Differential Regulation of Peroxisome Proliferator Activated Receptor γ1 (PPARγ1) and PPARγ2 Messenger RNA Expression in the Early Stages of Adipogenesis[1]

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Abstract

Adipocyte differentiation is driven by the expression and activation of three transcription factor families: the differentially expressed CAAT/enhancer binding proteins (C/EBPs) α, β, and δ; the helix-loop-helix adipocyte differentiation and determination factor-1; and peroxisome proliferator activated receptor γ (PPARγ), expressed as two isoforms, PPARγ1 and the adipocyte-specific PPARγ2. Overexpression of PPARγ can induce adipocyte differentiation; therefore, we analyzed the expression of the two PPARγ isoforms during early stages of differentiation to determine whether one was preferentially induced as an early determining event. Surprisingly, in the first 24 h, a 3–6-fold increase of PPARγ2 mRNA was observed, whereas PPARγ1 mRNA remained unchanged. PPARγ1 was induced 1 day later. Overexpression of C/EBPβ has also been shown to induce adipocyte differentiation. A C/EBP site was identified only in the human PPARγ2 promoter. Its deletion blunted the response of PPARγ2 promoter to cotransfected C/EBPβ or methylisobutylxanthine treatment. We hypothesize that PPARγ2 initiates adipocyte differentiation.

Introduction

Obesity is a major risk factor for several pathologies, such as type 2 diabetes, hypertension, and hyperlipidemia, and it is a major contributor to mortality and morbidity in industrialized countries (1). Obesity is characterized by a positive energy balance, which leads to abnormally elevated storage of triglycerides in adipocytes, resulting in adipocyte hypertrophy and hyperplasia (2). Adipocytes are derived from pluripotent stem cells, from which myocytes and chondrocytes also originate (3). The capacity of these precursor cells to differentiate is gradually reduced with age; however, they are still able to be recruited to adipocytes, even in the elderly (4). Therefore, an *in vitro* system suitable for studying adipogenesis is desirable. The mouse 3T3-L1 fibroblast cell line has been the most studied cell lines for this purpose because it can be differentiated into adipocytes by hormonal treatment (5). To date, no human preadipocyte transformed cell line is available, but human primary preadipocytes can be cultured *in vitro* and induced to differentiate into adipocytes (6).[5]

Over the last several years, the involvement of several transcription factors in adipocyte differentiation has been demonstrated (for review see Ref. 7). C/EBPα,[6] C/EBPβ, and C/EBPδ, members of the C/EBP family of transcription factors (for review see Ref. 8), are expressed at distinct times during adipogenesis. C/EBPβ and C/EBPδ are expressed very early and appear to be involved in the phase of mitotic clonal expansion that occurs just before the differentiation process begins (9). C/EBPβ and C/EBPδ activate the expression of the gene encoding C/EBPα (10). C/EBPα is expressed just after the clonal expansion phase and is able to drive the program of adipocyte differentiation (11, 12). Adipocyte differentiation and determination factor-1/sterol regulatory element-binding protein 1, a member of the helix-loop-helix transcription factor that is expressed in the early stages of differentiation, is capable of inducing fat accumulation and adipocyte differentiation when overexpressed in fibroblasts (13). In addition, PPARγ, a member of the PPAR subfamily of nuclear receptors, is expressed early in the adipocyte differentiation process and, when activated by a ligand, promotes adipocyte differentiation (11, 14–16). Both the mouse and the human PPARγ are expressed as two isoforms, PPARγ1 and PPARγ2 (15, 17–20). PPARγ2 is predominantly expressed in adipose tissue in both mouse (15) and human (18–20). Moreover, PPARγ genes have recently been shown to possess transcription activation capacities through the NH_2-terminal region, this effect being ligand independent (21). PPARγ2 was ~10 times more active than

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[6] The abbreviations used are: C/EBP, CAAT/enhancer binding protein; PPARγ, peroxisome proliferator activated receptor γ; RXR, retinoid X receptor; PPRE, PPAR response element; IBMX, 1-methyl-3-isobutylxanthine; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-PCR; DEX, dexamethasone; INS, insulin.

PPARγ1 in ligand-independent transcriptional activation, and this effect required INS (21). This data suggests that PPARγ1 and PPARγ2 may have different functions, with PPARγ1 being used when ligand is abundant and PPARγ2 being important under conditions of low ligand concentration, such as might occur in early adipocyte differentiation.

PPARγ appears to have multiple roles in adipocyte differentiation. In NIH-3T3 cells, ectopic expression of PPARγ accompanied by the addition of one of its ligands, pioglitazone, can halt the division of exponentially growing cells and induce adipocyte differentiation (22). In addition to its effect on cell cycle withdrawal, PPARγ also regulates a number of adipose tissue-specific genes by binding as a heterodimer with RXR to PPREs (reviewed in Ref. 23). ARE6 and ARE7, two sequence elements identified in the promoter of the gene coding for the lipid binding protein aP2, are PPREs that mediate the transcriptional response to PPARγ and are capable of interacting with both isoforms (15).

Although PPARγ1 is the most abundant isoform in preadipocytes, PPARγ2 is the adipocyte-specific form. Because PPARγ1 has been shown to be less transcriptionally active in some systems and PPARγ2 was a more potent ligand-independent activator, it was of interest to determine whether PPARγ2 was induced early in differentiation when ligand supply is thought to be low, thereby providing the necessary impetus to move the process forward. We set out to examine the regulation of the two isoforms during the differentiation process and specifically to attempt to identify the trigger for PPARγ2 expression in the mature adipocyte.

Our results demonstrate that PPARγ1 and PPARγ2 have distinctly different patterns of expression and that only PPARγ2 is directly up-regulated during the initiation of adipocyte differentiation by C/EBPβ or inducers of C/EBPβ expression or activity such as IBMX, suggesting a key role for PPARγ2 in driving the initiation of adipogenesis.

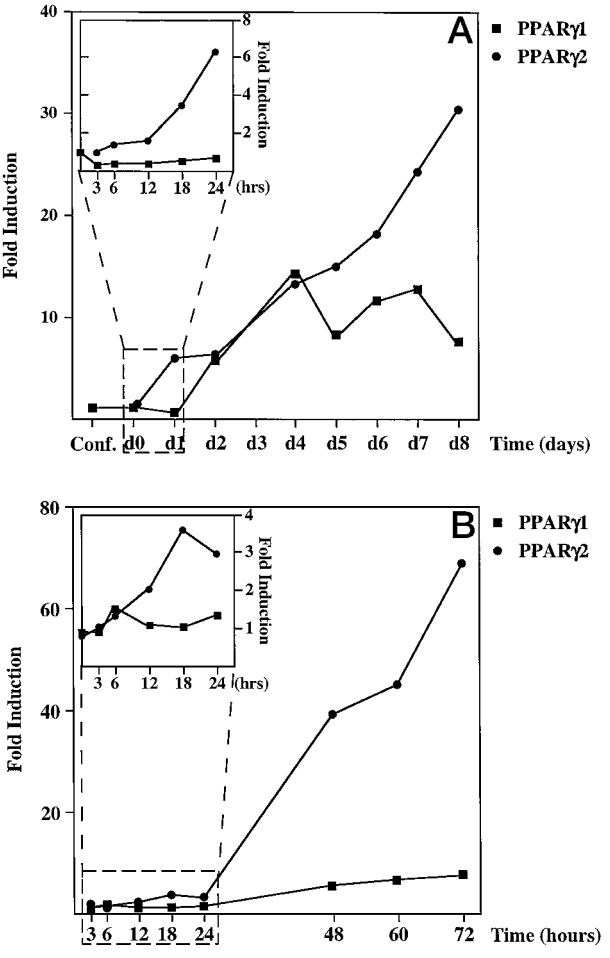

Fig. 1. Kinetic of expression of PPARγ1 and PPARγ2 mRNA during adipogenesis. *A*, 3T3-L1 preadipocytes, 2 days postconfluent, were treated with 10 μg/ml INS, 500 μM IBMX, and 1 μM DEX. Cells were harvested at the indicated times, and total mRNA was prepared as described in "Materials and Methods." PPARγ1 and PPARγ2 mRNAs and 28S rRNA were detected by RNase protection assay using 20 μg of total mRNA. The values obtained for PPARγs were normalized to those for 28S rRNA. *Data points*, average values of four different experiments. *B*, confluent human primary preadipocytes were treated as described in *A*, except that 1 μM BRL49653 was added. Cells were harvested at the indicated times, and total mRNA was prepared as described in "Materials and Methods." PPARγ1, PPARγ2, and β-microglobulin mRNA levels were quantified by competitive RT-PCR using 50 ng of RNA equivalent of cDNA. The values obtained for PPARγs were normalized to those for β-microglobulin. *Data points*, average values of two different experiments.

Results and Discussion

Induction of PPARγ mRNAs during Adipocyte Differentiation. As a first step to elucidating the regulation of the PPARγ isoforms, a time course experiment was performed. Mouse 3T3-L1 preadipocytes were cultured in DMEM containing 10% calf serum until 2 days postconfluency. Cells were then subjected to the differentiation protocol described in "Materials and Methods." Mouse PPARγ mRNA was assayed by an RNase protection assay in parallel with the 28S rRNA. PPARγ2 mRNA was first detectable 3 h after addition of the hormone mixture and was induced 6-fold compared to the first detected value by 24 h after induction of differentiation (Fig. 1*A*). Meanwhile, mRNA for PPARγ1 was initially slightly down-regulated in this period (Fig. 1*A*). After the first 24 h, PPARγ2 mRNA continued to increase, and after 8 days of differentiation, an increase of ~30-fold was observed (Fig. 1*A*). After the first quiescent 24 h, PPARγ1 mRNA became induced during the next 3 days, reaching a plateau with levels ~7-fold higher than the initial levels (Fig. 1*A*).

A similar experiment was next performed with human primary preadipocytes. Cells were cultured in DMEM/F10 (1:1) containing 10% FBS to confluency. The preadipocytes were then induced to differentiate as described in "Materials and Methods." Because of the low amount of mRNA obtained from these cells, PPARγ mRNA was assayed by competitive RT-PCR in parallel with the mRNA for the β-microglobulin as a normalization control. In the first 24 h, PPARγ2 was induced by 3–4-fold, whereas levels of PPARγ1 mRNA remained stable (Fig. 1*B*). Thereafter, both isoforms were induced. Strikingly, a 70-fold induction was observed in PPARγ2 expression, whereas PPARγ1 was up-regulated by 6-fold (Fig. 1*B*).

These results clearly demonstrate that one of the earliest events of both human and mouse adipocyte differentiation is the selective induction of PPARγ2 mRNA over PPARγ1,

which extends results previously described in rodent cell lines (15). PPARγ2 mRNA was detected in human preadipocytes, whereas it could not be detected in the mouse 3T3-L1 preadipocyte cell line. This is probably due to the higher sensitivity of the competitive RT-PCR *versus* the RNase protection assay used for the detection of mouse PPARγ mRNA. Nevertheless, the trace amount of PPARγ2 mRNA detected in human preadipocytes is at a level insufficent for adipocyte differentiation. Interestingly, previous studies in 3T3L1 cells suggested that RXRα is induced a few hours after differentiation is initiated (24). This coinduction of both partners of the heterodimer PPARγ2/RXRα suggests that they have a key role in the commitment of the cells to adipogenesis. Furthermore, because the relative levels of retinoic acid receptor, another heterodimeric partner of RXR, decrease upon adipocyte differentiation (25), a switch in RXR's partners could result in differential recruitment of corepressors and coactivators to DR-1-type PPREs (26). The results of this study clearly show that induction of PPARγ2 transcription appears very early after the initiation of differentiation. Considering the importance of PPARγ in adipocyte differentiation and the fact that obesity is associated with increased expression of PPARγ2 and not PPARγ1 in adipose tissue (20), we propose that up-regulation of PPARγ2 transcription may be a determining factor in the development of obesity through hyperplastic mechanisms.

PPARγ1 and PPARγ2 Promoter Activation Confirms Differential Regulation. To address the question of transcriptional regulation of the PPARγ isoforms *versus* mRNA stability, transfection assays using luciferase reporter constructs driven by the promoter for each PPARγ human isoform were performed. Because the human primary preadipocytes are difficult to transfect and because a similar induction was seen with both cell types, transfection assays were carried out in mouse 3T3-L1 cells. Briefly, 3T3-L1 preadipocytes were grown in regular medium 2 days postconfluency and then transfected for 3 h using Superfect reagent (Qiagen). Medium was then changed to the differentiation conditions. Thus, promoter assays were carried out in cells primed for differentiation. Luciferase activity was assayed 24 and 48 h after transfection. In the presence of 500 μM IBMX, 1 μM DEX, and 10 μg/ml INS, the human PPARγ2 promoter was up-regulated by 10-fold within the first 24 h (Fig. 2*A*), consistent with the mRNA data. Maximum induction of the PPARγ2 promoter activity was obtained when the combination IBMX/DEX was present (data not shown). This large induction of PPARγ2 persisted for 48 h (Fig. 2*B*). The human PPARγ1 promoter showed a different regulation. In presence of the hormone mixture, the human PPARγ1 promoter was down-regulated by 30% (Fig. 2*A*), consistent with the slight decrease obtained at the mRNA level. This inhibitory effect on PPARγ1 promoter activity was caused by IBMX because it was the only compound in the hormone mixture capable of repressing PPARγ1 promoter by itself (70% of control; data not shown). After 48 h of transfection, this down-regulation was no longer observed (Fig. 2*B*). Indeed, as a result of the combined effect of INS and DEX (data not shown), PPARγ1 promoter activity increased relative to control conditions, and the absolute level of expression was higher than that of PPARγ2 promoter (Fig. 2*B*).

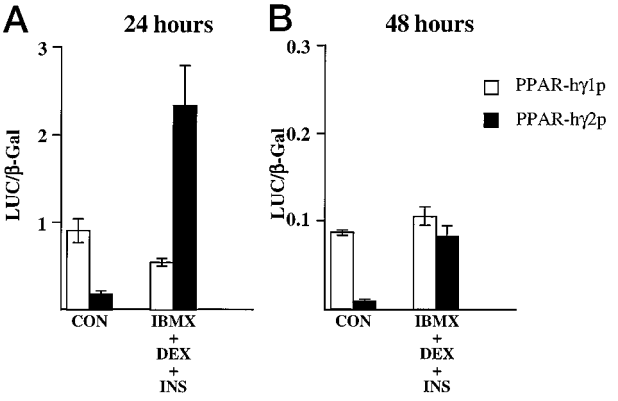

Fig. 2. Human PPARγ1 and PPARγ2 promoter activity in the early stage of adipocyte differentiation. 3T3-L1 preadipocytes 2 days postconfluency were transiently transfected with 2 μg of the indicated reporter plasmids and 1 μg of β-galactosidase plasmid as internal control. Cells were treated with 10 μg/ml INS, 500 μM IBMX, and 1 μM DEX or vehicle (*CON*). Luciferase activity was normalized to β-galactosidase activity. Over the time of transfection, for unknown reasons, luciferase activity declined, which explains the difference in scales in *A versus B*.

The results of the transfection study complement the mRNA analysis and demonstrate that PPARγ2 mRNA levels and promoter activity are strongly induced very early during adipocyte differentiation, further supporting the hypothesis that PPARγ2 is the key player in the initiation of adipogenesis. Human PPARγ1 expression appears to be more constitutive, being initially stable and then gradually increasing during the first 4 days of differentiation. The hormone cocktail showed a dramatic effect on PPARγ2 promoter activity but only a minor effect on the PPARγ1 promoter activity. However, both PPARγ1 and PPARγ2 mRNA levels are induced throughout adipogenesis with PPARγ2, preceding PPARγ1 by about 24 h. The increased level of PPARγ1 mRNA seen after the first 24 h is not driven by PPARγ and likely arises from other transcriptional regulatory pathways. Analysis of the 5′ flanking sequence of both genes did not reveal any consensus PPREs. Moreover, cotransfection of a vector expressing PPARγ did not induce expression from the human PPARγ promoter when ligand was added (data not shown).

C/EBPβ Increases PPARγ2 Promoter Activity. C/EBPβ expression has been shown to be induced by IBMX in 30A5 preadipocytes (27) and 3T3-L1 cells (28). Ectopic expression of C/EBPβ in NIH-3T3 cells was reported to induce PPARγ gene expression (29). Ectopically expressed C/EBPβ, however, required the addition of DEX to the culture medium to fully initiate the adipogenic program (29). In view of the strong preferential induction of the human PPARγ2 promoter by combination treatment with IBMX/DEX, it was investigated whether the up-regulation of the human PPARγ2 promoter during adipocyte differentiation could be mediated by C/EBPβ. Cotransfection experiments performed in growing 3T3-L1 cells showed that C/EBPβ induced the activity of PPARγ2 promoter (Fig. 3). In contrast, no effect was observed on the human PPARγ1 promoter (Fig. 3). Interest-

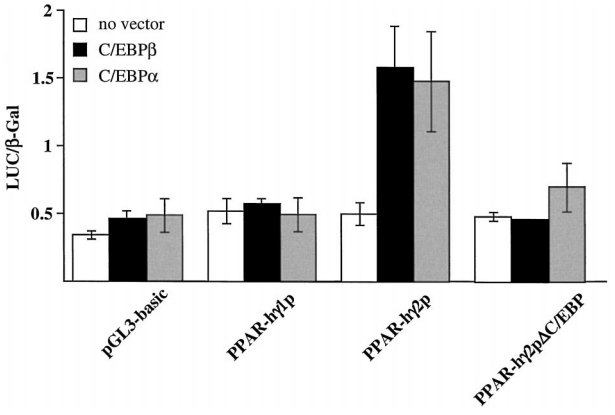

Fig. 3. C/EBPβ induces the human PPARγ2 promoter. Growing 3T3-L1 preadipocytes were transiently transfected with 2 μg of the indicated reporter plasmids, 200 ng C/EBPβ or C/EBPα, and 1 μg of β-galactosidase plasmid as internal control. After 24 h of transfection, cells were lysed, and luciferase activity was determined and normalized to β-galactosidase activity.

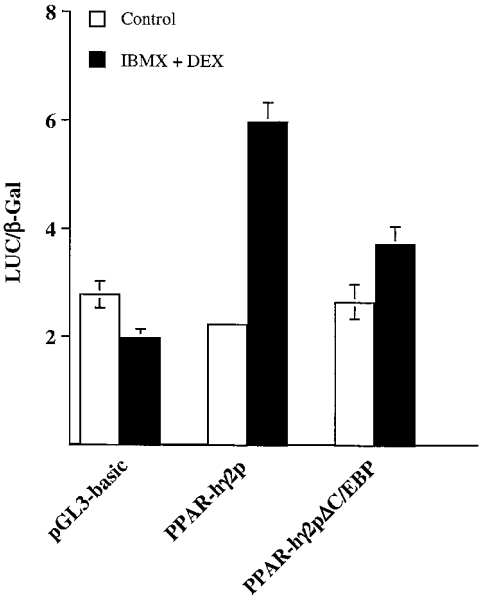

Fig. 4. C/EBPβ mediates the effect of IBMX and DEX on the human PPARγ2 promoter. Growing 3T3-L1 preadipocytes were transiently transfected with 2 μg of the indicated reporter plasmids and 1 μg of β-galactosidase plasmid as internal control. Cells were treated for 24 h with 500 μM IBMX and 1 μM DEX (*IBMX + DEX*) or vehicle (*Control*). Luciferase activity was normalized to β-galactosidase activity.

ingly, cotransfection of a expression vector for C/EBPα, not normally expressed early in differentiation but still able to bind the cognate C/EBP site, also resulted in the induction of only the human PPARγ2 promoter (Fig. 3). Sequence analysis confirmed the presence of a putative C/EBP site in the human PPARγ2 promoter, whereas no homology was found in the PPARγ1 promoter. To demonstrate that the effects of C/EBPβ were mediated by this sequence element, the putative C/EBPβ site was next deleted within the PPARγ2 promoter/reporter construct to generate the construct PPAR-hγ2pΔC/EBP. As shown in Fig. 3, when the PPAR-hγ2pΔC/EBP construct was examined in transfection experiments, no induction of promoter activity was observed in response to either C/EBPβ or C/EBPα. While this work was in progress and consistent with our observation on the human promoter, Clarke *et al.* (30) reported that deletion of a similar C/EBP site in the mouse PPARγ2 promoter reduced the inductive effects of C/EBPβ on the mouse PPARγ2 promoter (30). The identification of a C/EBP site within the mouse (30) and human PPARγ2 promoter provides a molecular basis for the observation that ectopic expression of C/EBPβ resulted in an induction of PPARγ mRNA (29). In addition, we extended the observation to show that C/EBPβ induction of PPARγ is specific to PPARγ2 and not PPARγ1 mRNA, thus supporting our hypothesis that PPARγ2 drives the initiation of differentiation. C/EBPα and C/EBPβ have different kinetics of expression during adipogenesis (28). The fact that both these factors were able to induce the human PPARγ2 promoter indicated that the C/EBP family was responsible for the expression of PPARγ2 throughout the process of adipocyte differentiation. C/EBPβ (and possibly C/EBPδ) drove the early induction of PPARγ2 when adipogenesis was initiated. Because C/EBPβ expression declined as differentiation progressed, the increased expression of C/EBPα would be able to maintain the expression of PPARγ2.

To unequivocally implicate C/EBPβ as the effector of the hormone mixture treatment on the human PPARγ2 promoter, transfection experiments were performed using both the human wild-type PPARγ2 promoter and the C/EBP binding site mutant construct. When IBMX and DEX were added in the medium, as expected, a increase in human PPARγ2 promoter activity was observed (Fig. 4). This induction was blunted when the PPARγ2 promoter construct that carried the mutation in the C/EBP site was used (Fig. 4). No significant induction in response to drug treatment was observed for the mutant human PPARγ2 promoter activity, thus ruling out the possibility of a cryptic glucocorticoid response element or other dominant regulatory site in the promoter. These data clearly demonstrated that endogenous C/EBPβ mediated the effect of IBMX on the human PPARγ2 promoter through the C/EBP site. C/EBPδ is expressed in early differentiation. It has been shown that C/EBPδ gene expression could be induced by DEX (27, 28). The fact that DEX was required for maximal activation of the human PPARγ2 promoter indicated a supporting a role for C/EBPδ in early maximal activation of PPARγ2 expression.

In conclusion, this study demonstrates that PPARγ1 and PPARγ2 are differentially regulated during adipocyte differentiation in human preadipocytes and in the mouse 3T3L1 preadipocyte cell line. The early induction observed for PPARγ2 relative to PPARγ1 suggests a key role for the PPARγ2 isoform in driving early events in adipogenesis. This is consistent with the fact that PPARγ2 isoform has greater ligand-independent transcriptional activity and is induced in the preadipocyte before the adipocyte differentiation and determination factor-1/sterol regulatory element-binding protein 1 pathway, which has been proposed to be important for ligand production (31). This work firmly establishes the

hypothesis that PPARγ2 is the important isoform in very early stages of adipogenesis and suggests that isoform activity differences may have profound biological impact. For example, if PPARγ2 can be prevented from being induced, would some or most adipocyte differentiation be inhibited? This work sets the stage for a more comprehensive analysis of the roles of PPARγ1 and PPARγ2 in adipogenesis and obesity. Reagents to selectively inhibit the expression or activity of either isoform have thus far been difficult to prepare and are the subject of ongoing work to elucidate a functional difference between these isoforms.

Materials and Methods

Cell Culture and Differentiation. 3T3-L1 preadipocytes (American Type Culture Collection) were cultured in growth medium containing DMEM and 10% bovine calf serum until they reached confluency. The medium was then changed, and the cells grown for 2 additional days. Adipocyte differentiation was then induced by replacing the growth medium by DMEM supplemented with 10% FBS, 500 μM IBMX (Sigma Chemical Co., St. Louis, MO), 1 μM DEX (Sigma), and 10 μg/ml INS (Sigma). Three days later, medium was replaced with DMEM supplemented with 10% FBS and 10 μg/ml INS, and cell were cultured for 2 additional days. Thereafter, the cells were refed at 48-h intervals with DMEM supplemented with 10% FBS only. The human primary preadipocytes were isolated by approved protocols from excised s.c. adipose tissue of a 37-year-old healthy female undergoing abdominal plastic surgery. The preadipocytes were prepared and cultured based on the procedures reported by Petruschke and Hauner (32). Adipocyte differentiation was induced by replacing the medium with DMEM/Ham's nutrient broth F-10 (1:1, v/v) supplemented with 10% FBS, 500 μM IBMX (Sigma), 1 μM DEX (Sigma), 10 μg/ml INS (Sigma), and 1 μM BRL49653.

RNase Protection Assay. Total mRNA from 3T3-L1 cells and human primary preadipocytes and adipocytes was isolated by the acid, guanidium thiocyanate, phenol, and chloroform method (33). A partial cDNA containing nucleotides 4–273 of mouse PPARγ was subcloned into the pCRII vector (Invitrogen). The pTRI-RNA-28S plasmid (Ambion) was used as a control. *Hin*dIII-linearized plasmids were used to make ^{32}P-labeled antisense riboprobes using the T7 RNA polymerase and Maxiscript *in vitro* transcription kit (Ambion). RNase protection assay was performed using the RPAII kit (Ambion). Band intensities were quantitated on a PhosphoImager (Molecular Dynamics).

Competitive RT-PCR Assay. Each RNA sample was reverse transcribed with oligo(dT) primers using the Superscript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Inc., Gaithersburg, MD) including a DNaseI pretreatment. Competitive PCR was performed on 50 ng of RNA equivalent of cDNA per 50 μl reaction in the presence of varying concentrations of competitor plasmid and using AmpliTaq Gold and the manufacturer's standard buffer (Perkin-Elmer/Roche Molecular Systems, Inc., Branchburg, NJ). Cycling was as described (34), with an additional first step of 10 min at 95°C to activate the AmpliTaq Gold. Amplified products were quantitated by agarose gel electrophoresis in the presence of EtBr, followed by analysis with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Competitor plasmids have been described (34, 35). All PPAR values were normalized to β-microglobulin values to adjust for differences in input RNA and efficiency of cDNA synthesis. PPARγ1 values were obtained by subtracting PPARγ2 from total PPARγ.

Transfection. To analyze the regulation of the human PPARγ promoters, the PPARγ1 and PPARγ2 promoters were each subcloned into the reporter vector pGL3 (Promega). Briefly, a 3Kb *Sac*I/*Xho*I (PPARγ1) and a 1-kb *Sma*I/*Kpn*I (PPARγ2) fragments were inserted in their respective sites in pGL3, resulting in pGL3-hγ1p and pGL3-hγ2p constructs (19). pGL3-hγ1p contains 3000 bp of 5′ promoter sequence, and pGL3-hγ2p contains 1000 bp. The pGL3-hγ2pΔC/EBP was constructed by deleting the CCAATT sequence located at the position −56 of the PPARγ2 promoter by splicing overlapping ends-PCR. 3T3-L1 transfections were carried out in 12-well plates using Superfect reagent (Qiagen). Luciferase and β-galactosidase assays were carried out as described (36).

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