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Functional Antagonism between CCAAT/Enhancer Binding Protein-α and Peroxisome Proliferator-activated Receptor-γ on the Leptin Promoter*

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The *ob* gene product, leptin, is a major hormonal regulator of appetite and fat cell mass. Recent work has suggested that the antidiabetic agents, the thiazolidinediones (TZ), which are also high affinity ligands of peroxisome proliferator-activated receptor-γ (PPARγ), inhibit leptin expression in rodents. To examine the effects of this class of drug on the leptin gene in adipocytes we performed Northern analysis on primary rat adipocytes cultured in the presence or absence of TZ. TZ reduced leptin mRNA levels by 75%. To determine whether this effect was mediated at the transcriptional level, we isolated 6510 base pairs of 5′-flanking sequence of the leptin promoter and studied reporter constructs in primary rat adipocytes and CV-1 cells. Sequence analysis demonstrated the presence of a consensus direct repeat with a 1-base-pair gap site between −3951 and −3939 as well as a consensus CCAAT/enhancer binding protein (C/EBP) site between −55 and −47. Our functional analysis in transfected primary rat adipocytes demonstrates that, despite the presence of a canonical direct repeat with a 1-base-pair gap site, TZ alone decreases reporter gene expression of leptin promoter constructs ranging from −6510 to +9 to −65 to +9. In CV-1 cells, which contain endogenous PPARγ, TZ treatment alone had little effect on these constructs. However, TZ treatment did inhibit C/EBPα-mediated transactivation of the leptin promoter. This down-regulation of leptin reporter constructs mapped to a −65 to +9 promoter fragment which binds C/EBPα in gel-mobility shift assays but does not bind PPARγ2 alone or as a heterodimer with 9-*cis*-retinoic acid receptor. Conversely, the promoter (−5400 to +24 base pairs) of the aP2 gene, another adipocyte-specific gene, was induced 7.3-fold by TZ. Co-transfection with C/EBPα minimally stimulated the aP2 promoter from basal levels but notably blocked activation by TZ. These data indicate that PPARγ and C/EBPα can functionally antagonize each other on at least two separate promoters and that this mechanism may explain the down-regulation of leptin expression by thiazolidinediones.

The identification of the leptin gene product (1) as a major hormonal regulator of appetite and fat cell mass provides a novel paradigm to explore the regulation of a fat-specific gene product which may be influenced by nutritional intake as well as other metabolic mediators. Recent studies have clearly demonstrated that the leptin gene is down-regulated by fasting (2) and up-regulated by obesity (3). Studies on fat cell gene expression have been enhanced by the identification of two specific classes of transcription factors: the CCAAT/enhancer binding proteins (C/EBPs)[1] (4, 5), members of the b-ZIP (basic DNA binding domain and a leucine zipper domain required for dimerization) family and PPARγ, a member of the peroxisomal proliferator activated receptor family of nuclear hormone receptors (6). The C/EBP isoforms are expressed at high levels in adipocytes and are induced during adipogenesis (7). Furthermore, C/EBPα has been demonstrated to play an important role in the differentiation of preadipocytes to adipocytes (8–10) and can convert fibroblasts into adipocytes (11). C/EBPβ can also induce adipocyte differentiation (12), possibly by inducing PPARγ (13), which contains C/EBP sites in its promoter (14). PPARγ isoforms are also potent triggers of the adipocyte differentiation cascade (15) and can synergize with C/EBPα to promote adipocyte differentiation (15) or the differentiation of myoblasts into adipocytes (16). In addition, C/EBPα and PPARγ can bind to the promoters and activate adipose-specific genes such as aP2 (6, 15, 17–18) and PEPCK (19–20).

From these previous observations logical candidate regulators of the leptin promoter include C/EBP isoforms and PPARγ. Recently, C/EBPα (21–22) has been identified as a transactivator of the leptin promoter working through a consensus C/EBP binding site in the proximal leptin promoter. This site mediates activation of the leptin promoter by co-transfected C/EBPα in primary rat adipocytes (21) and 3T3-L1 preadipocytes (22). Also, it has been recently established that the administration of the antidiabetic thiazolidinedione, which is a high affinity ligand for the PPARγ isoforms (23), down-regulates leptin expression in rodents (24) and in 3T3-L1 adipocytes (25). These data suggest that unlike other adipose tissue-specific genes such as aP2 and PEPCK, which are up-regulated by PPARγ (6, 20) in the presence of ligand, leptin may in fact be negatively regulated by PPARγ in the presence of its ligand.

The PPAR isoforms mediate positive effects on gene expression by binding to the hexamer sequence AGGTCA in a direct repeat formation spaced by 1 nucleotide (26, 27) with the reti-

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[1] The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; RXR, 9-*cis*-retinoic acid receptor; TZ, thiazolidinedione; DR+1, direct repeat with a 1-base-pair gap; PEPCK, phosphoenolpyruvate carboxykinase; kb, kilobase(s); bp, base pair(s); GST, glutathione *S*-transferase.

noid X receptor (RXR) as a heterodimer. Both ligands for the PPARs and RXR can activate transcription from this complex (28). Recently, a putative ligand for the PPARγ isoform has been identified as a metabolite of prostenoid J2 (29, 30), which can activate PPARγ in transient transfection assays and as well induce adipocyte differentiation in fibroblasts. In contrast to positive regulation by PPAR isoforms, little is known about negative regulation. The PPARs can down-regulate thyroid hormone-responsive promoters by competing for available RXR (31, 32); furthermore, fibrates, which can activate gene expression through PPARs, have been shown to down-regulate the human apolipoprotein A-1 promoter, but the mechanism remains unclear (33). Whether specific negative peroxisome proliferator-activated receptor response elements or cross-competition between PPARs with other positively acting transcription factors occurs is unknown.

Other members of the steroid/thyroid hormone receptor superfamily mediate negative regulation by 1) binding to specific negative response elements, as is the case for the thyroid hormone receptor on a number of specific genes (34–38); 2) interfering through protein-protein interactions or direct competition for DNA binding with positively acting transcription factors such as c-*jun* or C/EBP (39–42); and 3) as recently reported, by competition for limiting co-factors such as CREB-binding protein (CBP)/p300 (43).

In this paper we examine the role of PPARγ in the regulation of the leptin promoter and demonstrate that, despite the presence of a consensus DR+1 binding site located between −3951 and −3939 of the mouse 5′-flanking sequence, PPARγ2 mediates down-regulation of the leptin promoter by inhibiting C/EBPα-mediated transactivation. This mechanism may explain the down-regulation of leptin expression in rodents by the thiazolidinediones.

MATERIALS AND METHODS

Preparation and Treatment of Rat Adipocytes—Adipocytes were isolated from male Wistar rats using the procedure previously described with modifications (44). Briefly, epididymal adipose tissue was minced and digested in Dulbecco's modified Eagle's (DME)-Ham's F-12 medium (v:v, 1:1) containing 1 mg/ml collagenase (Worthington) and 1% bovine serum albumin at 37 °C for 1 h. The adipocytes were strained through nylon mesh and washed three times with the DME-Ham's F-12 medium containing 1% bovine serum albumin. Packed adipocytes (4 ml) were added to 20 ml of DME-Ham's F-12 medium containing 2% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.), and the incubation was carried out at 37 °C in 5% CO_2 for 16 h. Insulin, AD-5075 (5-methyl-2-phenyl-4-oxazolyl-2-hydroxyethoxy-benzyl-2,4-thiazolidinedione) were added to the culture medium, and the incubation was continued for 24 h.

Isolation of RNA and Northern Blot Analysis—Total RNA was prepared from cells using the method of Chomczynski and Sacchi (45). RNA concentrations were quantitated by absorbance at 260 nM. For Northern analyses, 10-μg aliquots of RNA were denatured in formamide/formaldehyde and electrophoresed in formaldehyde-containing 1% agarose gels. RNA was transferred to Hybond™-N membranes (Amersham Corp.) by capillary blotting. Prehybridization and hybridization with ^{32}P-labeled cDNA probes (for leptin or aP2) were carried out as described (24). After washing the membranes, the hybridization signals were analyzed with a PhosphorImager (Molecular Dynamics). Each time point was repeated at least two times in duplicate.

Isolation of the Leptin Promoter and Construction of Luciferase Constructs—Two overlapping P1 clones (Genome Systems, St. Louis, MO) representing the mouse leptin gene and 5′ and 3′ sequences were isolated. 6.5 kb of 5′-flanking sequence including the transcription start site and TATAA box were identified. The transcription start site was confirmed by 5′ rapid amplification of cDNA ends from adipose tissue. A similar transcription start site has been identified by other investigators (21, 22). The promoter region was subjected to dideoxy sequencing between −6.51 kb and +9 bp.

Leptin promoter-luciferase constructs were created by initially shuttling a *Bam*HI fragment containing 765 bp of the promoter into PGEM 3Z. Subsequently −454 to +9 and −765 to +9 fragments were introduced into the luciferase vector pA_3Luc, which contains a trimerized SV40 poly(A) termination to prevent transcriptional readthrough (46, 47) as *Kpn*I-*Hin*dIII and *Sma*I-*Hin*dIII fragments, respectively. The −6510 bp construct was created by excising the region from −454 to −6510 as a *Kpn*I fragment from a genomic subclone and introducing it into the *Kpn*I site of the −454 to +9 construct. The −3821 bp construct was created using an internal *Hin*dIII site and a *Hin*dIII site in pA_3Luc. The −159 to +9 construct was created by *Xho*I digestion of the −454 construct followed by blunt-ending using Klenow and ligating to the *Sma*I site of pA_3Luc. The −65 to +9 construct was cloned using a similar strategy employing *Eae*I. Site-directed mutagenesis of the C/EBP site (see below for oligonucleotide sequence) between −55 and −47 was performed in context of the −454 to +9 construct using synthetic oligonucleotides and restriction enzyme selection (48). The aP2 promoter (−5400 bp to +24) was cloned into the *Sma*I site of pA_3Luc using *Hin*cII and *Sma*I sites. To create the −249 aP2 construct, the parental aP2 plasmid was digested using Ppu101 and *Hin*dIII, and the insert was cloned into the *Sma*I and *Hin*dIII sites of pA_3Luc. The RSV 180 construct contains 180 bp of the Rous sarcoma virus long terminal repeat in pA_3Luc. The sequences encompassing the leptin DR+1 (from −3951 to −3939, see below) were inserted as a *Hin*dIII fragment either as one or two copies upstream of 109 bp of the thymidine kinase promoter (TK109) in pA_3Luc. TK109 pA_3Luc was constructed by inserting the TK promoter from PT109 (49) into pA_3Luc (a gift of T. Nagaya). All promoter constructs were confirmed by restriction endonuclease digestion and dideoxy sequencing. Plasmids for transfection were prepared using column purification (Qiagen) and were generally subjected to at least two separate plasmid preparations.

Generation of Expression Vectors—The MSV rat C/EBPα expression vector was a gift from Alan Friedman (4). An *Eco*RI-*Bss*HII fragment encompassing the complete C/EBP cDNA sequence was subsequently cloned into PGEM 3Z, pSV-SPORT (BioResearch Laboratories), and pBK-CMV (Stratagene). The mouse PPARγ2 isoform was cloned into pSV-SPORT. All expression vectors were confirmed by restriction endonuclease digestion and dideoxy sequencing. Plasmids for transfection were prepared using column purification (Qiagen) and were generally subjected to at least two separate plasmid preparations.

Cell Culture and Transient Transfection—CV-1 and NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with L-glutamine, 10% fetal calf serum, 100 μg/ml penicillin, 0.25 μg/ml streptomycin, and amphotericin. Transient transfections were performed in six-well plates with each well receiving 1.67 μg of reporter and 417 ng of expression vector using the calcium phosphate technique. Amount of expression vector DNA was kept constant within experiments using $pKCR_2$ (similar SV40 early promoter to pSV-SPORT). Each well also received 0.4 μg of a β-galactosidase expression plasmid to control for transfection efficiency. 15–18 h after transfection the cells were washed with phosphate-buffered saline and refed with Dulbecco's modified Eagle's medium with 10% steroid hormone-depleted fetal bovine serum and the indicated concentration of BRL 49653. To remove steroid and thyroid hormones, fetal bovine serum was treated for 24 h at 4 °C with 50 mg/ml activated charcoal (Sigma) and 30 mg/ml anion exchange resin (type AGX-8, analytical grade, Bio-Rad). After centrifugation, anion exchange resin was added again for 5 more hours. The resulting fetal bovine serum was spun again and filtered before use. 40–44 h after transfection the cells were harvested in extraction buffer (38) and assayed for both luciferase and β-galactosidase activity (Tropix). Luciferase activity was corrected for β-galactosidase activity as indicated. All points were repeated in triplicate, and each experiment was repeated at least twice.

Primary rat adipocytes were harvested by isolating white adipose tissue cells from epididymal fat pads of Sprague-Dawley rats. Isolated adipocytes were transfected by electroporation as described previously (50) with 6 μg of either RSV 180 or leptin promoter luciferase constructs. A β-galactosidase expression vector was not introduced into the primary rat adipocytes. Two hours after transfection Dulbecco's modified Eagle's medium with 3.5% bovine serum albumin (final concentration) was added, and the cells were treated with 1 μM BRL 49653 or dimethylsulfoxide carrier alone. 20 h after transfection the cells were harvested for luciferase activity. Statistical analysis was performed using Student's *t* test comparing treated groups to dimethylsulfoxide carrier alone.

Electrophoretic Gel-Mobility Shift Assays—Wild type and mutant overlapping oligonucleotides encompassing the C/EBP site from −67 to −36 (wild type 5′-TGGCCGGACAGTTGCGCAAGTGGCACTGGGG-3′: mutant 5′-TGGCCGGACAGTTGC**AC**G**G**GTGGCACTGGGG-3′) and the DR+1 site at −3951 kb (wild type 5′-ACGTAGAAGCTTGAAATGAGGTAAAAGGTCAGAGTCCAAGCTT-3′) were radiolabeled by fill-

ing in with Klenow and [^{32}P]deoxycytodine triphosphate (3000 μCi/mmol). The −65 to +9 fragment was prepared by *Bam*HI digestion of the pA_3Luc reporter construct and was radiolabeled with Klenow. Unincorporated ^{32}P was removed by Sephadex G-25 chromatography, and each oligonucleotide was subsequently gel-purified. *In vitro*-translated C/EBPα, PPARγ2, and RXRα were prepared in rabbit reticulocyte lysate, and protein production was analyzed by ^{35}S incorporation and direct visualization on SDS-polyacrylamide gel electrophoresis. 3–5 μl of *in vitro*-translated protein were incubated in 10 μl of binding buffer (20% glycerol, 20 mM Hepes, pH 7.6, and 50 mM KCl), 1 mM dithiothreitol, 1 μg of poly(dI-dC), and 0.1 μg of salmon sperm DNA. Incubations were carried out at room temperature for 25 min. Gel-mobility shifts were resolved on 5% nondenaturing polyacrylamide gels and visualized after autoradiography.

Western Blotting—CV-1 cells were transfected with 15 μg of different C/EBPα expression vectors as described above. Cells were treated in triplicate with either BRL 49653 or dimethylsulfoxide carrier for 18–24 h. Whole cell extracts were generated in 600 μl of RIPA buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μl/ml phenylmethylsulfonyl fluoride (10 mg/ml) and 10 μl/ml sodium orthovanadate (100 mM)). 40 μg of protein from each well was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Primary antibody (C/EBP rabbit polyclonal, Santa Cruz Biotechnology) probing was performed according to the manufacturer's instructions, and the secondary antibody specificity was elicited by Amersham ECL. C/EBP-specific bands were quantified with a PhosphorImager. Each experiment was performed in triplicate.

Expression and Purification of GST-C/EBPα—The b-ZIP region of human C/EBPα was cloned in frame with the glutathione *S*-transferase moiety in PGEX-2TK (a gift of D.-E. Zhang). The recombinant protein and GST itself were induced in *Escherichia coli* DH5α at 32 °C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. The GST proteins were purified on glutathione- agarose beads (Sigma) as described elsewhere (51) and analyzed on SDS-polyacrylamide gel electrophoresis. Interaction assays were performed by incubating equal amounts of GST-C/EBP or GST protein alone immobilized on glutathione-agarose beads with 5 μl of ^{35}S-labeled *in vitro*-translated PPARγ2 or C/EBPα for 2 h in 500 μl of interaction buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 0.3% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) at 4 °C with gentle rocking. The protein-GST beads were washed four times with the same buffer. The resulting bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

RESULTS

Thiazolidinediones Down-regulate Leptin mRNA in Primary Rat Adipocytes—In previously reported work (24) it was demonstrated that the chronic administration of a TZ down-regulates leptin mRNA in rodent models. To confirm that this effect was mediated by a direct effect of TZ on adipocytes, we studied the regulation of leptin gene expression using primary rat adipocytes. In the absence of added insulin, leptin mRNA levels were low and not significantly affected by the TZ agonist AD-5075. The effect of insulin to augment leptin expression (Fig. 1) was consistent with data reported by Saladin *et al.* (52). In the context of insulin incubation, AD-5075 caused a 75% decrease in leptin mRNA levels (Fig. 1). Similar data on the regulation of leptin mRNA by the thiazolidinediones were seen by Kallen and Lazar in 3T3-L1 adipocytes (25). Furthermore, these authors demonstrated that this effect was not due to changes in mRNA stability. In contrast to the effects on leptin, TZ stimulated aP2 expression by approximately 2-fold in the absence of insulin and 6-fold in insulin-treated cells (Fig. 1, *panel 2*). In that TZs are high affinity ligands for PPARγ and that the aP2 promoter contains two positive peroxisome proliferator-activated receptor response elements between −5.4 and −4.9 kb (6), these data indicate that liganded PPARγ has differential effects on aP2 and leptin gene expression.

Structure of the Mouse Leptin Promoter—To facilitate our analysis of the transcriptional regulation of leptin we isolated two overlapping P1 clones which include the entire leptin gene as well as 5′- and 3′-flanking sequences. Our analysis agrees with other reports concerning the genomic structure and initiation of transcription (21, 22). We subsequently analyzed the 5′-flanking sequence of the leptin gene. As outlined in Fig. 2, a consensus C/EBP site exists between −55 and −47 and a consensus DR+1 between −3951 and −3939 bp. Other sites with partial homology to C/EBP sites were also identified. No other exact consensus sites for members of the steroid/thyroid receptor superfamily were found between −6510 and +9 of the leptin promoter.

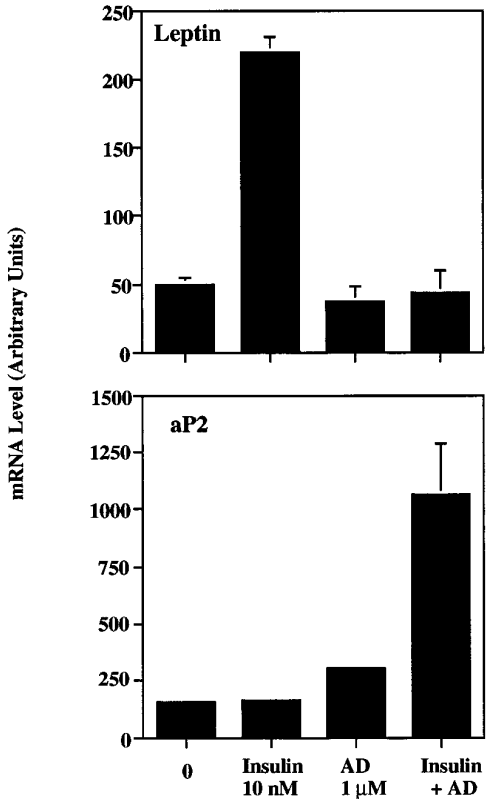


FIG. 1. **Regulation of leptin and aP2 mRNA in primary adipocytes.** Primary rat adipocytes were exposed to insulin, the thiazolidinedione AD-5075, or both for 24 h. RNA was isolated as described and subjected to Northern analysis and hybridization using a leptin or aP2 cDNA probe. mRNA levels were quantified using a PhosphorImager (Molecular Dynamics), and each point was repeated at least twice. Data are presented as the means ± S.E.

The Leptin Promoter Is Down-regulated by Thiazolidinediones in Primary Rat Adipocytes—In that the TZ compounds down-regulate leptin mRNA levels in primary rat adipocytes, we then determined the transcriptional effects of TZ on leptin promoter-constructs transiently transfected into primary rat adipocytes. As Fig. 3*A* shows, leptin promoter constructs were expressed well above background in transfected rat adipocytes (background, 250–300 light units). However, their expression was markedly below the RSV control (70,508 light units). Also, another full-length fat-specific gene promoter, aP2, was expressed at levels slightly above the −454 to +9 leptin promoter construct. Mutagenesis of the proximal C/EBP binding site in context of either the −454 construct (Fig. 3*A*) or the −6510 bp leptin construct (data not shown) caused reporter gene activity to be dramatically reduced indicating that this C/EBP site is essential for full promoter activity in transiently transfected primary rat adipocytes. The addition of 1 μM BRL 49653, a potent and selective TZ and PPARγ ligand, caused significant negative regulation of all the leptin promoter constructs (Fig. 3*B*). In contrast, the −249 aP2 construct was unaffected while the full-length aP2 construct which contains the two DR+1 elements was slightly induced. These data indicate that negative transcriptional regulation of the leptin gene by PPARγ2

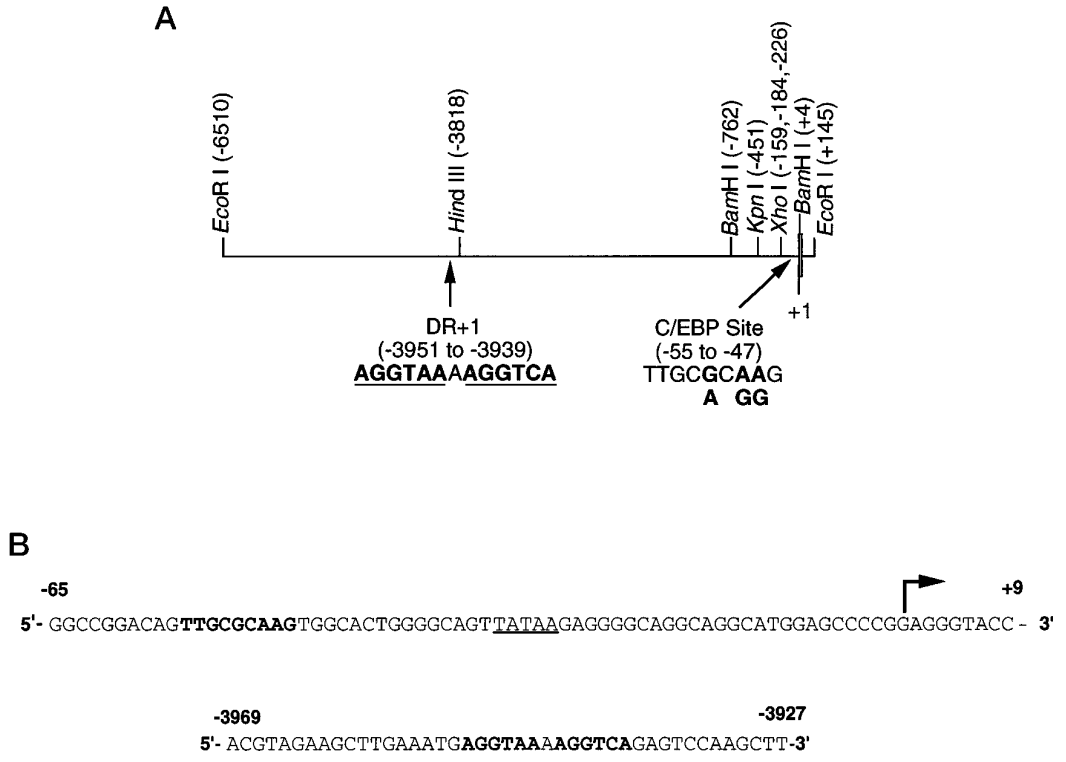


FIG. 2. **Structure of the leptin promoter.** 6.51 kb of 5′-flanking sequence was isolated as described. *A,* the location of restriction endonuclease sites used for constructing many of the reporter constructs. Also shown is the C/EBP site between −55 and −47 and the 3 bases changed to create the mutant site used in both functional analyses and gel-mobility shift assays and the DR+1 site between −3951 and −3939 bp. *B,* the sequence of the proximal promoter region between −65 and +9 and the sequence surrounding the DR+1 site between −3969 and −3927. The *arrow* corresponds to the transcription start site as described previously (22). The entire 5′-flanking sequence (−6510 to +9) was subjected to dideoxy sequencing and has been submitted to submitted to the GenBank™/EMBL Data Bank with the accession number U65742.

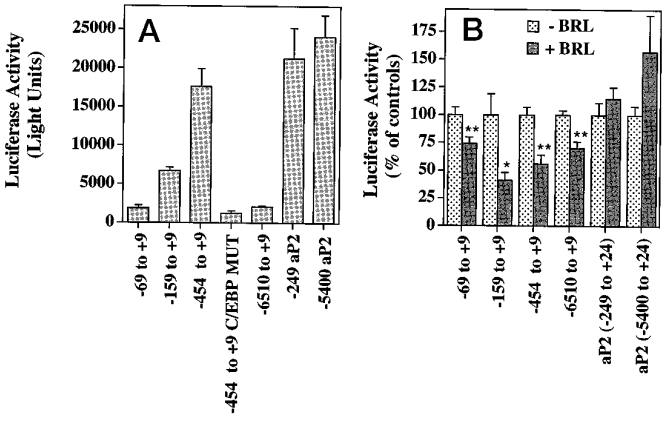


FIG. 3. **Function of leptin promoter-luciferase constructs in isolated primary rat adipocytes.** *A,* primary rat adipocytes were transfected with a variety of leptin promoter constructs, aP2 promoter constructs, and RSV 180 Luc. The data are shown as actual light units after the subtraction of background (250–300 light units). −454 to +9 C/EBP MUT contains the mutated C/EBP site. RSV 180 had a mean activity of 70,508 light units. Each point is the mean of at least nine separate determinations. *B,* the effect of 1 μM BRL 49653 was determined on each of the constructs examined in *A*, and the results are expressed as percent of activity in the presence of dimethylsulfoxide carrier alone. Each point is the mean of at least nine separate determinations, and *p* values were determined using Student's *t* test. (*$p < 0.02$, **$p < 0.01$ *versus* carrier alone).

appears to be mediated by elements within the proximal leptin promoter.

Leptin Promoter Function in Heterologous Cells—To ascertain the effect of C/EBPα and PPARγ2 on the leptin promoter, we performed transient transfections in CV-1 cells, an African green monkey kidney cell line used by many investigators to study steroid hormone action. Recently, these cells were found to contain active PPARγ (30). All leptin promoter constructs had very low basal activity (300–1000 light units) and did not respond appreciably to the thiazolidinedione BRL 49653 (data not shown). We hypothesized that the leptin DR+1 site at position −3951 to −3939 may not support basal activity in these cells and that the lack of reporter activity of leptin promoter constructs was due to an absence of C/EBPα from this cell line. In contrast, the full-length aP2 promoter demonstrated a much higher basal activity (70,000–75,000 light units) and was induced considerably in response to BRL 49653 consistent with the presence of endogenous PPARγ and RXR within this cell line (see Fig. 6*C*). These data support the use of CV-1 cells as a model system to study regulation of PPARγ-responsive genes. When C/EBPα was co-transfected into CV-1 cells, leptin promoter constructs from −6510 to +9 down to −65 to +9 were induced from 17–73-fold (Fig. 4). Absolute levels of expression of the leptin promoter constructs were similar (10,000-25,000 light units) when induced by C/EBPα except for the full-length construct whose induced expression was never above 5000 light units. Mutagenesis of the proximal C/EBP site in context of the −454 to +9 construct (−454 to +9 C/EBP MUT, Fig. 4) caused a near complete loss of C/EBP induction. The viral control, RSVLuc, was not up-regulated by C/EBPα. Surprisingly, the full-length aP2 promoter was only mildly induced (1.5-fold) by C/EBPα despite the presence of a functional C/EBP site within the proximal promoter (17, 18).

These data demonstrate that the proximal C/EBP site within the leptin promoter is critical for C/EBP-induced activation and that the far upstream DR+1 site does not appear to contribute to basal activity of the leptin promoter.

PPARγ2 Binds the Leptin DR+1 Site—Given the consensus nature of the leptin DR+1 site at −3951 bp, we were surprised at its lack of ability to functionally direct transcription in

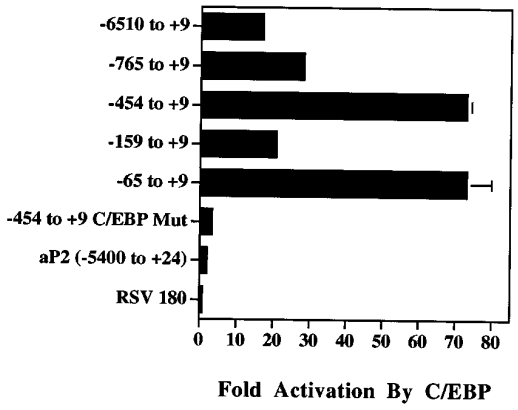


FIG. 4. **C/EBPα transactivates the leptin promoter through its consensus C/EBP site.** CV-1 cells were co-transfected with either 1.6 μg of the indicated leptin reporter or the full-length aP2 promoter linked to luciferase (−5400 to +24) or RSV 180 linked to luciferase and either 417 ng of pSV-C/EBPα or blank expression vector. The −454 to +9 C/EBP MUT construct contains the mutant C/EBP site in context of the −454 reporter construct. The data are presented as fold stimulation over basal expression (without C/EBP) ± S.E. Shown is a representative experiment performed in triplicate, which was repeated at least twice with similar results.

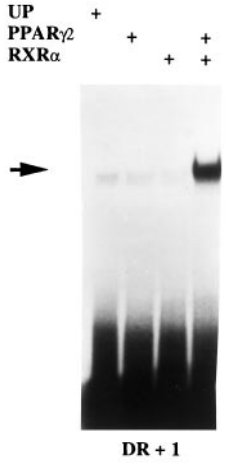


FIG. 5. **The leptin promoter DR+1 binds PPARγ2/RXR heterodimers.** Oligonucleotides encompassing the DR+1 site at −3.9 kb of the leptin promoter (Fig. 2) were radiolabeled and incubated with 3 μl of *in vitro*-translated PPARγ2, RXRα, or both. The specific heterodimer band is indicated by an *arrow*. *UP,* unprogrammed reticulocyte lysate.

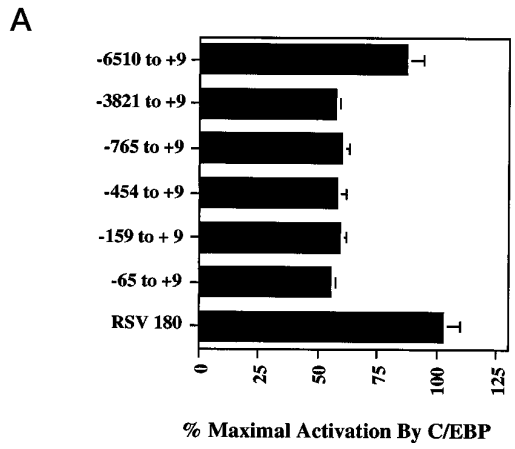
response to BRL 49653 in context of the −6510 to +9 leptin construct. To assess its ability to interact with PPARγ2, we performed gel-mobility shift assays on the DR+1 site and surrounding sequences. Because of its structural similarity to other DR+1s, we were not surprised to see strong binding of PPARγ2/RXR heterodimers to this element (Fig. 5). Given this binding, we then inserted the corresponding oligonucleotides upstream of TK109 luciferase. A single copy of the leptin DR+1 was induced up to 3-fold by 10 μM BRL 49653, while two copies directed up to 8-fold induction of the reporter construct (data not shown). The TK109 promoter itself was not induced by 10 μM BRL 49653. These data indicate that the leptin DR+1 is able to function as a PPAR response element when positioned close to a heterologous promoter.

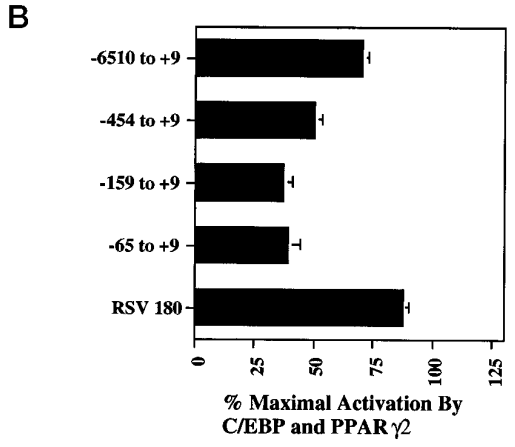
Thiazolidinediones Inhibit C/EBP-Induced Activation of the Leptin Promoter—PPARγ2 and C/EBPα are important determinants of fat cell differentiation and both bind to the promoters of a number of fat-specific genes including leptin, as our data demonstrate. Therefore we studied the effects of C/EBPα and PPARγ2 coexpression on the leptin promoter. When 10 μM BRL 49653 was added to CV-1 cells co-transfected with pSV-C/EBPα expression vector, leptin promoter induction by C/EBP was blocked by 40–50% on constructs from −3821 bp to +9 down to −65 to +9 (Fig. 6*A*). Similar data were obtained with BRL 49653 concentrations ranging from 1 μM to 50 μM. The full-length reporter construct, which includes the DR+1 site, was not as greatly reduced (its activation was attenuated by 10–15%). The −454 to +9 construct with the mutant C/EBP site was expressed at near background levels, making any determination of the effect of BRL 49653 difficult to interpret. RSV 180 Luc was not appreciably affected in the presence of co-transfected C/EBP. The addition of equal amounts of pSV-PPARγ2 increased the negative effect of BRL 49653 on all constructs (Fig. 6*B*). In another heterologous cell line, NIH-3T3 cells, which have little PPARγ, an effect of TZ on both positive and negative regulation on the aP2 and leptin promoters, respectively, could not be seen unless PPARγ2 was co-transfected (data not shown). This implies that the effect of TZ is mediated principally through PPARγ. Thus, these data strongly suggest that PPARγ, in the presence of its ligand, can inhibit C/EBP induction of reporter activity through a direct protein-protein interaction, competition for a common co-factor, or by binding to a non-canonical PPAR site. We also examined the effect of 9-*cis*-retinoic on C/EBP activation of the −454 to +9 leptin construct in order to examine the effect of ligand-activated endogenous RXR on the C/EBP effect. In contrast to BRL 49653, 1 μM 9-*cis*-retinoic acid failed to decrease leptin promoter activity (data not shown), indicating that the negative regulation of the leptin promoter appears to be specific to endogenous PPARγ.

Further confirmation of mutual antagonism between C/EBPα and PPARγ was gained by assessing the effect of co-transfected C/EBPα on the full-length aP2 reporter construct, in that co-transfected C/EBP should be able to block the positive effect of endogenous PPARγ. As shown in Fig. 6*C*, the aP2 promoter was induced 7.3-fold in CV-1 cells by BRL 49653 through endogenous PPARγ receptors (*top*). Co-transfection of C/EBPα (Fig. 6*C*, *center*) reduced BRL 49653-induced activation both in terms of fold activation (only 1.4-fold) and overall activity (by approximately 65%). Co-transfected PPARγ2 increased both the overall response to BRL 49653 (by 2.5 times) and also substantially increased basal activity in the absence of added ligand (Fig. 6*C, bottom*).

To rule out an effect of BRL 49653 on the pSV-C/EBP expression plasmid we performed Western blots on CV-1 whole cell extracts in the presence and absence of BRL 49653 and found no difference in C/EBP amount in the presence or absence of ligand (data not shown). Similar experiments performed using a MSV-C/EBP expression plasmid demonstrated a down-regulation of C/EBP expression by BRL 49653. Thus, the MSV C/EBP expression plasmid was not used.

C/EBPα but not PPARγ2 Binds to the Minimal Promoter Region Down-Regulated by Thiazolidinedione—As our functional data demonstrate inhibition of C/EBP activity by PPARγ2 and its ligand, we examined the ability of PPARγ2 and C/EBP to bind to the region of the promoter which mediates inhibition by BRL 49653. We performed gel-mobility shift assays using either the immediate region surrounding the C/EBP site (data not shown) or the entire region spanning −65 to +9 as probes (Fig. 2*B*), which encompasses the canonical C/EBP site (between −55 and −47). As shown in Fig. 7, *in vitro*-translated C/EBPα bound this region of the promoter and all binding was lost when the site was mutated (data not shown). Furthermore, PPARγ2 and RXR did not bind to this region of the promoter (Fig. 7) as a heterodimer (*lanes 4* and *5*) or alone (*lanes 3* and *6*). When C/EBPα and PPARγ2 were added to-





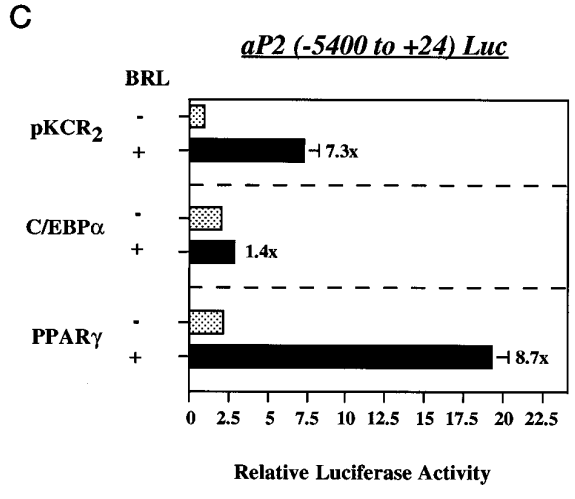


FIG. 6. **Functional antagonism between C/EBPα and PPARγ2 on the leptin and aP2 promoters.** CV-1 cells were transfected as described and treated for 16–20 h with 10 μM BRL 49653 before being assayed for luciferase and β-galactosidase activity. *A,* effect of BRL 49653 on C/EBP activation of leptin promoter-luciferase constructs. The indicated reporters (1.6 μg) were co-transfected with the pSV-C/EBPα (417 ng) expression vector as described previously and treated with BRL 49653. The results are quantified as the percent maximal stimulation by C/EBP, where 100% is the maximum expression of the indicated construct. The data presented are the means ± S.E. of at least two separate transfections performed in triplicate. Empty expression vector was included in certain experiments with no effect on down-regulation by BRL 46953. *B,* effect of BRL 49653 on C/EBP activation of leptin promoter-luciferase constructs in the presence of additional PPARγ2. A similar paradigm was used, except that the cells were also co-transfected with equal amounts of a pSV-PPARγ2 expression vector. The data are expressed as above where 100% is the maximum expression of the indicated construct in the absence of ligand and the presence of co-transfected C/EBPα and PPARγ2 and represents the means ± S.E. of at least two separate transfections performed in triplicate. *C,* effect of C/EBP or additional PPARγ2 on the aP2-luciferase construct (−5400 to +24) in the presence or absence of BRL 49653. The full-length aP2 reporter (−5400 to +24) was co-transfected with blank expression vector, pSV-C/EBPα, or pSV-PPARγ2. The data are shown as relative luciferase activity, where basal expression in the presence of blank expression vector is shown at a value of 1. The data are from a representative experiment performed in triplicate ± S.E. which was repeated at least twice with similar results.

together, C/EBPα binding was decreased (data not shown). However, this finding was most likely not specific, as both *in vitro*-translated RXR and thyroid hormone receptor caused a similar decrease in C/EBP binding.

The inability of PPARγ2 to bind to the proximal leptin promoter in the gel-mobility shift studies suggests that PPARγ2-mediated inhibition occurs through direct interactions with C/EBP or by competition for a critical co-factor. To examine further the possibility of a direct protein-protein interaction we performed GST pull-down experiments using the b-ZIP domain of C/EBPα fused to GST (51). Our data demonstrate that radiolabeled PPARγ2 could not interact with GST-C/EBP either in the presence or absence of its ligand, while radiolabeled C/EBP could interact with GST-C/EBP, consistent with its ability to form a homodimer (data not shown). Further evidence of a lack of interaction between these two transcription factors was garnered from a yeast two-hybrid system where no interaction was seen between a PPARγ bait construct (containing the C terminus) and the full C/EBPα protein used as the prey.[2]

DISCUSSION

Leptin is an adipocyte-secreted hormone which communicates the status of fat stores to the brain, and as such leptin is the afferent signal for a feedback loop whose function is to regulate fat stores. For this reason, regulation of leptin gene expression is critical in maintaining normal body fat content. However, little is known about the transcriptional regulation of leptin gene expression. Previous studies have demonstrated an important role for C/EBPα in regulating the leptin promoter (21–22). The 5′-flanking sequence of leptin possesses a C/EBPα binding site (mouse promoter, −55 to −47) and co-transfection of C/EBPα expression plasmids transactivates leptin promoter-reporter constructs (21–22). Mutation of the C/EBPα site within the leptin promoter results in a loss of transactivation by C/EBPα (Ref. 21 and this study). Finally, this C/EBPα site appears to be critical for expression in transiently transfected primary rat adipocytes, as promoter activity is almost completely lost when the site is destroyed (53) (Fig. 3*A*). These data imply that the C/EBPα site may be necessary for adipocyte-specific expression of the leptin gene.

PPARγ, an important cell-specific regulator of adipocyte gene expression, is another transcription factor which could control leptin gene expression. Of note, PPARs are activated directly and indirectly by metabolites of fat (29, 30, 54–57). This is potentially significant, as many studies have demonstrated that leptin mRNA levels are proportional to the size of fat stores, suggesting that leptin gene expression is somehow linked to adipocyte triglyceride content (58, 59). Of interest, treatment of rodents *in vivo* with a thiazolidinedione (TZ), a PPARγ agonist, reduces leptin mRNA and serum protein levels demonstrating a role for PPARγ in regulating leptin gene expression (24). Recent work has extended these studies and dem-

[2] C. W. Park and B. M. Spiegelman, unpublished data.

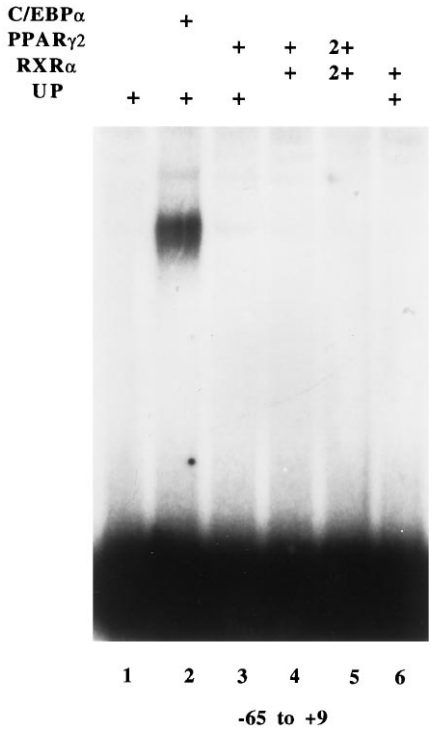


FIG. 7. **C/EBPα but not PPARγ2 or PPARγ2/RXR heterodimers bind to the proximal leptin promoter.** A fragment encompassing from −65 to +9 of the leptin promoter (Fig. 2) was radiolabeled and incubated with *in vitro*-translated C/EBPα, PPARγ2, and RXRα. *Lane 2* shows specific C/EBP binding. *2+* refers to 6 μl of reticulocyte lysate rather than 3 μl. *UP,* unprogrammed reticulocyte lysate.

onstrated that leptin mRNA levels in 3T3-L1 adipocytes are down-regulated at the transcriptional level by the thiazolidinediones (25), and 3 kb of the human leptin promoter has been shown to be negatively regulated by BRL 49653 in primary adipocytes and 3T3-L1 preadipocytes (60). However, neither of these studies examined the possible mechanisms by which PPARγ mediates negative regulation of the leptin promoter.

In the present study, TZ treatment of cultured primary rat adipocytes was found to reduce leptin mRNA levels, indicating that negative regulation by TZ is due to a direct effect on adipocytes. Furthermore, the reduction in leptin mRNA levels appears to result at least in part from negative effects on gene transcription as TZ reduced activity of leptin promoter-reporter gene constructs in primary rat adipocytes. Of note, negative regulation by TZ mapped to the proximal leptin promoter (−65 to +9), a region which binds C/EBPα but not PPARγ and RXR, suggesting that negative regulation by PPARγ occurred without DNA binding. Negative regulation by TZ was also observed in CV-1 cells, a heterologous cell line which expresses endogenous PPARγ but not C/EBPα. However, this effect required co-transfection of a C/EBPα expression plasmid suggesting that negative regulation by liganded PPARγ might be mediated via functional antagonism of C/EBPα. C/EBPα, as shown above, is required for leptin promoter activity in primary rat adipocytes. In addition to the effects of C/EBPα and PPARγ on the leptin promoter, we also found functional antagonism between C/EBPα and liganded PPARγ on the aP2 promoter in CV-1 cells, where co-transfection of a C/EBPα expression plasmid markedly blunted the sizable stimulatory effect of TZ on aP2 promoter activity.

Functional antagonism between PPARγ and C/EBPα could be mediated by a direct protein-protein interaction between these two transcription factors or by competition for a limiting critical co-factor such as CBP/p300 (43). As GST-fusion protein pull-down assays and yeast two-hybrid analyses failed to detect a direct interaction between PPARγ and C/EBPα, we favor the latter hypothesis. When functional antagonism occurs following co-transfection of transcription factors, it is usually thought to be due to "unphysiologic squelching" caused by unnaturally high levels of transcription factors. However, such unphysiologic squelching cannot explain the inhibitory effects of liganded PPARγ on leptin gene expression, since negative regulation by TZ in primary rat adipocytes and CV-1 cells occurred in the presence of endogenous PPARγ and did not require the addition of exogenous PPARγ.

In the present study, a canonical DR+1 site was observed at position −3951 to −3939 of the murine leptin promoter. This site binds PPARγ/RXR heterodimers and is a positive peroxisome proliferator-activated receptor response element when linked to a heterologous promoter. Indeed, the murine leptin 5′-flanking region bears resemblance to other adipocyte genes, such as aP2 and PEPCK, in that it contains a functional C/EBPα binding site within the proximal promoter as well as a functional PPARγ binding site further upstream. Although inclusion of this site in leptin reporter gene constructs attenuated the negative regulation by TZ, the dominant role of PPARγ on leptin expression appears to be inhibitory in both primary rat adipocytes and heterologous cell lines. This contrasts with net positive regulation of the aP2 or PEPCK promoter. The reason for this is presently unknown but might be explained by the presence of two PPARγ binding sites within the aP2 and PEPCK 5′-flanking regions, while only one was found within the leptin 5′ region. Alternatively, additional elements may be present within the leptin 5′ region which modify transactivation by PPARγ.

In light of recent observations demonstrating positive synergy between PPARγ and C/EBPα on fat cell differentiation (15), it is somewhat paradoxical that PPARγ would functionally antagonize C/EBPα action on the leptin promoter. However, closer examination indicates that these two observations need not be mutually exclusive. The mechanisms by which PPARγ and C/EBPα synergize to accelerate fat cell differentiation are unknown but could be explained by a number of possibilities. First, PPARγ and C/EBPα could synergistically cooperate on the promoters of adipocyte genes possessing binding sites for both transcription factors (*i.e.* aP2 and PEPCK). While this is plausible, it should be noted that such synergistic effects on promoter-reporter gene constructs have not been reported. Indeed, in the present study, C/EBPα not only failed to synergize with PPARγ on the aP2 promoter but actually antagonized the effect of TZ (Fig. 6*C*). Alternatively, PPARγ and C/EBPα could each separately induce additional, presently unknown, important transcription factors which then might cooperate on 5′-flanking regions of adipocyte genes. Finally, PPARγ and C/EBPα might each be responsible for increasing expression of subsets of genes which together are required for efficient fat cell differentiation. These latter two possibilities are not at odds with functional antagonism of PPARγ and C/EBPα on leptin gene expression.

In summary, the present study reports that TZ treatment of primary rat adipocytes decrease leptin mRNA levels. This effect appears to be due, at least in part, to functional antagonism of liganded PPARγ on C/EBPα transactivation of the leptin promoter. Identification of the precise mechanism for this antagonism will be the subject of future investigations.

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Functional Antagonism between CCAAT/Enhancer Binding Protein-α and Peroxisome Proliferator-activated Receptor-γ on the Leptin Promoter

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