full-length LPL promoter/luciferase reporter construct and 10 μg each of PPARγ2/RXRα expression constructs in bulk and equal numbers of cells cultured in individual wells on a 24 well plate. Individual wells were treated with agents at the following concentrations: 9-*cis* retinoic acid (9-Cis RA), 1 μM; docosahexaenoic acid (DHA), 10 μM; fenofibrate (FF), 150 μM; pioglitazone (PIO), 10 μM; BRL49653 (BRL), 1 μM; 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (Wy14643), 10 μM; 5,8,11,14-eicosatetraynoic acid (ETYA), 50 μM. Data represents the mean ± SE of n = 3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.

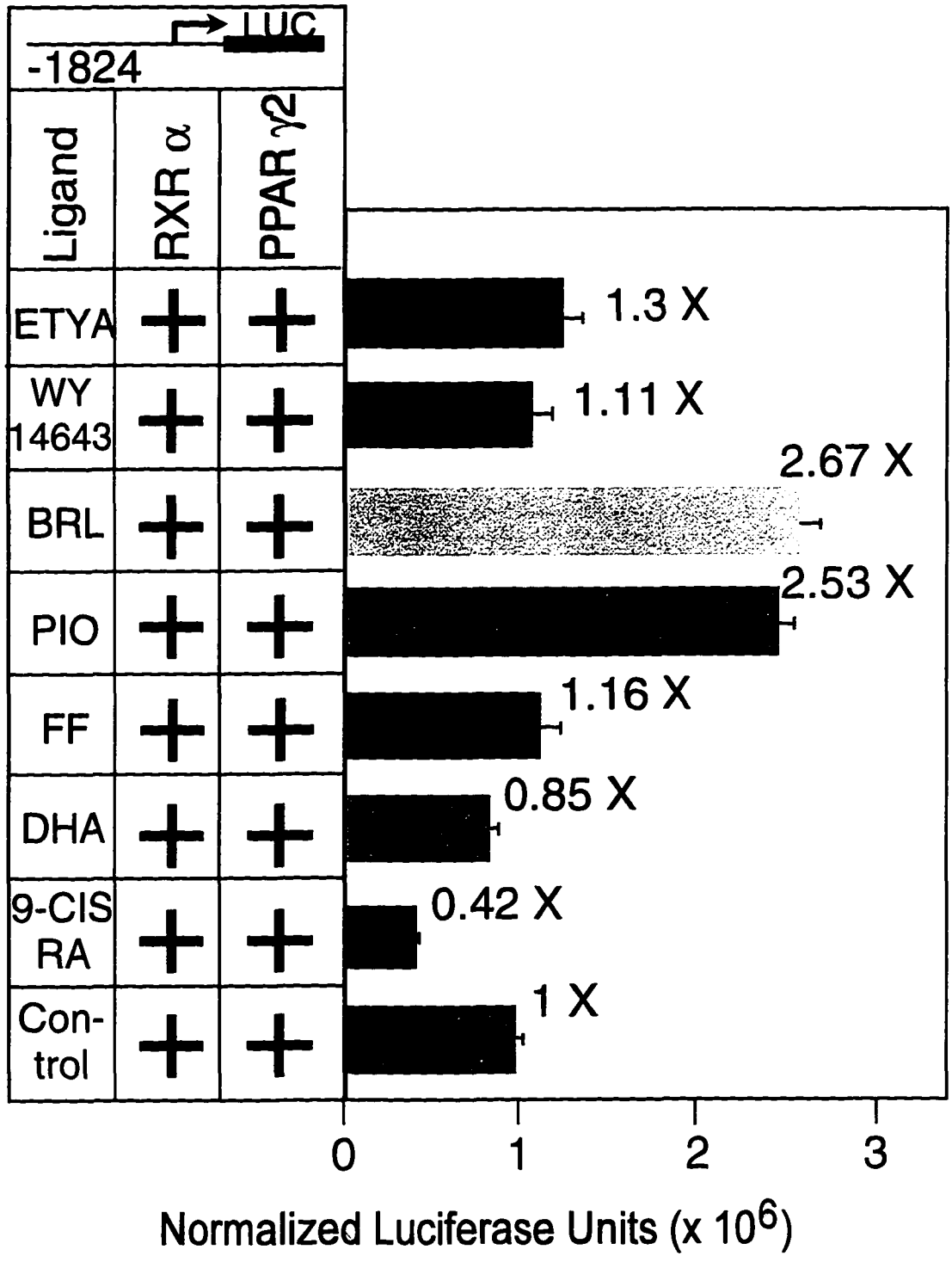
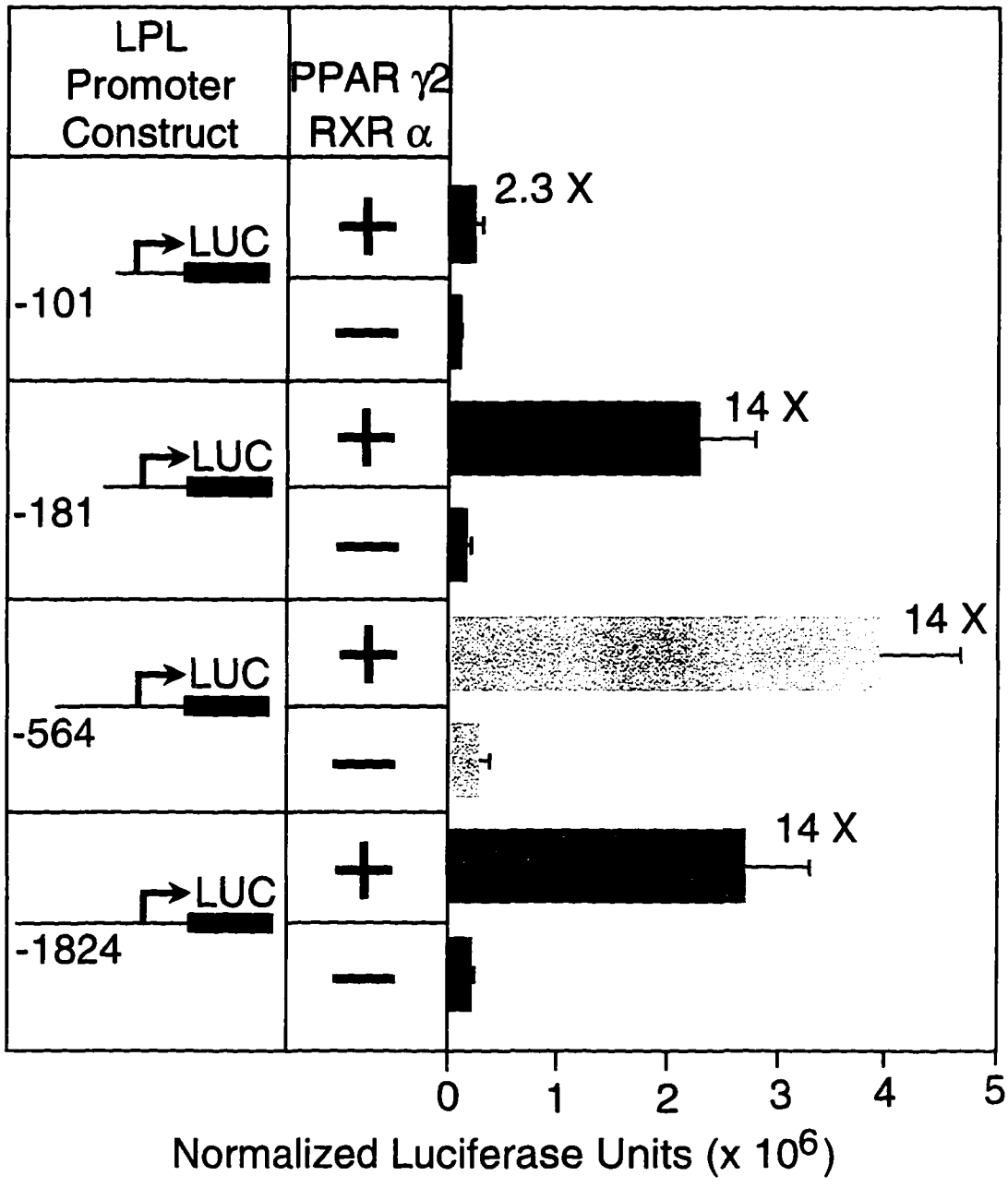
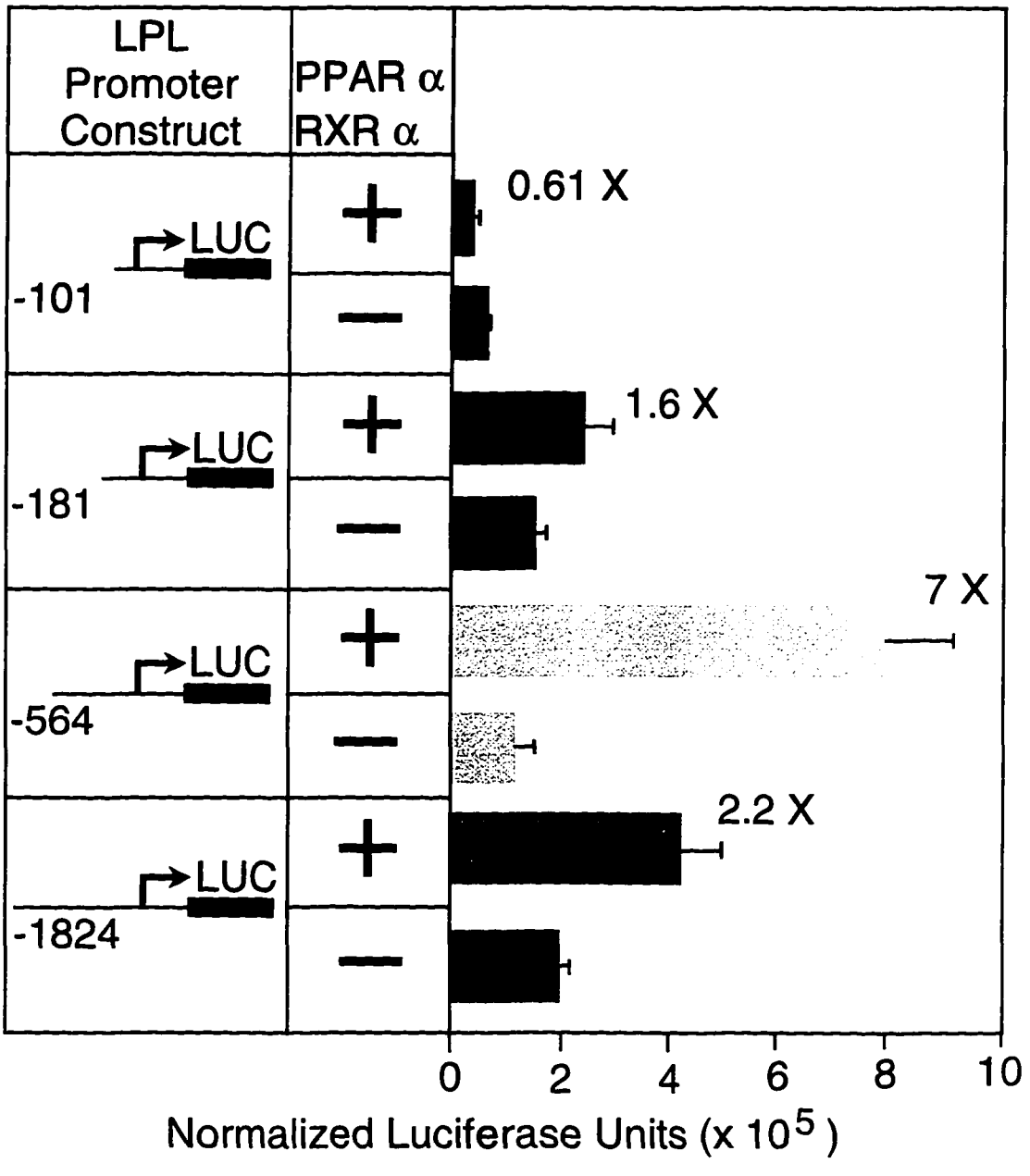

LUC
-1824
Ligand
RXR α
PPAR γ2
ETYA
WY 14643
BRL
PIO
FF
DHA
9-CIS RA
Con-trol
1.3 X
1.11 X
2.67 X
2.53 X
1.16 X
0.85 X
0.42 X
1 X
0
1
2
3
Normalized Luciferase Units (x 10^6)

Figure 3. Deletion analysis of the LPL promoter: activation by each PPAR heterodimer. 1 μg of deletion constructs linking from -1824 bp, -564 bp, -181 bp and -101 bp to +187 bp of the LPL promoter to the luciferase reporter gene were cotransfected into 293T cells (5 x 10^5/ 35 mm plate) in the absence or presence of 4 μg each of the RXRα and individual PPAR expression constructs. Cotransfections were performed with PPARγ2 **(A)**, PPARα **(B)**, and PPARδ **(C)** expression vectors. Results are normalized relative to protein concentration (87.5 μg/rxn) and represent the mean ± SE of n=3 experiments. The fold-induction was determined relative to the baseline activity of each reporter construct in the absence of RXR and PPAR expression constructs and is indicated by numbers at the end of the bar graphs. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.


LPL
Promoter
Construct
PPAR γ2
RXR α
LUC
-101
LUC
-181
LUC
-564
LUC
-1824
+
−
2.3 X
14 X
14 X
14 X
0
1
2
3
4
5
Normalized Luciferase Units (x 10^6)


LPL Promoter Construct
PPAR α
RXR α
LUC
-101
-181
-564
-1824
+
−
0.61 X
1.6 X
7 X
2.2 X
0
2
4
6
8
10
Normalized Luciferase Units (x 10^5)

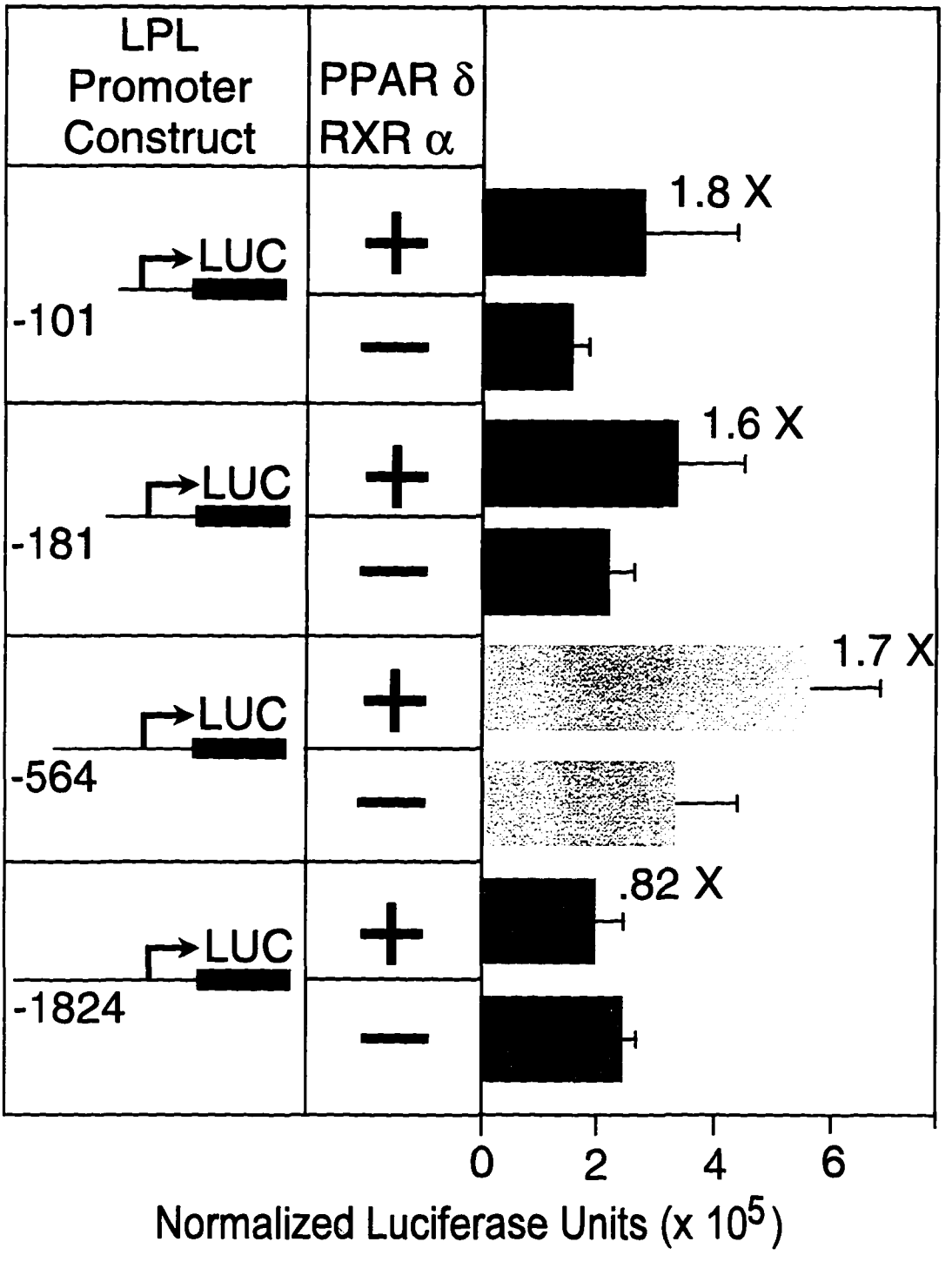

LPL Promoter Construct
PPAR δ RXR α
LUC
-101
+
−
1.8 X
LUC
-181
+
−
1.6 X
LUC
-564
+
−
1.7 X
LUC
-1824
+
−
.82 X
0
2
4
6
Normalized Luciferase Units (x 10^5)

Figure 4. EMSA analysis of the LPL promoter. A 67 bp fragment spanning -181 bp to -113 bp of the LPL promoter was used as a probe in EMSA experiments with recombinant RXRα and PPARγ2 proteins in the absence and presence of specific (LPL, PPREop) and non-specific (NfκB, C/EBP) double stranded oligonucleotide competitors. "Free" represents the free labeled probe while "A" and "B" represent the slow and fast migrating protein/DNA complexes. Representative of n =2 experiments.

RXRα	–	+	–	+	+	+	+	+	+	+	+	+
PPARγ2	–	–	+	+	+	+	+	+	+	+	+	+
LPL (ng)					16	3						
NFκB (ng)							16	3				
PPREop (ng)									16	3		
C/EBP (ng)											16	3

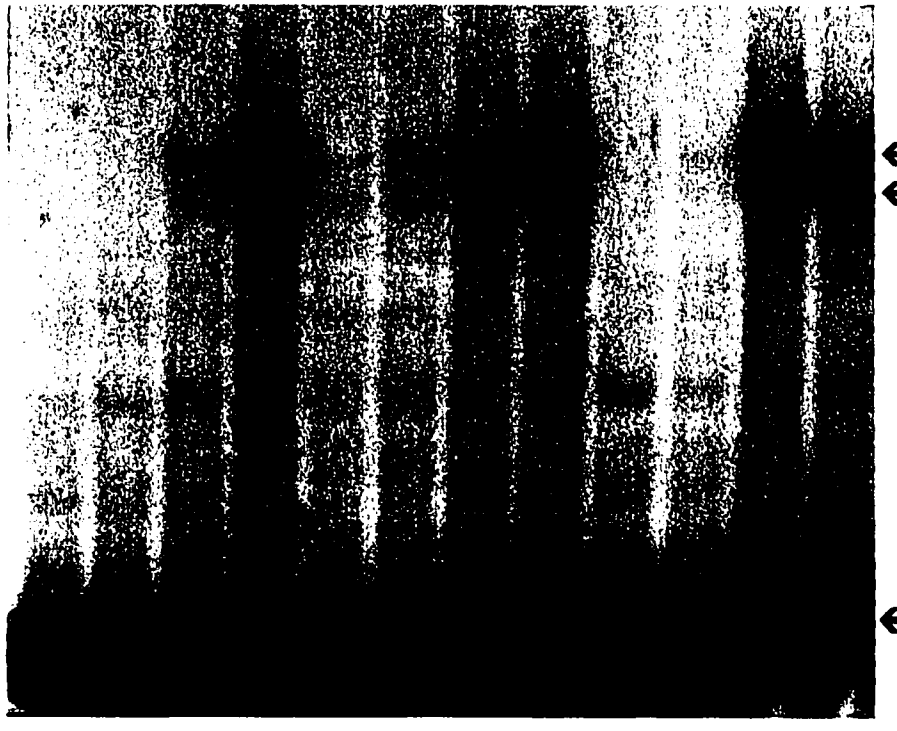
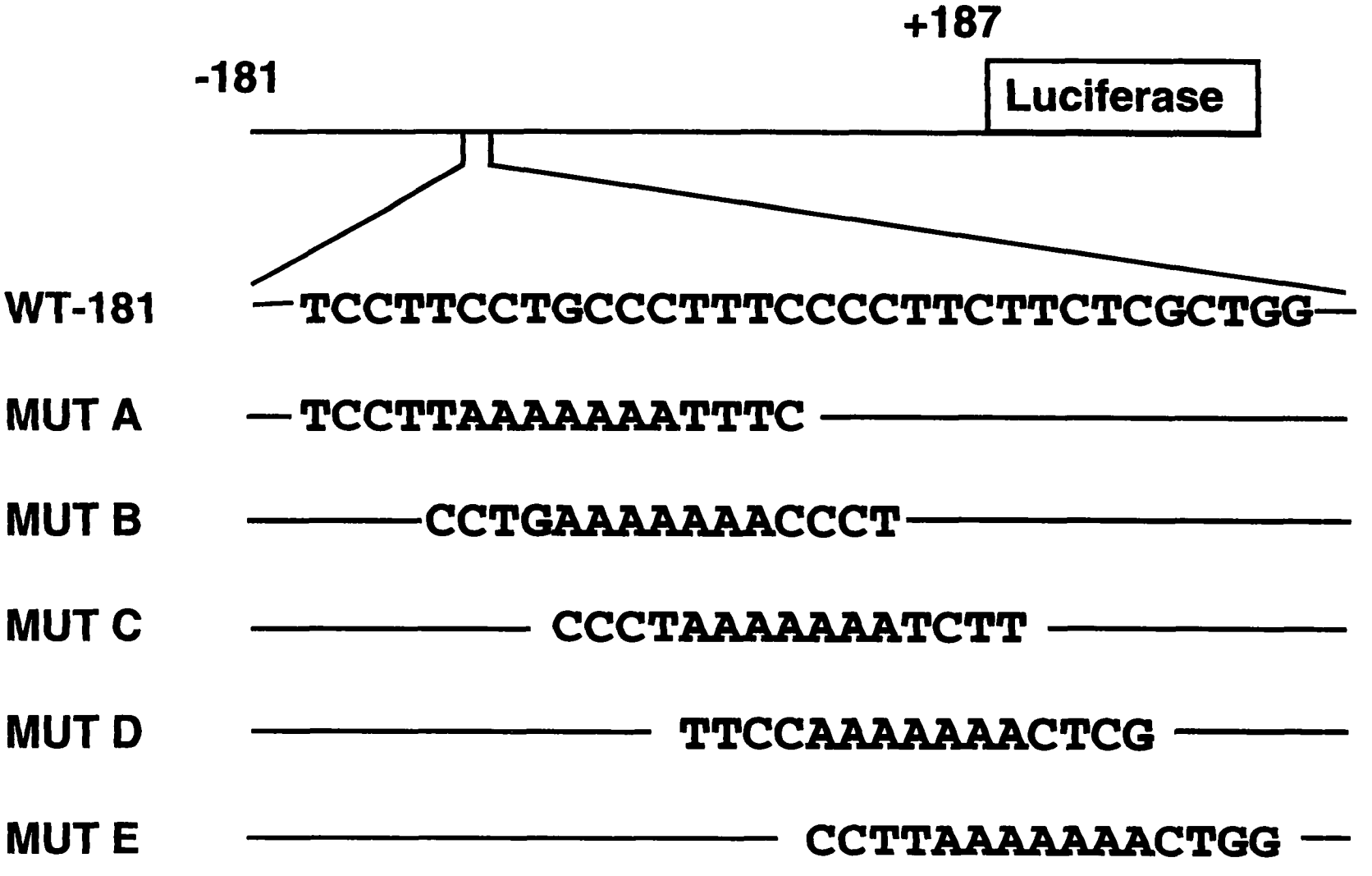
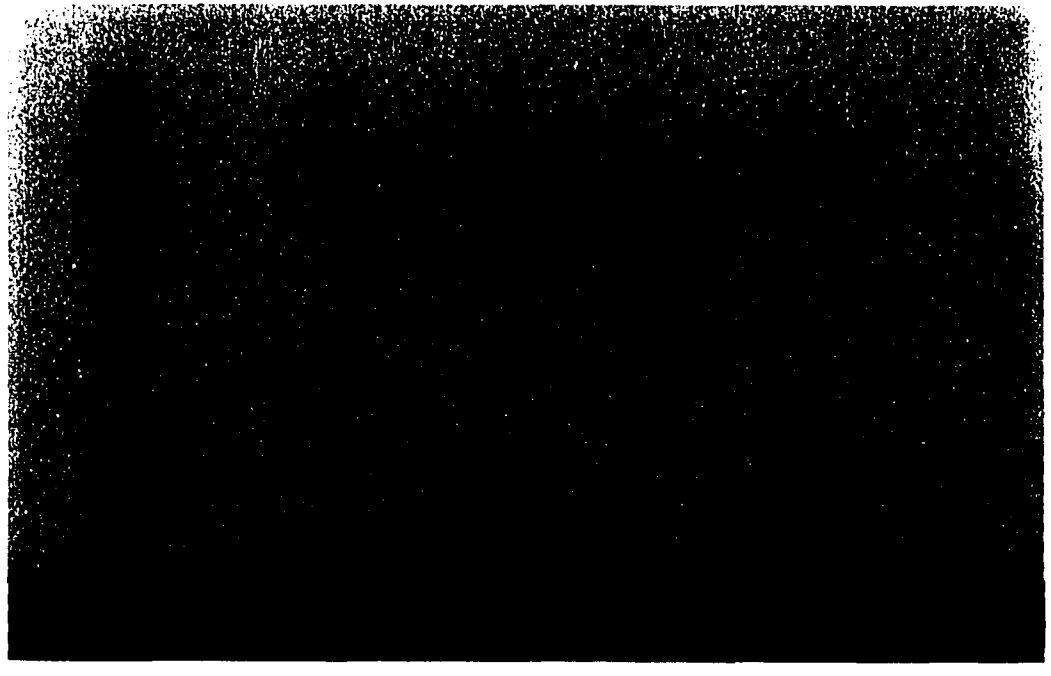


Figure 5. Effect of mutations of the PPARγ2 recognition element on EMSA and cotransfection assays. Mutations introduced into the LPL promoter spanning -171 bp to -149 bp are outlined in **(A)**. DNA fragments containing each of the mutations were used as competitors in EMSA assays performed using the 67 bp wild-type LPL promoter probe employed in Figure 4 **(B)**. Reporter constructs linking -181 bp to +187 bp of the LPL promoter to the luciferase reporter were prepared with either the wild type (WT) or mutant (MUT) DNA sequences. 1 μg of these were transfected in the absence and presence of 4 μg each of the RXRα and PPARγ2 expression constructs on 35 mm dishes containing 5 x 10^5 293T cells. Fold induction was determined relative to the baseline activity of each reporter construct in the absence of RXR and PPAR expression constructs. Data is normalized relative to protein concentration (87.5 μg/assay) and represents the mean ± SE of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed **(C)**.


+187
-181
Luciferase
WT-181
TCCTTCCTGCCCTTTCCCCTTCTTCTCGCTGG
MUT A
TCCTTAAAAAAATTTC
MUT B
CCTGAAAAAAACCCT
MUT C
CCCTAAAAAAATCTT
MUT D
TTCCAAAAAAACTCG
MUT E
CCTTAAAAAAACTGG

RXRα		+	+	+	+	+	+	+	+	+	+	+	+	+
PPARγ2		+	+	+	+	+	+	+	+	+	+	+	+	+
WT (ng)			20	100										
Mut A (ng)					20	100								
Mut B (ng)							20	100						
Mut C (ng)									20	100				
Mut D (ng)											20	100		
Mut E (ng)													20	100



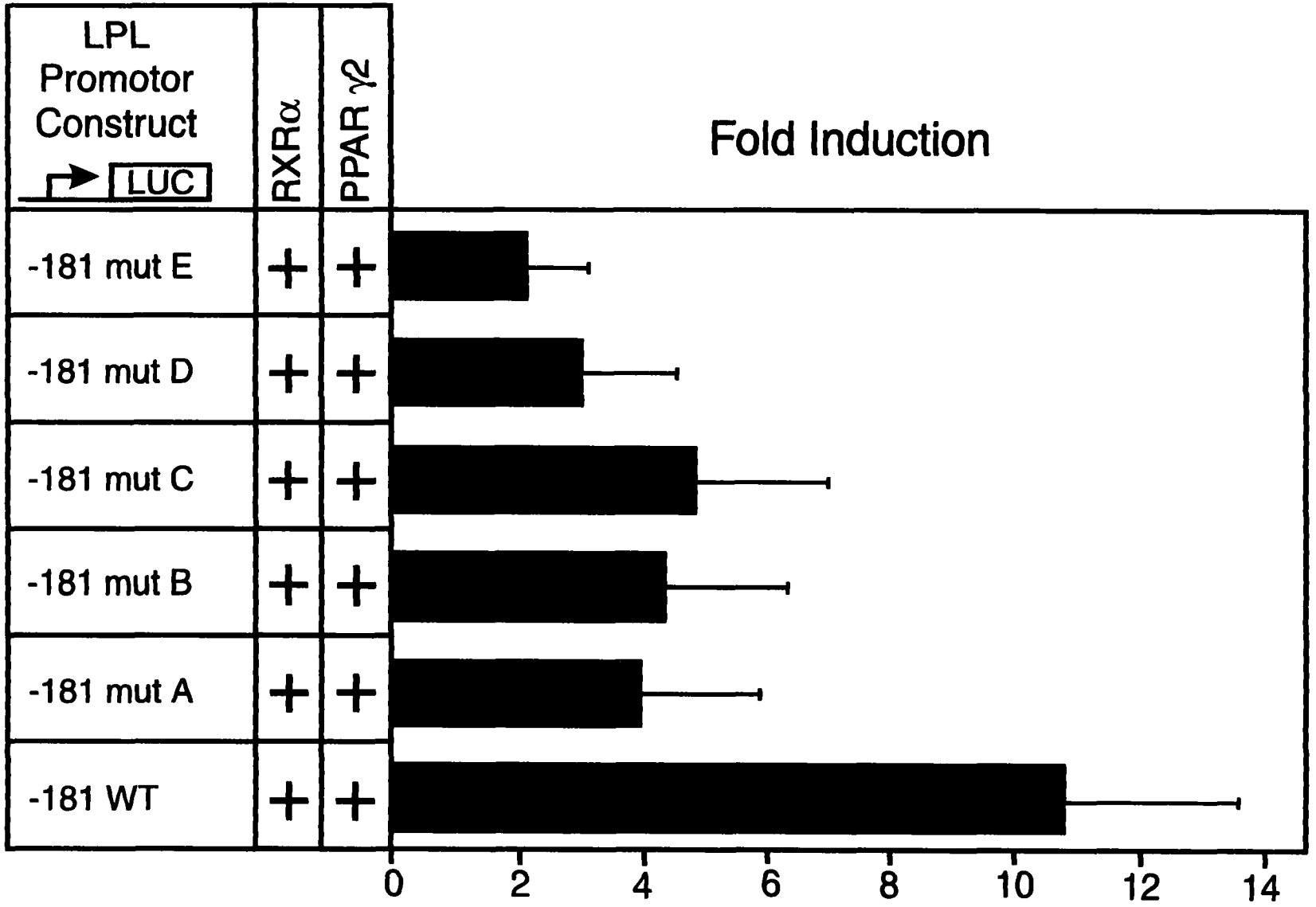
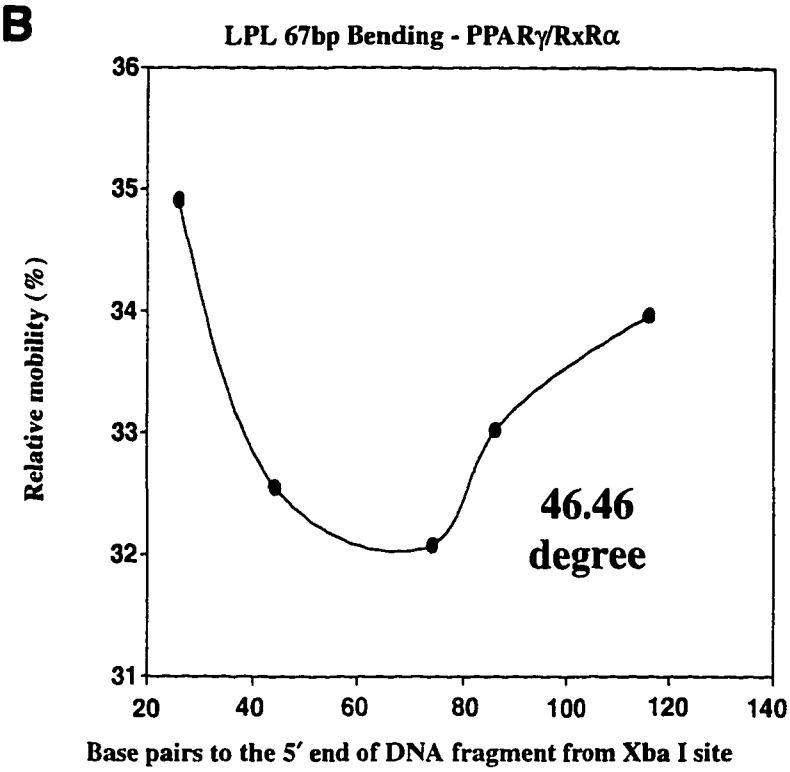

LPL
Promotor
Construct
LUC
RXRα
PPAR γ2
Fold Induction
-181 mut E
-181 mut D
-181 mut C
-181 mut B
-181 mut A
-181 WT
+
0
2
4
6
8
10
12
14

Figure 6. Circular permutation analysis of DNA distortion by PPARγ2/RXRα heterodimers. Circular permutation analysis was performed using the 67 bp LPL promoter and an optimal PPRE sequence subcloned into the pBend2 vector. Restriction digests with a battery of enzymes generated a single sized DNA fragment in which the protein recognition site was located at varying distances from the ends. The resulting DNA/protein complexes were analyzed by EMSA **(A & C)** and the relative mobility's examined **(B & D)**.

6A

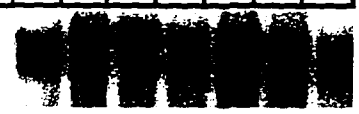
LPL P-bend	+	+	+	+	+
PPAR + RxR	+	+	+	+	+
Bam HI					+
Ssp I				+	
Eco Rv			+		
Xho I		+			
Bgl II	+				

6B



6C

PPREop-P-bend	+	+	+	+	+	+	+
PPAR + RxR	+	+	+	+	+	+	+
Bam HI							+
Rsa I						+	
Ssp I					+		
Eco Rv				+			
Xho I			+				
Spe I		+					
Bgl II	+						



6D

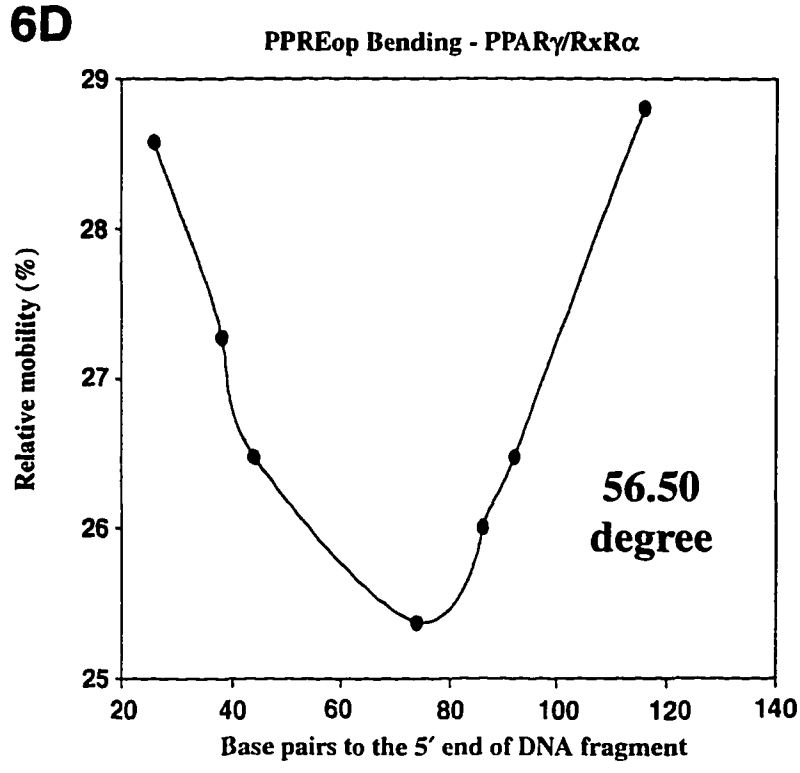
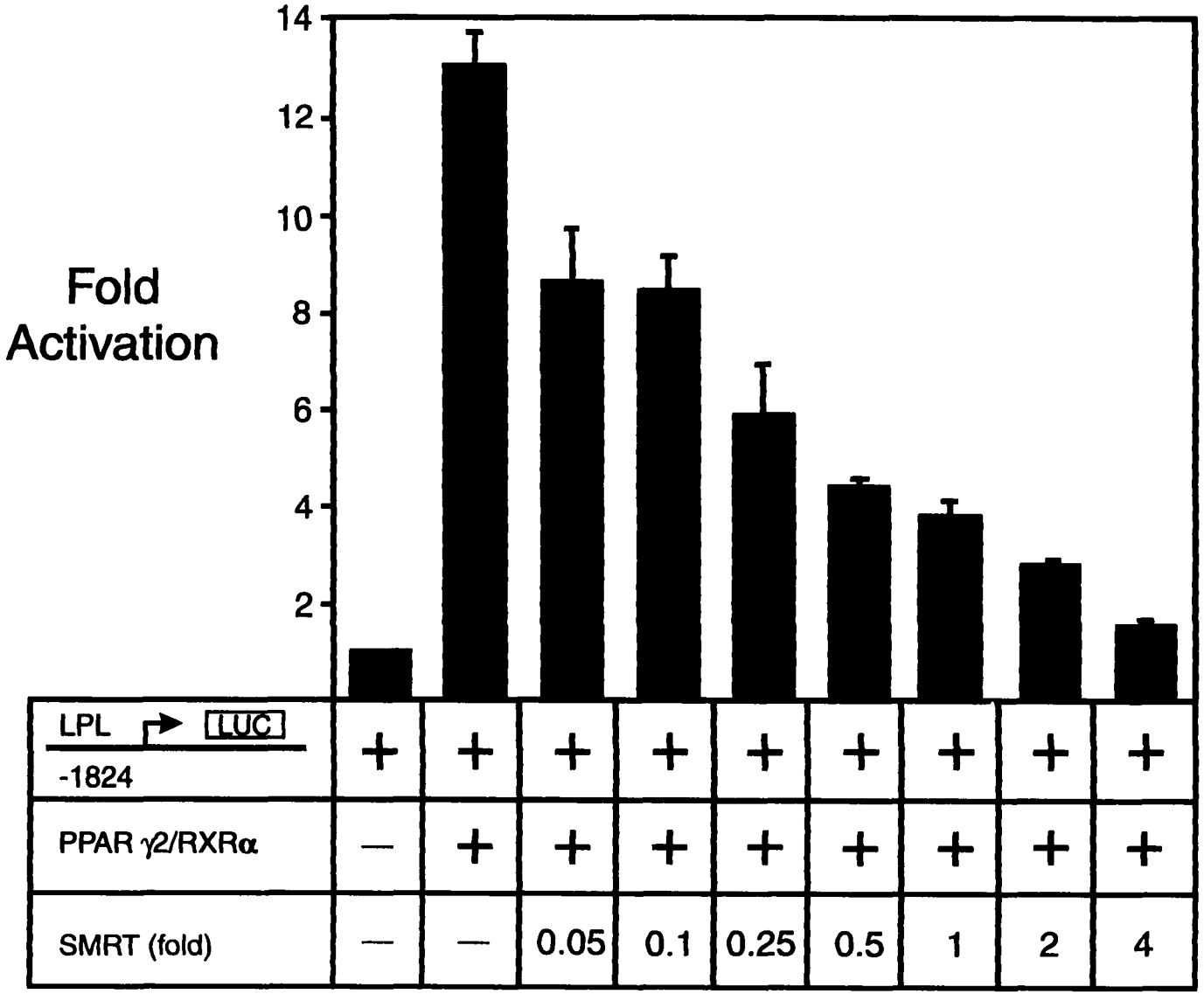


Figure 7. SMRT inhibits the activation of the LPL promoter by the PPARγ2/RXRα heterodimer while SRC-1 enhances activation in the presence of ligand. 293T cells (5 x 10^5/35 mm plate) were cotransfected with the 1 μg of the -1824 to +187 LPL/luciferase reporter construct with or without 4 μg each of the PPARγ2 and RXRα expression vectors in the presence of increasing concentrations (0.2 - 16 μg) of the SMRT expression construct (**7A**). 1 μg of the -1824 LPL promoter was cotransfected with 4 μg each of the PPARγ2 and/or RXRα expression constructs in the presence or absence of a 2-fold concentration (8 μg) of SRC-1 (relative to the concentration of PPAR and RXR) plus or minus addition of 5 μM BRL 49653 (**7B**). Fold induction was determined relative to the baseline activity of the -1824 LPL promoter/luciferase reporter construct in the absence of added expression constructs. Data is normalized relative to protein concentration (87.5 μg/assay) and represents the mean of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.


Fold Activation
14
12
10
8
6
4
2
LPL
LUC
-1824
PPAR γ2/RXRα
SMRT (fold)
+ + + + + + + + +
— + + + + + + + +
— — 0.05 0.1 0.25 0.5 1 2 4

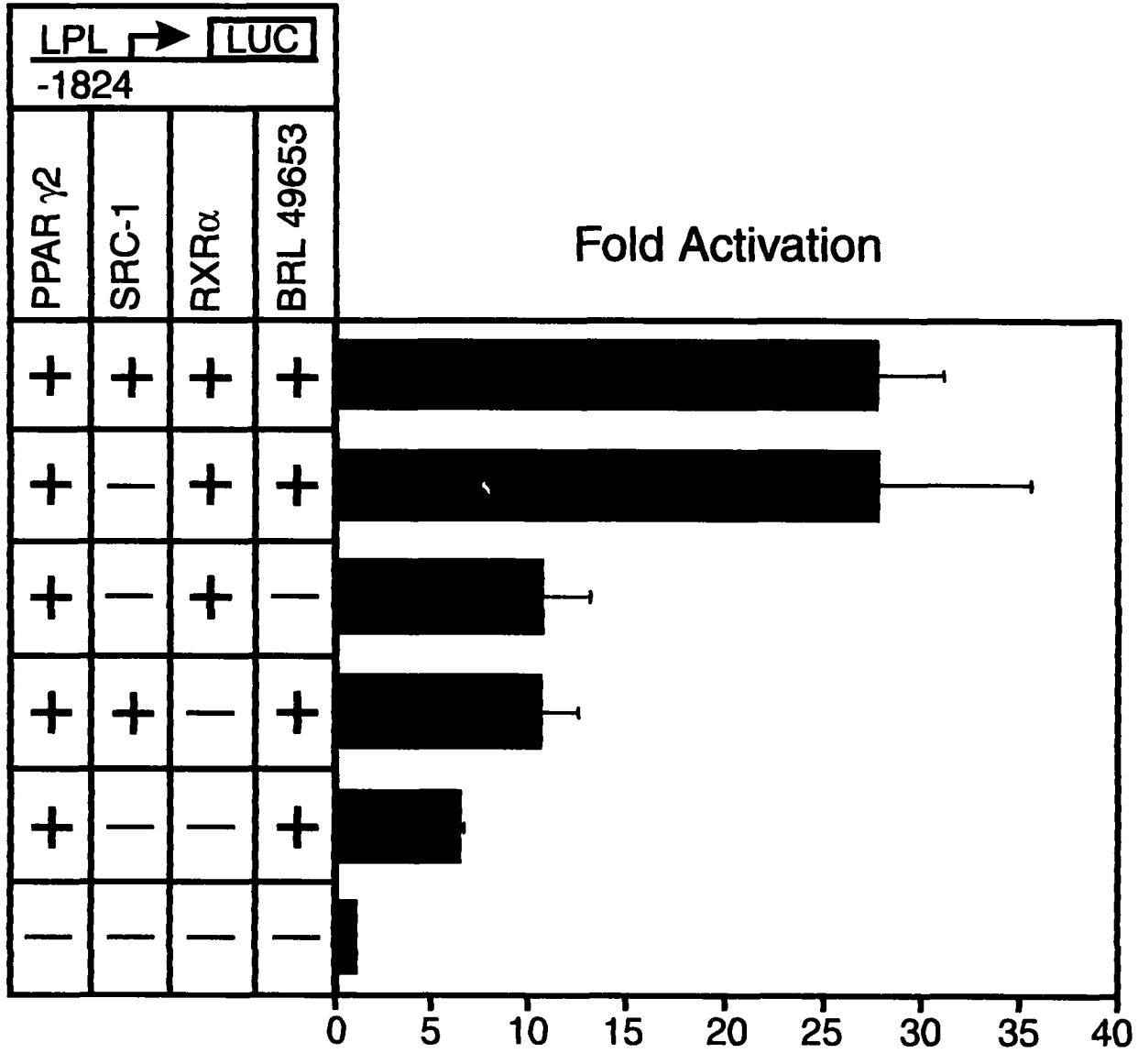
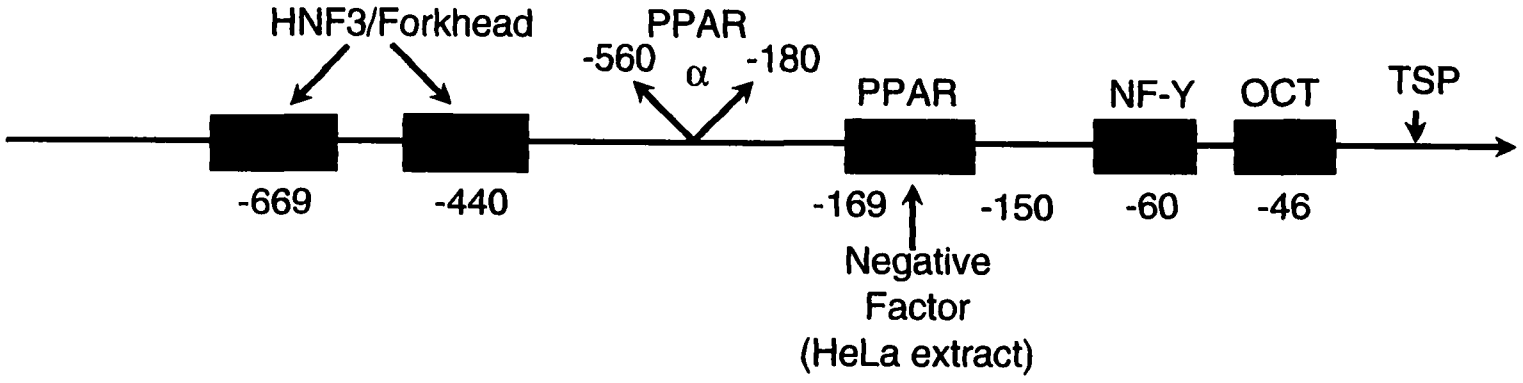

LPL
LUC
-1824
PPAR γ2
SRC-1
RXRα
BRL 49653
Fold Activation
0
5
10
15
20
25
30
35
40

Figure 8. Map of known transcriptional regulatory sites within the LPL promoter.

The location of known transcriptional regulatory protein binding sites within the LPL promoter is outlined. Abbreviations: HNF3, hepatic nuclear factor 3; Oct1, octamer binding protein 1; NF-Y, nuclear factor-Y; PPAR, peroxisome proliferator activated receptor; TSP, transcriptional start point.

Lipoprotein Lipase Promoter



Title: Chicken Ovalbumin Upstream Promoter Transcription Factors are Present during Adipogenesis and act with Peroxisome Proliferator Activated Receptor γ, Retinoic Acid Receptor α and the Co-regulators of Steroid Function SMRT and SRC-1 to Regulate Transcription from the Murine Lipoprotein Lipase Promoter

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Running Title: Coup-TF Regulation of LPL Transcription

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Key Words: Adipocytes, Bone Marrow Stroma, Lipoprotein Lipase, Peroxisome Proliferator Activated Receptors, Steroid Receptors, Thiazolidinediones, Transcription

Footnote: This work is submitted as partial fullfillment of the Ph.D. thesis requirement for C.E.R. in the Department of Zoology, University of Oklahoma.

ABSTRACT

Regulation of lipoprotein lipase gene expression during adipogenesis is intricate and involves many tissue-specific transcription factors. Coup-TFs are orphan members of the nuclear hormone receptor family that have been shown to down-regulate induction by other nuclear hormone receptors involved in differentiation. The peroxisome proliferator activated receptors induce adipocyte differentiation in response to ligand treatment. The coregulators of nuclear hormone receptor action SMRT (silencing mediator for retinoid and thyroid-hormone receptors) and SRC-1 (steroid receptor coactivator) are thought to coregulate nuclear receptor action in a positive or negative fashion. With the discovery of a PPARγ2 recognition element within the LPL promoter, we examined the ability of members of the Coup-TF family of nuclear hormone receptor molecules and the coregulators SMRT and SRC-1 to modulate this effect. Utilizing a -1824 bp to +187 bp portion of the murine LPL promoter attached to a luciferase reporter construct, we determined that addition of Coup-TFII (ARP-1) to the PPARγ2/RXRα heterodimer in transfection assay increased activation of the LPL promoter by greater than 7 fold. The effect was greater than the activation observed by PPARγ2/RXRα in the presence of thiazolidinedione ligands. Coup-TFI (Ear3) did not significantly activate the LPL promoter more than the level seen with PPARγ2/RXRα alone. Ear2 activated the PPARγ2/RXRα system about 50% as well as Coup-TFII (ARP-1). The activation of the PPARγ2/RXRα heterodimer by Coup-TFII (ARP-1) is dose-dependent. When thiazolidinediones are added to the PPARγ2/RXRα/Coup-TFII (ARP-1) transfection, activation is further increased. The corepressor protein SMRT inhibits the activation of

the LPL promoter by the PPARγ2/RXRα/Coup-TFII (ARP-1) proteins in a dose-dependent manner. The coactivator protein SRC-1 does not further activate the PPARγ2/RXRα/Coup-TFII (ARP-1) system in either the presence or absence of added ligand. Based on deletion analysis and EMSA and transfection analysis of mutants of the LPL promoter, the PPARγ2/ RXRα/ Coup-TFII (ARP-1) mediated activation of the LPL promoter is dependent on a polypyrimidine tract from -171 to -156 bp of the LPL promoter that contains the PPARγ2/RXRα recognition sequence. The polypyrimidine DNA sequence is novel in that it appears to contain overlapping DR-1 elements and the sequence resembles the polypyrimidine sequences identified as single stranded DNA binding sites within the promoters of the adipsin and c-*myc* genes. These data support a role for Coup-TF family members as well as the coregulators SMRT and SRC-1 in modulation of the PPARγ2/RXRα mediated regulation of LPL transcription.

INTRODUCTION

The enzyme lipoprotein lipase (LPL: EC 3.1.1.34) hydrolyzes triglycerides into free fatty acids for transport across cell membranes and is responsible for the clearance of chylomicrons from the bloodstream (36,37). Hereditary deficiencies of LPL activity cause increased morbidity in man due to cardiovascular disease (36) and in murine models where the LPL gene has been inactivated by homologous recombination, death results during the neonatal period due to an overwhelming hypertriglyceridemia (21).

The LPL gene is one of the earliest markers of adipocyte differentiation, with its mRNA level increased in less than 4 hours after preadipocytes are exposed to adipogenic agonists (26). It is expressed in numerous tissues including a high level of developmental expression in heart, brown adipose, mammary tissue and also in skeletal muscle (37). There is a medium level of developmental expression in lung and brain, with a low level of expression in liver, kidney and spleen (37). In transgenic mouse models expressing portions of the LPL promoter fused to a luciferase reporter gene, brown adipose tissue activity was 269-fold and brain 200-fold higher than the level of promoter activity in the liver (44). The human and murine LPL genomic genes have been cloned (28,38,58,70,153) and exhibit greater than 65% identity within the first 1.5 kb of the transcriptional start point (TSP) (58). This suggests that there is evolutionary conservation of regulatory sequences through this area of the promoter (58). Some of the transcription factors that regulate the LPL promoter in this region have been identified. These include the octamer binding proteins, the CAAT box binding protein nuclear factor

Y (NF-Y), the hepatic nuclear factor-3/forkhead and the peroxisome proliferator activated receptors α and γ2 which all increase the transcriptional rate of the LPL promoter (25,38,101,113)

The Chicken Ovalbumin Upstream Promoter Transcription Factor (Coup-TFI) is an orphan receptor that was originally cloned based on its ability to promote transcription of the chicken ovalbumin gene (107,116,142). It was independently cloned via homology to *erb* A and called *erb* A related protein 3 (Ear3) (92). Somewhat later, Coup-TFII (ARP-1) was cloned from a HeLa cell cDNA library through its homology to hCoup-TFI (141) and from a placental library as apolipoprotein AI regulatory protein-1 (ARP-1) (78). Another family member, *erb*A related protein 2 (Ear2) was cloned in the same way as Ear3 (92). In humans, Coups are expressed in a variety of cell lines (92). Coup-TFs are highly expressed in organs such as lung, testis, prostate, skin, intestine, pancreas, stomach, and salivary gland (62,85,108) and have recently been shown to be present in adipocytes (11). The importance of the Coup-TFs is suggested in studies on the homozygous null mutations of the Coup-TFI and Coup-TFII genes. In both cases, the null mutation is lethal (83).

It is becoming apparent that nuclear hormone receptors are further regulated in their actions by proteins that act as coactivators and corepressors of their function. These proteins may serve as links between other transcription factors and the transcriptional apparatus (see (57) for reviews). SMRT (silencing mediator for retinoid and thyroid hormone receptor) (18) was isolated by yeast two-hybrid screening using an

unliganded hRXRα ligand binding domain fusion protein as bait. It is considered a corepressor protein because it interacts with unliganded nuclear hormone receptors to transmit a repressive signal to the transcriptional apparatus. It is thought that binding of ligand to the nuclear receptor results in dissociation of the corepressor. When this occurs, proteins known as coactivators of steroid receptor function such as SRC-1 (104) are able to bind to the nuclear receptor and enhance transcription. SRC-1 was isolated by a yeast two-hybrid screening of a human cDNA library using the ligand binding domain of the human progesterone receptor as bait (104). It is widely distributed among different cell types and enhances the activity of ligands that are bound by nuclear hormone receptors. Recently, it was cloned in the mouse (mSRC-1) and demonstrated to be a coactivator of PPARγ activation of a PPAR response element in the presence of ligand (155).

Because Coup-TFs have been generally described as inactivators of transcription (23,41,77,78,83,100,103,139,144), and more infrequently as transcriptional activators (86,112) we set out to determine how they interact with the PPARγ2/RXRα activating system of the LPL gene promoter. Based on our results of Coup-TFII (ARP-1) enhancing PPARγ2/RXRα induced activation of the LPL promoter, and because coregulators of nuclear hormone receptor action are becoming recognized as important players in transcriptional events, we tested if the proteins SMRT or SRC-1 interacted with the Coup-TF/PPARγ2/RXRα system.

MATERIALS AND METHODS

Materials: All reagents were purchased from Fisher Scientific (Dallas, TX) or Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Oligonucleotides were synthesized by Dr. Ken Jackson, Oklahoma Center for Molecular Biology, Oklahoma City, OK.

Plasmid Constructs: Constructs containing portions of the wild type mouse LPL promoter fused to the luciferase reporter gene were prepared in the p19Luc vector (provided by D. R. Helinski, UCSD) (32) as previously described (44). Mutations in the murine LPL promoter were introduced as described . Eukaryotic expression vectors for PPARγ2 and RXRα (a gift from Dr. Ron Evans, Salk Institute (87)) were prepared as described . Coup-TFII (Arp-1), Coup-TFI (Ear3) and Ear2 (Kindly provided by Dr. John Ladias, Harvard) (78) were excised from their original vectors, the fragments ligated to BstXI linkers and subcloned into the BstXI site of the pEF-BOS vector (94). Eukaryotic expression vectors for SMRT (silencing mediator for retinoid and thyroid-hormone receptors) and SRC-1 (steroid receptor coactivator-1) were prepared as previously described (18,104). Recombinant PPARγ2 and RXRα were prepared and purified as described . A recombinant bacterial expression construct for Coup-TFII (Arp-1) protein was prepared by creating a expression vector containing the Coup-TFII coding sequence with a c-*myc* tag and affinity purifying the protein.

Transient Transfections: The human embryonic kidney cell line, 293T (49) (obtained courtesy of Kenji Oritani, OMRF), was maintained in Dulbecco's Modifed Eagle's

Medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin 100 units/ml and streptomycin 100 µg/ml. A total of 8 X 10^4 cells in a 2 ml volume were plated in 35 mm dishes 18 hours prior to transfection. A calcium phosphate/DNA co-precipitate was prepared by mixing a total of up to 24.4 µg of DNA in 1/10 TE (1mM Tris-HCl (pH 8.0); 0.1 mM EDTA (pH 8.0)), adding 23 µl of 2M $CaCl_2$ with 183 µl of 2X HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2PO_4, 12 mM dextrose, 50 mM HEPES pH 7.05)25 minutes prior to equal additions to two 35 mm plates. Following an overnight incubation, the cells were fed with fresh medium, incubated an additional 24 hours and harvested in a 100 µl volume of 25 mM glycylglycine, 15 mM $MgSO_4$, 1 mM dithiothreitol and 1% Triton X-100. Protein concentrations were determined by the bicinchonic acid method (Pierce, Rockford, IL) and adjusted to 3.5 µg/µl. Luciferase assays were performed over a 20 second period using a 25 µl (87.5 µg) aliquot of protein and 100 µl of reaction buffer (0.5 mM D-luciferin, 2.5 mM ATP, 7.5 mM $MgSO_4$, 100 mM KH_2PO_4) in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA) as previously described (44).

Protein expression and purification: Recombinant PPARγ2 and RXRα His-tagged proteins were expressed and purified as described . Recombinant Coup-TFII (ARP-1) c-*myc* tagged proteins were expressed and purified by attaching a c-*myc* tag to the Coup-TFII (ARP-1) cDNA and expressing the fusion protein in a bacterial system. A lysate was prepared from the induced bacterial culture and the proteins isolated by ammonium sulfate precipitation. The precipitated proteins were then affinity purified over a c-*myc* column.

Electromobility shift assays (EMSA): DNA electromobility shift assays were performed with a 67 bp HindIII/SspI DNA fragment spanning bp -181 to -113 of the LPL promoter. DNA labeling was performed using T4 polynucleotide kinase and γ^{32}P-ATP (ICN, Irvine, CA). Probes were labeled to a specific activity of 10^5-10^6 cpm/pmol. Reactions were conducted in a 30 µl volume containing 10 mM Tris-HCl (pH 8.0), 0.1 M KCl, 0.05% NP40, 1 mM DTT, 6% glycerol, with one or more of the following: 1 ng PPARγ, 0.2 ng RXRα, 0.5 ng Coup-TFII (Arp-1), and 2-10 X 10^5 cpm of probe for a 20 min period at room temperature. Samples were separated on a 5% acrylamide/bis-acrylamide (24:1) gel by electrophoresis at 100 v for 3 hours. Gels were dried at 80° C for 90 min and exposed on Kodak XAR film for 18 hours without an intensifying screen.

RESULTS

Accumulation of Coup-TFII message with adipocyte differentiation: The temporal-dependent expression of Coup-TFII mRNA in pre-adipocytes and adipocytes was examined in methylisobutylxanthine /hydrocortisone / indomethacin (MHI) or 1,25-dihydroxyvitamin D_3 (VD_3) treated BMS-2 preadipocytes (**Figure 1**). Confluent BMS-2 cultures were treated with standard medium alone (control) or medium supplemented with either MHI or VD_3. Reverse transcriptase PCR analyses were performed using total RNA harvested daily from the cells after treatment initiation (**Figure 1**). PCR was performed with PPARγ2 and Coup-TFII (ARP-1) specific primers. PCR was also performed on the RNAs with β-actin primers to control for the relative RNA loading between lanes. Treatment with the adipogenic cocktail MHI but not the osteogenic VD_3 increased the amount of Coup-TFII mRNA relative to control.

Members of the Coup-TF family act in concert with PPARγ2 and RXRα to activate the LPL promoter: The next experiments were designed to ask if any of the Coup-TF family (Coup-TFI, Coup-TFII, Ear2), alone or in combination with RXRα, PPARγ2 or PPARγ2/RXRα , regulated transcription from the full length (-1824 bp to +187 bp) LPL promoter. The experiments were carried out in the human embryonic kidney cell line, 293T, because of its ease of transcription and low level of LPL expression in its tissue of origin (5). Cotransfection with expression constructs for the different Coup-TF family members with or without PPARγ2, RXRα or the combination PPARγ2/RXRα revealed

the following pattern of LPL transcriptional regulation. With no added Coup-TF vector, the PPARγ2/RXRα heterodimer induced the highest transcriptional rate as previously described (**Figure 2**). Cotransfection with PPARγ2 alone resulted in statistically significant induction over baseline while transfection with an RXRα vector did not activate significantly over baseline as previously described . Of the Coup-TF family members transfected alone with the LPL promoter construct, only Coup-TFII (Arp-I) and Ear2 significantly activated the LPL promoter over baseline. In combinations with either PPARγ2 or RXRα, the Coup-TF family members activated the LPL promoter only minimally with respect to baseline activation. However, when both PPARγ2 and RXRα were present with a Coup-TF family member, activation increased for all three Coup-TFs with Coup-TFII (ARP-1) activating the highest at over 100 fold, Ear2 next with 50 fold activation and finally Coup-TFI (Ear3) activating 25 fold which was not significantly different from the activation seen with PPARγ2/RXRα alone.

Dose-dependent increase in the PPARγ2/RXRα activation of the LPL promoter by Coup-TFII: To confirm the positive regulatory effect of Coup-TFII (ARP-1), cotransfections were performed with a constant concentration of the PPARγ2/RXRα expression vectors and increasing concentration of the Coup-TFII (ARP-1) vector (**Figure 3**). At the highest concentration, the Coup-TFII (ARP-1) vector increased transcription of the -1824 to +187 LPL promoter by 7.5 times the level of activation by PPARγ2/RXRα alone. These findings demonstrate that the Coup-TFII (ARP-1) protein can positively enhance PPARγ2/RXRα activation of the LPL promoter.

Deletion analysis of the LPL promoter localizes the region of Coup-TFII/PPARγ2/RXRα interaction: To localize the position of Coup-TF recognition elements and to determine if negative regulatory elements might be present, we analyzed a series of luciferase reporter constructs with progressive deletions of the LPL promoter between -1824 bp and -101 bp 5' to the transcriptional start point (TSP) (**Figure 4**). Each reporter construct was transfected alone or cotransfected with the appropriate expression constructs for the PPARγ2/RXRα heterodimer and Coup-TF protein. With Coup-TFII (ARP-1) (**Figure 4a**), deletion of the promoter construct from -1824 to -564 bp and to -181 from the TSP resulted in a decrease in activation of approximately 50% (from >80 fold to approximately 40 fold). Further deletion to -101 bp from the TSP resulted in a decrease in activation of the promoter construct to the level seen with Coup-TFII alone from the -1824 LPL promoter/luciferase reporter construct. Coup-TFI (Ear3) activated the -1824 LPL/luciferase reporter at a level similar to PPARγ2/RXRα alone (**Figure 4b**). Successive deletion of the promoter to -564, then -181 from the TSP resulted in slightly increased activation of the promoter with the combination of Coup-TFI (Ear3)/PPARγ2/RXRα with each deletion in a manner similar to the effect observed with PPARα/RXRα and PPARδ/RXRα indicating a tendency toward a release of inhibition from the -1824 promoter construct. Ear2 (**Figure 4c**) activated the deleted LPL promoter constructs in a manner nearly identical to Coup-TFI (Ear3) except that it demonstrated a higher activation of the -1824 construct.

EMSA analysis identifies a region of the LPL promoter that is bound by Coup-TFII; transfection analysis reveals that Coup-TF binding to the element may not be important to function: Experiments were performed to determine if Coup-TFII (ARP-1) binds the LPL promoter in the same region as PPARγ2/RXRα . Electromobility shift assays were performed using a DNA probe spanning bp -181 to -113 of the LPL promoter and bacterially expressed, c-*myc* tagged Coup-TFII protein (**Figure 5**). A series of five overlapping mutations spanning -171 to -149 of the 5' flanking region (**Figure 5A**) were used as competitors in EMSA analysis against recombinant Coup-TFII (ARP-1) protein (**Figure 5B**). None of the mutations specifically competed for DNA binding except for those spanning bp -162 to -149 (Mutants D and E, **Figure 5B**). In cotransfection analysis, none of the mutants decreased the Coup-TFII (ARP-1)/PPARγ2/RXRα-induced activation except for mutant D which repressed activation by 40% relative to the wild type control (**Figure 5C**). To test if Coup-TFII (ARP-1) could bind the LPL DNA element simultaneously with the PPARγ2/RXRα heterodimer, EMSA experiments were performed where recombinant PPARγ2 and RXRα proteins were added with recombinant Coup-TFII (ARP-1) protein and the 67 bp LPL probe (**Figure 5D**). During competition experiments with the LPL mutant competitors, separate bands of differing mobility are present for each complex (**Figure 5D**) indicating that under these conditions the three proteins do not bind the LPL promoter simultaneously. These experiments demonstrate that Coup-TFII (ARP-1) binds a DNA element that spans from -180 bp to -163 bp from the LPL TSP that includes part of the PPARγ2./RXRα recognition element -169 to -155 and an additional region from -180 to -170. There is also evidence that Coup-TFII (ARP-1) does not bind the DNA element at the same time as the PPARγ2/RXRα heterodimer.

Coregulators of steroid receptor action modify the ability of Coup-TFII (ARP-1) to activate the LPL promoter in concert with PPARγ2/RXRα: To determine if coregulators of steroid receptor action interact with the Coup-TFII/PPARγ2/RXRα system, a corepressor protein SMRT (silencing mediator for retinoid and thyroid-hormone receptors) and a coactivator protein, human steroid receptor coactivator (hSRC-1) were expressed in 293T cells with Coup-TFII (ARP-1)/PPARγ2/RXRα and the -1824 LPL promoter construct (**Figure 6**). To confirm the regulatory effect of the SMRT protein, cotransfections were performed with a constant amount of the PPARγ2, RXRα and Coup-TFII (ARP-1) expression vectors as indicated, with increasing amounts of the SMRT expression vector with the -1824 LPL/luciferase reporter construct (**figure 6A**). The SMRT vector inhibited transcription from the LPL promoter to nearly baseline level of expression of the LPL promoter construct at the highest dose. This finding demonstrates that the SMRT protein can disrupt the ability of Coup-TFII (ARP-1) to enhance the activation of the LPL promoter by PPARγ2/RXRα. In experiments with hSRC-1, increasing concentration of hSRC-1 with a constant concentration of PPARγ2, RXRα and Coup-TFII (ARP-1) in the absence of added ligand demonstrated no significant increase in activation of the LPL promoter (data not shown). When Coup-TFII (ARP-1) was transfected with PPARγ2 and RXRα, activation of the LPL promoter was observed similar to the level observed with PPARγ2 and RXRα in the presence of BRL49653 (**Figure 6B**). When BRL 49653 was added to the PPARγ2/RXRα/Coup-TFII (ARP-1) transfection, induction of the LPL promoter was further increased. Addition of a two-

fold excess of SRC-1 to the BRL treated PPARγ2/RXRα/Coup-TFII (ARP-1) transfections reduced activation of the LPL promoter to the level seen before addition of ligand.

DISCUSSION

The enzyme lipoprotein lipase is an important enzyme that governs the clearance of plasma triglycerides and chylomicrons. It is expressed in a wide variety of tissues including adipocytes, macrophages, lactating mammary tissue and the central nervous tissue. The varying expression of LPL message in different tissue types is a direct reflection of the complex regulation of its promoter by the orchestrated input of numerous transcription factors (reviewed in Enerback and Gimble, (37)). The octamer proteins bind the sequence ATTTGCAT at bp -46 in both the human (28,70) and murine (58,153) LPL promoters and at bp -42 in the chicken (24) LPL promoter. When this site or its flanking regions are mutated, expression is reduced by 75% (25,113).

Coup-TFs are orphan nuclear hormone receptors that bind a variety of AGGTCA repeats and repress the actions of many other steroid hormone receptors. Coup-TFs inhibit expression of two genes that are considered to be antagonistic to adipose differentiation, myoD, a master-regulator gene of myogenesis (99), and bone morphogenetic protein 4 (BMP4), a cytokine that induces bone growth (39). Generally, Coup-TFs are recognized as proteins that downregulate expression from target genes but evidence is accumulating that Coup-TFs may positively regulate certain systems. We have discovered that Coup-TFII (ARP-1) helps the PPARγ2/RXRα heterodimer to increase activation of the promoter of the LPL gene, a marker of adipogenic differentiation. We believe this occurs independently of its ability to bind to the region of the DR-1 that PPARγ2/RXRα bind. In general, binding of Coup-TF to a promoter response element is antagonistic to expression

of the gene product. Baes et al. (3) concluded that Coup-TF antagonized the PPARα/RXRα induced activation of the malic enzyme gene promoter by binding to the Malic enzyme PPRE and that reversal of this effect depended on the presence or absence of 9-Cis RA. In the hydratase-dehydrogenase promoter, Miyata et al. (93) found that Coup-TFI binds to its PPRE and antagonizes PPAR-dependent activation. Brodie et al (11) in the first report of Coup-TF presence in adipocytes demonstrated that inhibitors of preadipocyte differentiation induced Coup-TF to bind to a PPAR/RXR response element, and repress transcription. We believe that the activation observed in the LPL promoter by Coup-TFII (ARP-1)/PPARγ2/RXRα is not occurring through direct binding of Coup-TF to the LPL PPRE but instead through protein/protein interactions involving the transcriptional apparatus. This is supported by recent evidence from Marcus et al. (89) who found through yeast two hybrid cloning, a cellular factor that binds Coup-TFII *in vitro* and allows it to function as a transcriptional activator independent of DNA binding. The factor is identical to a recently described ligand of the tyrosine kinase signaling molecule $p56^{lck}$ (63). The factor, called ORCA (orphan receptor coactivator) appears to mediate interactions between mitogenic and nuclear hormone receptor signal transduction pathways (89). In their hands, ORCA selectively activated Coup-TFII (ARP-1) versus Coup-TFI (Ear3) in cotransfection experiments with the hydratase-dehydrogenase PPRE (93). ORCA had a stimulatory effect on Coup-TFI (Ear3) but the effect was not as pronounced as the effect on Coup-TFII (ARP-1). These results are similar to our own in the LPL promoter where we find minor activation of the promoter by Coup-TFI (Ear3) over the effect of PPARγ2/RXRα but a dramatic increase in activation of the system by Coup-TFII (ARP-1). We hypothesize that Coup-TFII (ARP-1) induction of the

PPARγ2/RXRα system occurs through such a mechanism of interaction with transcriptional cofactors. Alternatively, Coup-TFs could be acting directly through parts of the transcriptional apparatus. It has been shown that Coup-TF associates with the Octamer binding proteins (112)and that it binds TFIIB (60) implying that it can help stabilize the transcriptional apparatus for other nuclear hormone receptors including PPARγ2/RXRα.

Based on this data, we can add Coup-TFII and the coregulators of nuclear hormone receptor action SMRT and SRC-1 to the numbers of transcription factors that regulate LPL gene regulation. The coactivation of the promoter by Coup-TFII/PPARγ2/RXRα is a novel finding in that there is evidence of activation by COUP-TFII in the absence of Coup-TF binding of DNA. .

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FIGURE LEGENDS

Figure 1. PCR analysis of Coup-TFII and PPARγ2 mRNA levels during adipogenesis. Total RNA was prepared from BMS2 cells 6 days after treatment was initiated. BMS2 cells were plated at a density of 4 X 10^6 and were cultured in standard medium alone (Control), with the classical adipogenic agonists (methylisobutylxanthine, hydrocortisone, indomethacin: MHI), or with 1, 25-dihydroxy vitamin D_3. 2 μg of total RNA was reverse transcribed for each sample and amplified with oligonucleotide primers specific for PPARγ2 and β-actin or Coup-TFII (ARP-1). The β-actin gene was used as a control. Aliquots of each reaction were examined on 6% acrylamide gels to compare the relative signal intensity. A ϕX174 HaeIII DNA size marker was run on the gel to confirm the size of the expected fragments.

Treatment	Control Plasmid				+				Ø
	Control	+				+			
	MHI		+				+		
	VD3			+				+	
Primers	βactin	+	+	+					
	PPAR γ2	+	+	+					
	Coup-TFII (ARP-1)				+	+	+	+	

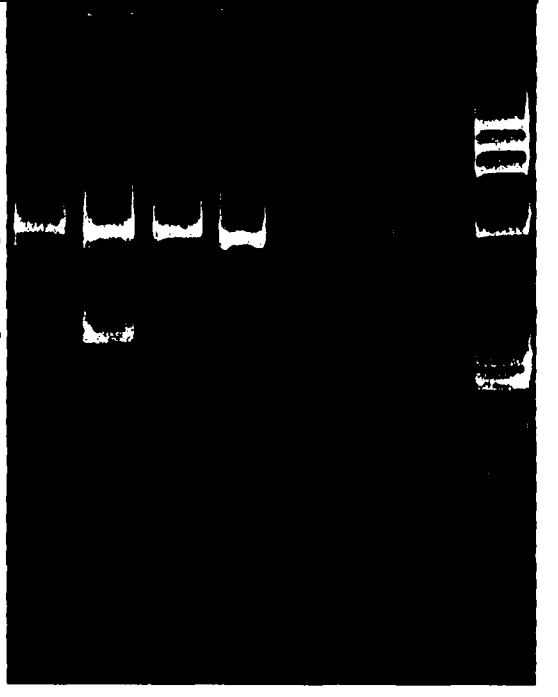


Figure 2. Activation of the lipoprotein lipase gene promoter by Coup-TFs in conjunction with PPARγ2 and RXRα. Cultures of 293T cells (5 x 10^5/35 mm plate) were transfected with 1 μg of the full-length -1824 to +187 LPL promoter/luciferase reporter construct by calcium phosphate coprecipitation. Cells were additionally transfected with 4 μg each of pEF-BOS expression vectors containing either PPARγ2 ± RXRα ± Coup-TFII (ARP-1), Coup-TFI (Ear3), or Ear2 and cultured in the presence of fetal bovine serum. Fold activation is calculated relative to the luciferase baseline activity in the absence of nuclear hormone receptor expression constructs, defined as "1". Data is normalized relative to a constant protein concentration per assay (87.5μg) and represents the mean ± S.E. of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.

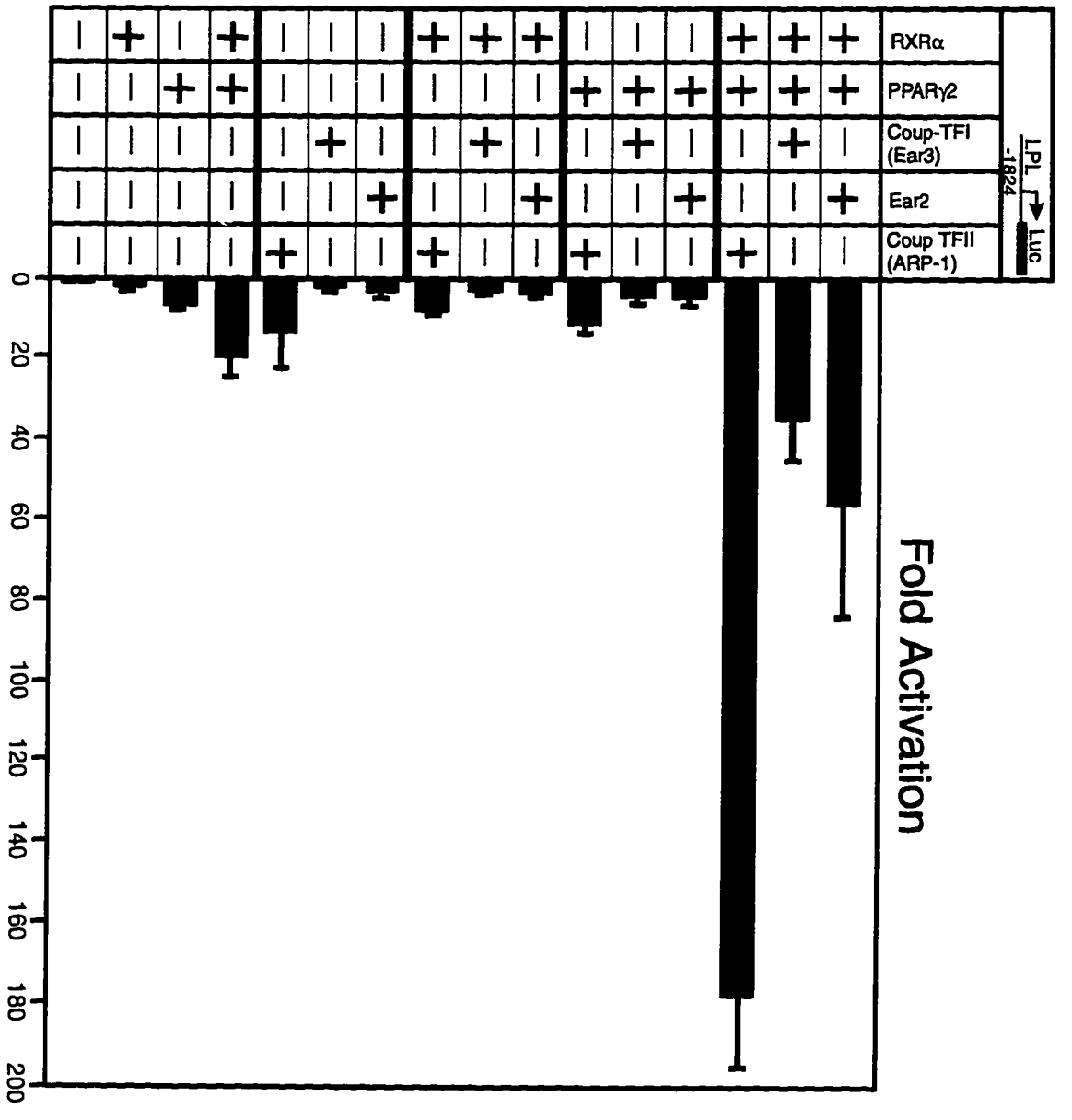

RXRα
PPARγ2
Coup-TFI (Ear3)
Ear2
Coup TFII (ARP-1)
LPL -1824
Luc
0
20
40
60
80
100
120
140
160
180
200
Fold Activation

Figure 3. Dose-dependent activation of the LPL promoter by Coup-TFII (ARP-1) in the presence of PPARγ2 and RXRα. Cultures of 293T cells (5 x 10^5/35 mm plate) were cotransfected with 1 μg of the full-length LPL promoter/luciferase reporter construct and 4 μg each of the PPARγ2 and RXRα expression constructs and increasing concentrations (0.4 - 16 μg) of the Coup-TFII (ARP-1) expression construct. The total amount of DNA in each transfection was kept constant by the addition of the appropriate amount of the empty pEF-BOS vector. Fold activation is calculated relative to the luciferase baseline activity in the absence of nuclear hormone receptor expression constructs, defined as "1". Data is normalized relative to a constant protein concentration per assay (87.5μg) and represents the mean ± S.E. of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.

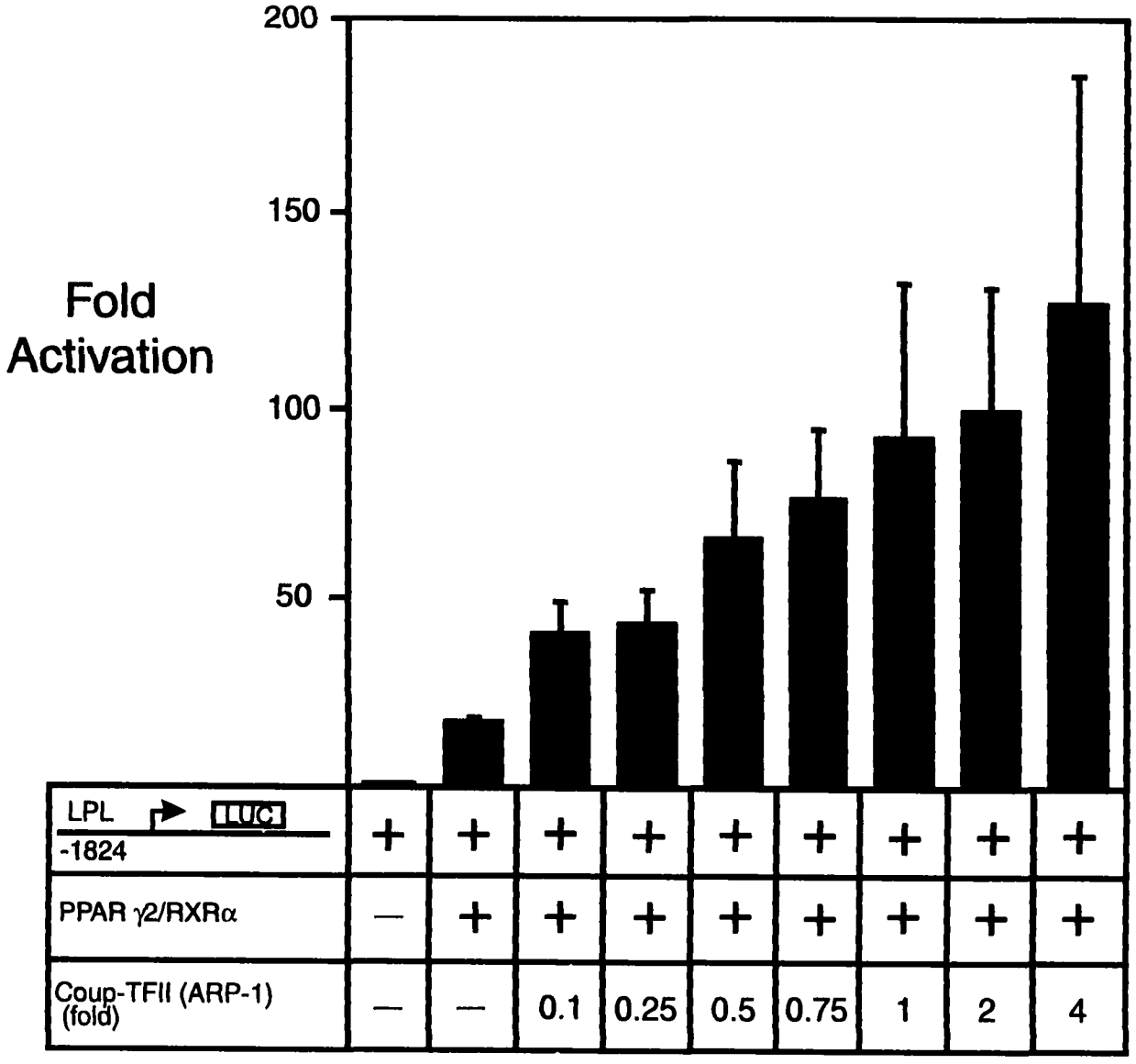
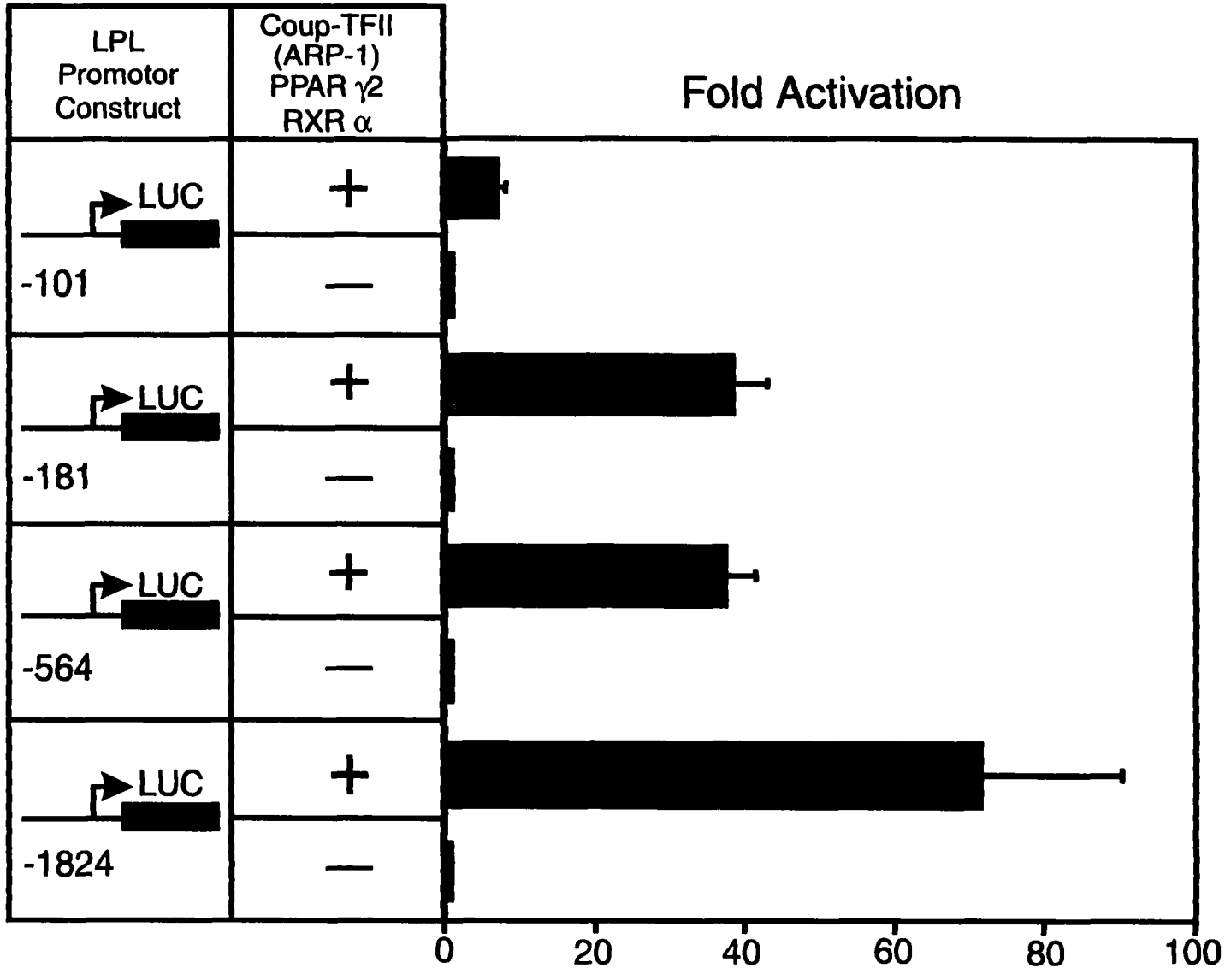
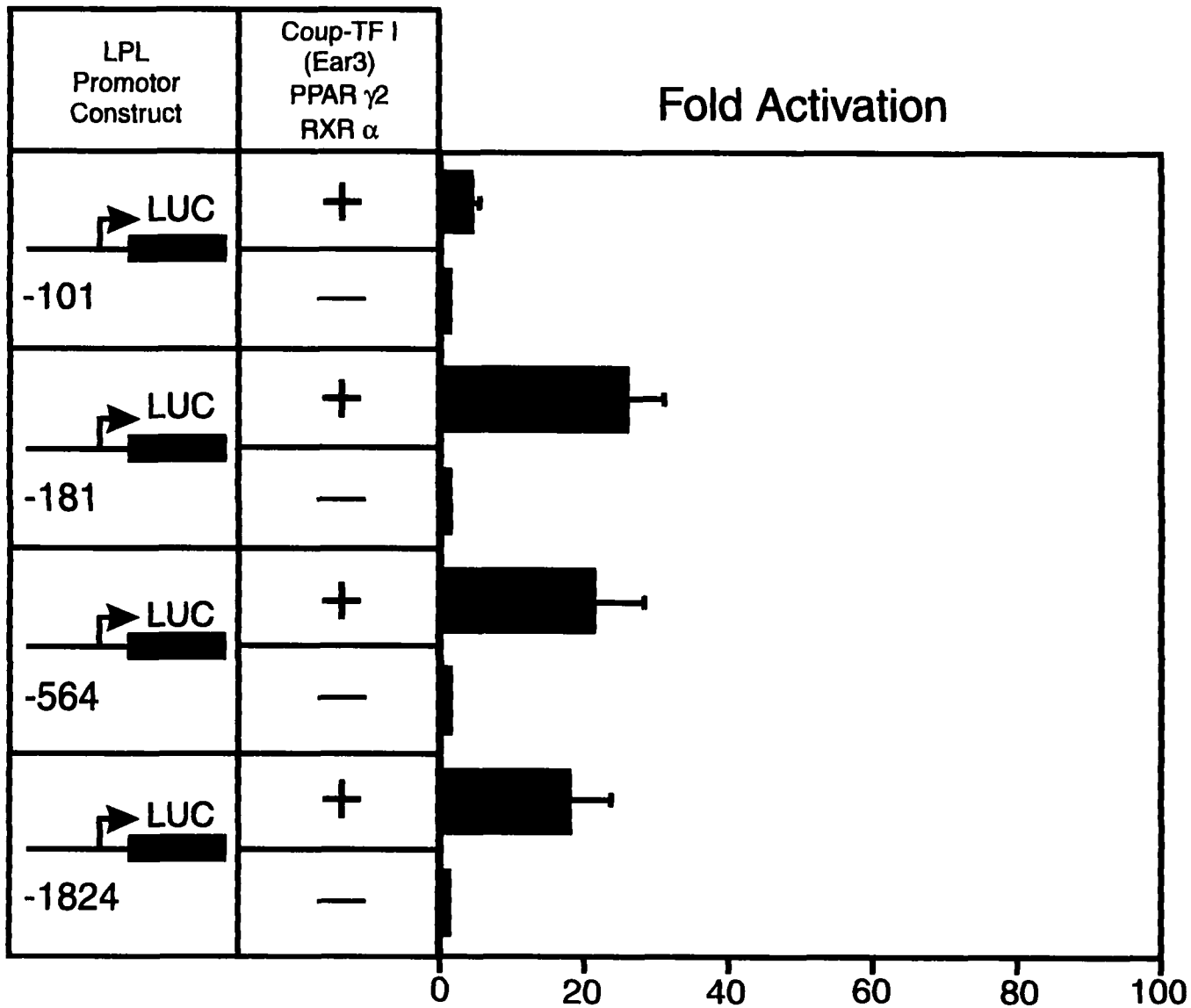

Fold Activation
200
150
100
50
LPL -1824 LUCI
PPAR γ2/RXRα
Coup-TFII (ARP-1) (fold)
+ + + + + + + + +
— + + + + + + + +
— — 0.1 0.25 0.5 0.75 1 2 4

Figure 4. Deletion analysis of the LPL promoter: activation by each Coup-TF family member in the presence of PPARγ2 and RXRα. 1 μg each of deletion constructs that link portions of the LPL promoter from -1824 bp, -564 bp, -181 bp, and -101 bp to +187 to the luciferase reporter gene were cotransfected into 293T cells (5 x 10^5/35 mm plate) in the presence or absence of 4 μg each of PPARγ2, RXRα and one of the Coup-TF family member(Coup-TFII (ARP-1)(**A**), Coup-TFI (Ear3)(**B**), or Ear2 (**C**)) expression constructs. Results are normalized to protein concentration (87.5 μg/rxn) and represent the mean ± SE of n=3 experiments. Fold induction was determined relative to the baseline activity of each reporter construct in the absence of nuclear hormone receptor expression constructs. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.


LPL
Promotor
Construct
Coup-TFII
(ARP-1)
PPAR γ2
RXR α
Fold Activation
LUC
-101
LUC
-181
LUC
-564
LUC
-1824
+
—
+
—
+
—
+
—
0
20
40
60
80
100


LPL
Promotor
Construct
Coup-TF I
(Ear3)
PPAR γ2
RXR α
Fold Activation
LUC
-101
LUC
-181
LUC
-564
LUC
-1824
+
—
0
20
40
60
80
100

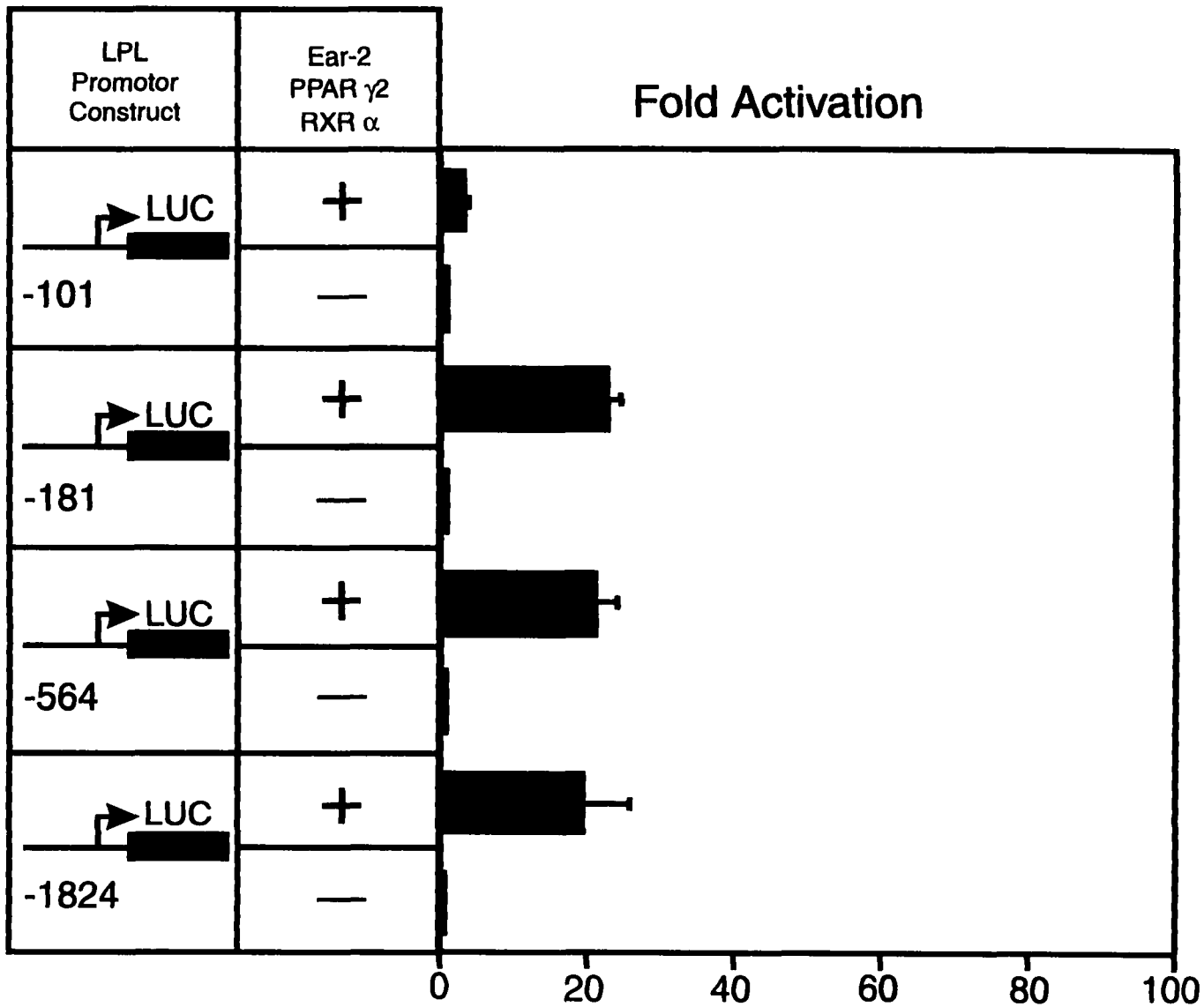
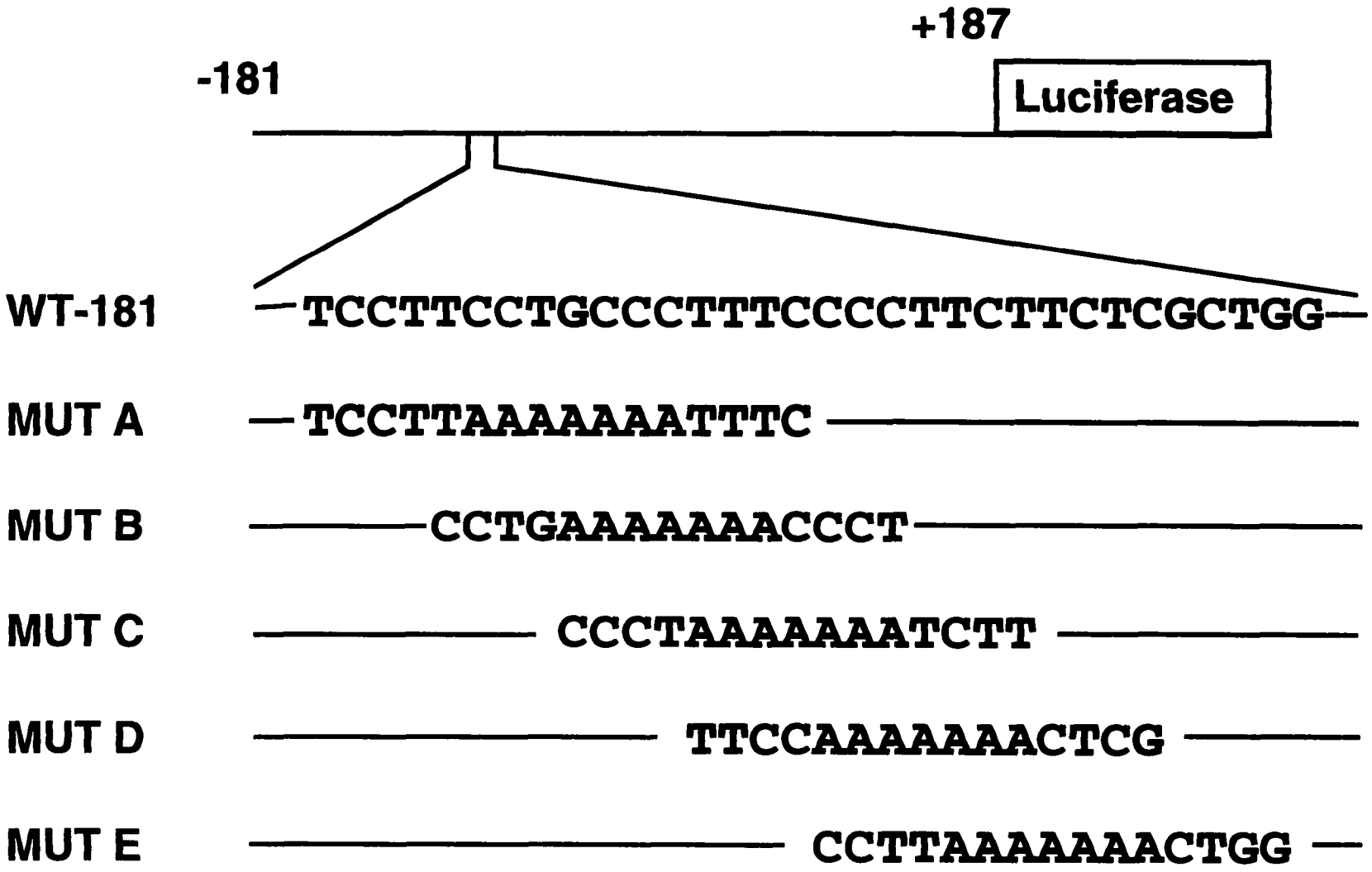
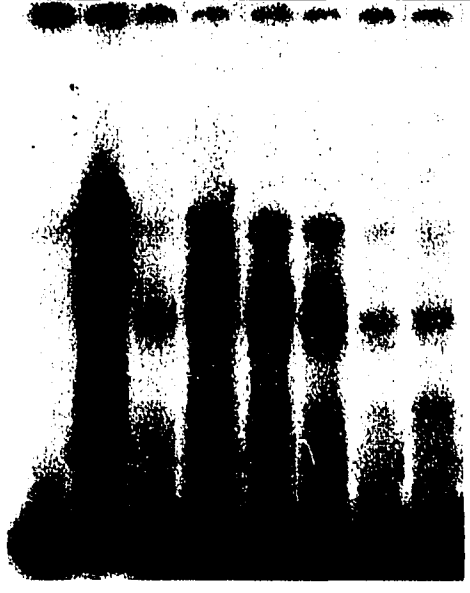
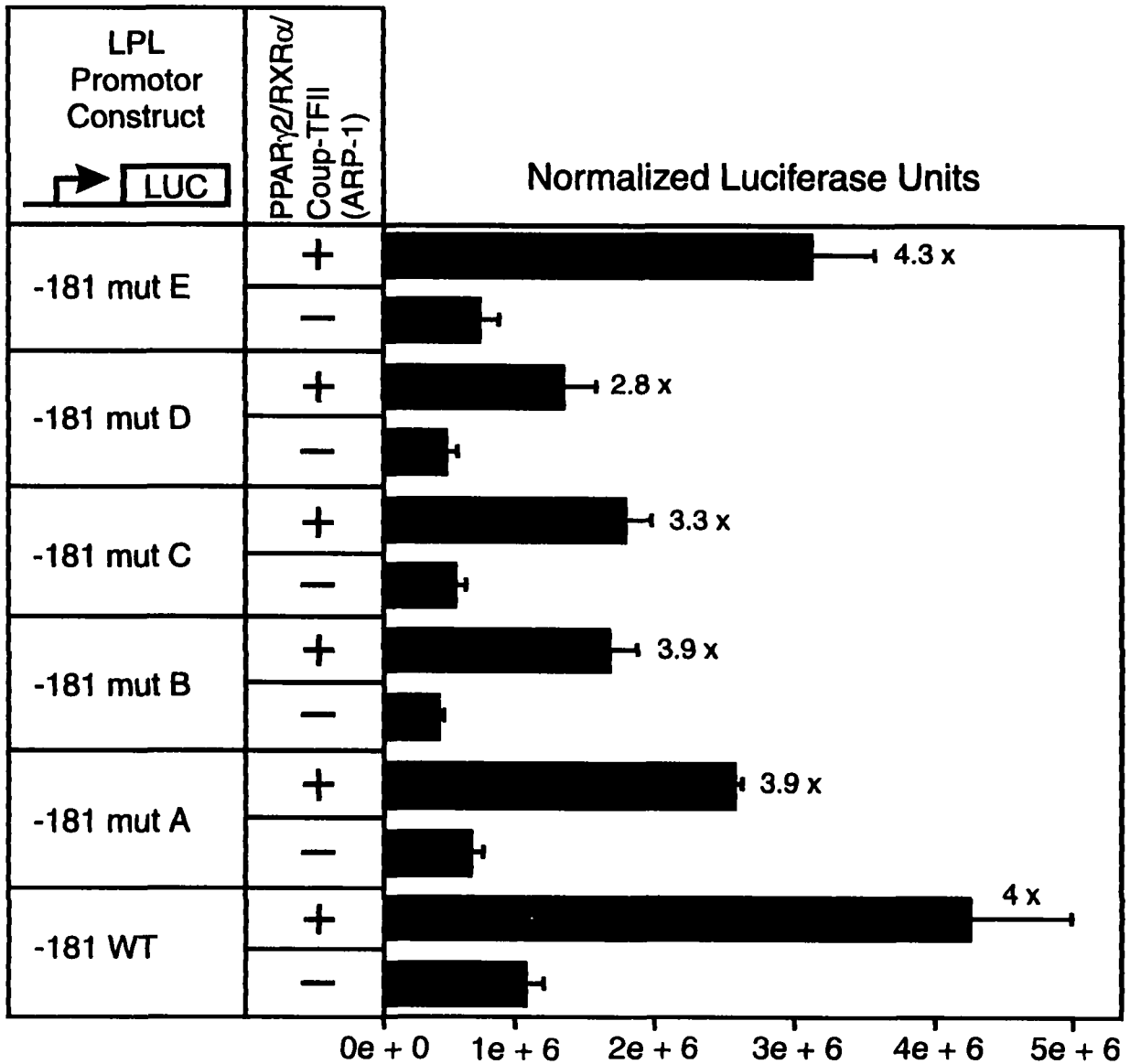

LPL
Promotor
Construct
Ear-2
PPAR γ2
RXR α
Fold Activation
LUC
-101
+
—
LUC
-181
+
—
LUC
-564
+
—
LUC
-1824
+
—
0
20
40
60
80
100

Figure 5. Effect of mutations of the PPARγ2 recognition element on EMSA and cotransfection assays with Coup-TFII (ARP-1). Mutations of the LPL promoter that span bp -171 to -149 are outlined in (**A**). DNA fragments that contain each of the mutations were used as competitors in EMSA assays performed using a 67 bp wild-type LPL promoter probe spanning -181 to -113 of the LPL promoter (**B**) & (**D**). 1 μg each of reporter constructs that link -181 bp to +187 bp of the mutant (MUT) and wild-type (WT) DNA sequences to the luciferase reporter were transfected into 293T cells (5 x 10^5/35 mm plate) ± 4 μg each of the PPARγ2, RXRα and Coup-TFII (ARP-1) expression constructs. Fold induction was determined relative to the baseline activity of each reporter construct in the absence of nuclear hormone receptor expression constructs. Data is normalized relative to protein concentration (87.5 μg/assay) and represents the mean ± SE of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed (**C**).


+187
-181
Luciferase
WT-181
TCCTTCCTGCCCTTTCCCCTTCTTCTCGCTGG
MUT A
TCCTTAAAAAAATTTC
MUT B
CCTGAAAAAAACCCT
MUT C
CCCTAAAAAAATCTT
MUT D
TTCCAAAAAAACTCG
MUT E
CCTTAAAAAAACTGG

							+	mut E
						+		mut D
					+			mut C
				+				mut B
			+					mut A
		+						WT
	+	+	+	+	+	+	+	Coup-TFII (ARP-1)
+	+	+	+	+	+	+	+	67 BP LPL Probe




LPL
Promotor
Construct
LUC
PPARγ2/RXRα/
Coup-TFII
(ARP-1)
Normalized Luciferase Units
-181 mut E
-181 mut D
-181 mut C
-181 mut B
-181 mut A
-181 WT
+
−
4.3 x
2.8 x
3.3 x
3.9 x
3.9 x
4 x
0e + 0
1e + 6
2e + 6
3e + 6
4e + 6
5e + 6

							+	mut E
						+		mut D
					+			mut C
				+				mut B
			+					mut A
		+						WT
	+	+	+	+	+	+	+	PPAR/RXR/Coup-TFII (ARP-1)
+	+	+	+	+	+	+	+	67 BP LPL Probe

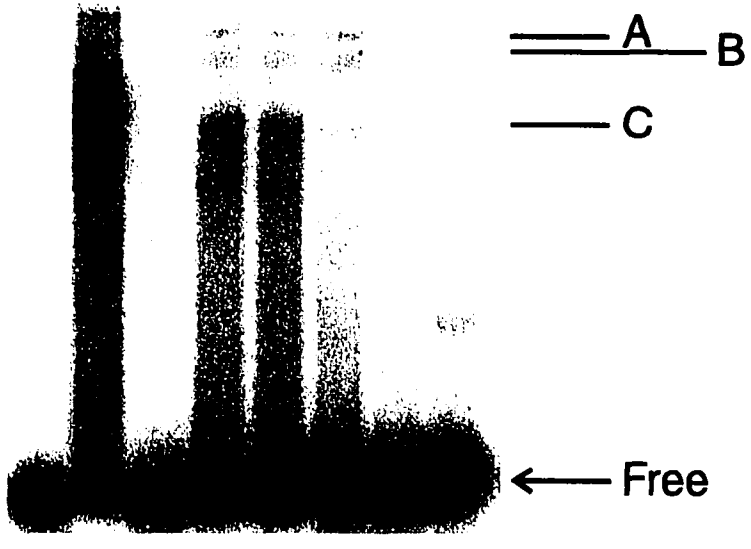
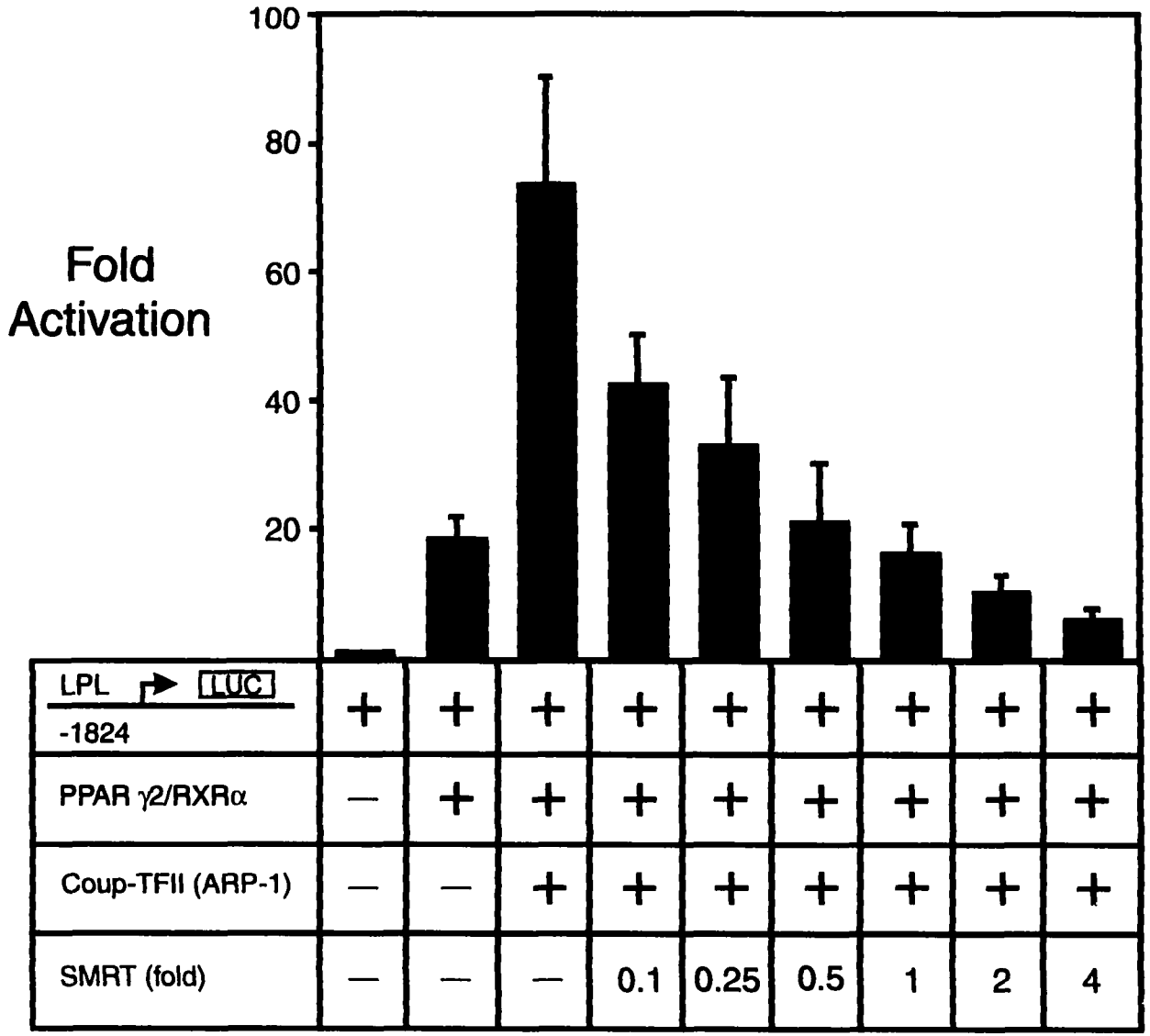
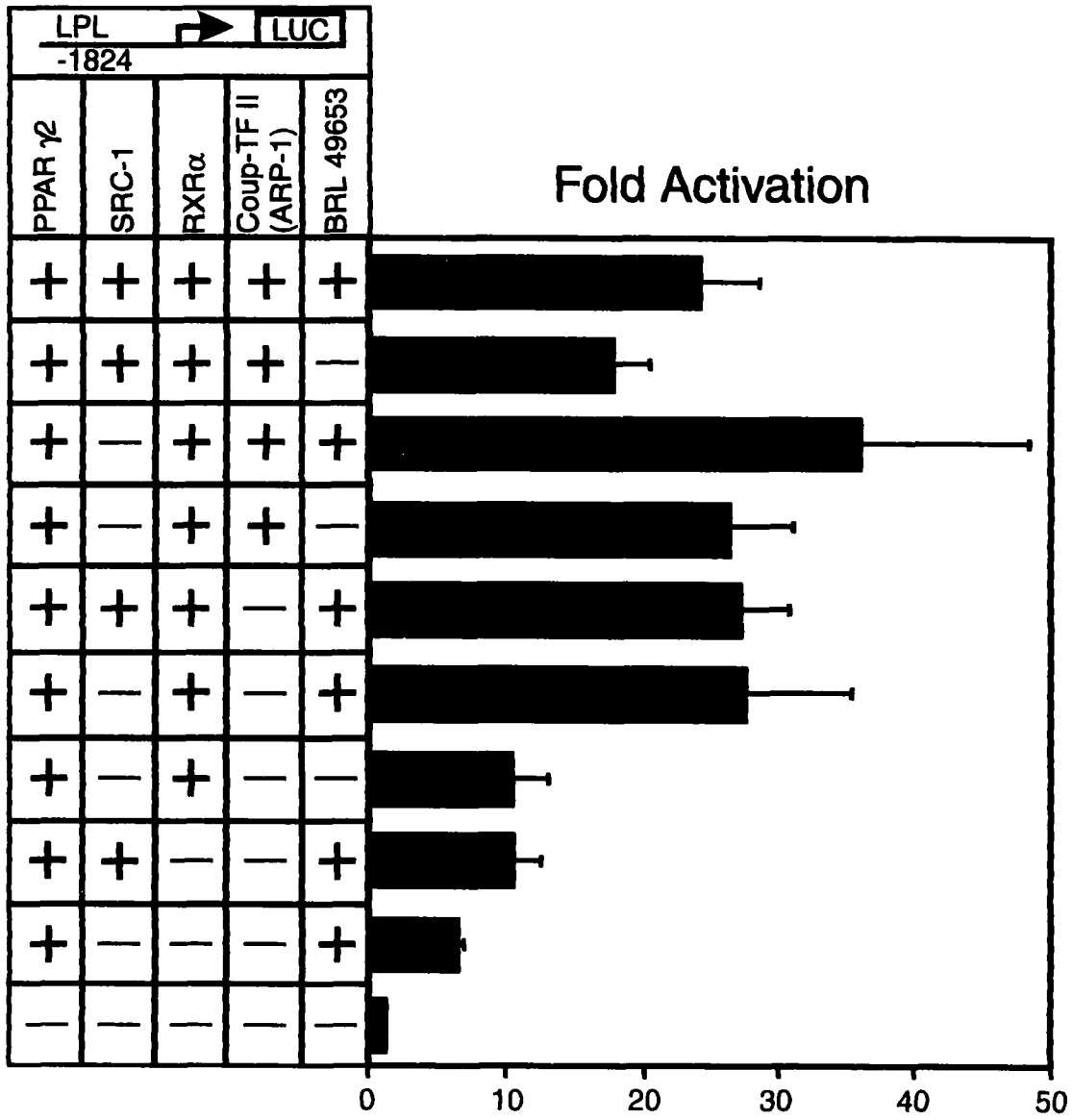


Figure 6. SMRT and SRC-1 modulate the effect of Coup-TFII (ARP-1) on PPARγ2/RXRα induction of the lipoprotein lipase promoter: effect of thiazolidinedione treatment. 293T cells (5 x 10^5/35 mm plate) were cotransfected with 1 μg of the -1824 to +187 LPL/luciferase reporter construct ± 4 μg each of the PPARγ2, RXRα and Coup-TFII (ARP-1) expression vectors in the presence of increasing concentrations (0.4 - 16 μg) of the SMRT expression construct. The total amount of DNA in each transfection was kept constant by the addition of the appropriate amount of the empty pEF-BOS vector (**A**). 1 μg of the -1824 LPL promoter was cotransfected into 5 x 10^5 293T cells/35 mm dish with 4 μg each of PPARγ2 ± RXRα ± Coup-TFII (ARP-1) ± a 2X (8 μg) concentration of SRC-1 in the presence or absence of 5 μM BRL 49653. The total amount of DNA in the transfections was kept constant by adding the appropriate concentration of the empty pEF-BOS vector (**B**). Fold induction was determined relative to the baseline activity of the -1824 LPL promoter/luciferase reporter construct in the absence of added expression constructs. Data is normalized relative to protein concentration (87.5 μg/assay) and represents the mean ± SE of n=3 experiments. Data were analyzed by One Way ANOVA and the Student-Neuman Keuls multiple comparison test was performed.


Fold Activation
100
80
60
40
20
LPL -1824 LUC
PPAR γ2/RXRα
Coup-TFII (ARP-1)
SMRT (fold)
+ + + + + + + + +
— + + + + + + + +
— — + + + + + + +
— — — 0.1 0.25 0.5 1 2 4


LPL
-1824
LUC
PPAR γ2
SRC-1
RXRα
Coup-TF II (ARP-1)
BRL 49653
Fold Activation
0
10
20
30
40
50

SUMMARY

The purpose of this thesis has been to investigate the molecular mechanisms governing the process of nuclear hormone receptor regulation of adipogenesis in the bone marrow stroma and the transcriptional regulation of the enzyme lipoprotein lipase, a marker of adipogenesis. The work put together in the thesis has established that members of the nuclear hormone receptor family called peroxisome proliferator activated receptors (PPARs) do, in the presence of ligand and the retinoid X receptor, activate the adipogenic pathway in bone marrow preadipocytes. The molecular processes that occur during this differentiation event were investigated using the model of the lipoprotein lipase gene promoter as the system for study. It is demonstrated that PPARγ2 and PPARα in the presence of RXRα activate the LPL promoter and that activation increases in the presence of PPARγ2 ligands. The DNA binding element to which PPARγ2 binds is identified through mutant electromobility shift assay and transfection analysis.

In the work, we also determine that a member of the chicken ovalbumin upstream promoter transcription factor (Coup-TF) subfamily of nuclear hormone receptors, Coup-TFII, acts as an auxiliary cofactor for PPARγ2 and RXRα in activation of the LPL promoter. We determine that the effect is not through Coup-TFII binding to the LPL DNA element, but through another mechanism of interaction with the transcriptional apparatus. In attempting to determine the means through which Coup-TFII helps activate

the LPL promoter, we employ the coregulators of nuclear hormone receptor action SMRT (silencing mediator for retinoid and thyroid hormone receptor) and steroid receptor coactivator 1 (SRC-1). We determine that SMRT inhibits the activation of the LPL promoter by the PPARγ2/RXRα system in the absence of ligand. We find that it also totally inhibits the ability of Coup-TFII to assist in activation of the LPL promoter. SRC-1 helps to activate the liganded PPARγ2 receptor in the absence of RXRα. The activation is on the order of that seen by PPARγ2 with RXRα in the absence of added ligand. Addition of SRC-1 to the liganded PPARγ2/RXRα complex does not further increase activation. Addition of SRC-1 to the Coup-TFII/PPARγ2/RXRα system also has no effect on the activation of the LPL promoter. We interpret this to mean that coactivators do interact with the nuclear hormone receptor system, and they do so in a manner that is dependent on which other factors are present in the system as well as whether ligand has bound and changed the conformation of the receptor.

We determine that Coup-TFs may play a role in the adipogenic pathway by helping to activate the LPL promoter. We recognize that Coup-TFs are found abundantly in neural tissue and that the LPL promoter is very active in neural tissue. Thus, we hypothesize that Coup-TFs may also play a role in activation of the LPL promoter in neural tissue.

Future directions of this work include a search for additional coactivating factors for this system involving screening of preadipocyte and adipocyte lineages. With the BMS-2 cell, we believe we have a system that will enhance discovery of new PPAR and Coup-TF interacting factors since both of these molecules appear to play an important role in the

adipocyte. An examination of the adipogenic process in Coup-TF null mice might lead to further understanding of the adipogenic differentiation pathway.

This dissertation has attempted to answer novel questions about the regulation of the LPL promoter in adipogenesis by members of the nuclear hormone receptor family and the actions of coregulators of these receptors on this effect. We feel that the findings are important because of the implications they carry in terms of human obesity, hemopoiesis, osteogenesis and atherogenesis. We hope that our findings help to elucidate the molecular mechanisms governing these conditions so that, one day, therapeutic interventions may be devised for human disease.

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Appendix I: Steroid Receptor cDNAs

INSERT	soln #	SPECIE	VECTOR	PROMOTER	in pEF-BOS	soln #
ARP-1 (COUP-TFII) Source: John Ladias. JBC 269 5944-51. 1994.	5073	human	PMT2	Adeno Major	yes	5270
Coup-TFI (ligand binding domain -) Source: Ming-Jer Tsai. Baylor COM	6100	mouse	pABgal	SV40	no	
EAR2 Source: John Ladias. JBC 269 5944-51. 1994	5071	human	PMT2	"	yes	5928
EAR3 (COUP-TFI) Source: John Ladias. JBC 269 5944-51. 1994	5072	human	PMT2	"	yes	5930
PPARα Source: Frank Gonzalez NCI NIH.	5021	human	PSG5	T7/SV40	no	
PPARα Source: Steve Kliewer. GLAXO Pharmaceuticals	5396	mouse	PECE	SV40	yes	5639
PPARβ/δ (hNUC-1) Source: Schmidt et al. Mol. Endo. 6(4) 1634. Merck Pharm.	5052	human	PJ3Ω	SV40	no	
PPARβ/δ Source: Steve Kliewer. GLAXO Pharmaceuticals	5387	mouse	PSG5	T7/SV40	yes	5642
PPARγ1 Source: Bert O'Malley. Baylor University	5085	mouse	bluescript	T7	yes	5224
PPARγ2 Source: Steve Kliewer. GLAXO Pharmaceuticals	5083	mouse	PSG5	T7/SV40	yes	5637
PPARγ2 Source: direct PCR of 5083	5492	mouse	bluescript	T7	yes	5636
PPARγ2 Source: Mitchell Lazar. UPenn	6117	mouse	pCMX	T7/CMV	no	

INSERT	soln #	SPECIE	VECTOR	PROMOTER	in pEF-BOS	soln #
PPARγ2SA Source: Mitchell Lazar. UPenn	6116	mouse	pCMX	T7/CMV	no	
RAR Source: ATCC	3970	human	PTZ19R	T7	yes	5222
RXRα Source: Ron Evans. Salk Institute	5204	mouse	pCMX	T7/CMV	yes	5312
RXRα Source: direct PCR of 5204	5493	mouse	bluescript	T7	no	
RXRβ Source: Ron Evans. Salk Institute	5373	mouse	pCMX	T7/CMV	no	
RXRγ Source: Ron Evans. Salk Institute	5374	mouse	pCMX	T7/CMV	no	
SMRT Source: Zafar Nawaz. Baylor COM	6119	mouse	pABDgal	CMV	no	
SRC-1 Source: Sergio Onate. Baylor COM	6138	human	pCR3.1	T7/CMV	no	
VDR Source: ATCC	3972	human	PGEM3	T7	yes	5163