mPPARγ2: tissue-specific regulator of an adipocyte enhancer

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Previously, we have isolated and characterized an enhancer from the 5′-flanking region of the adipocyte P2 (aP2) gene that directs high-level adipocyte-specific gene expression in both cultured cells and transgenic mice. The key regulator of this enhancer is a cell type-restricted nuclear factor termed ARF6. Target sequences for ARF6 in the aP2 enhancer exhibit homology to a direct repeat of hormone response elements (HREs) spaced by one nucleotide; this motif (DR-1) has been demonstrated previously to be the preferred binding site for heterodimers of the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor (PPAR). We have cloned a novel member of the peroxisome proliferator-activated receptor family designated mPPARγ2, and we demonstrate that a heterodimeric complex of mPPARγ2 and RXRα constitute a functional ARF6 complex. Expression of mPPARγ2 is induced very early during the differentiation of several cultured adipocyte cell lines and is strikingly adipose-specific in vivo. mPPARγ2 and RXRα form heterodimers on ARF6-binding sites in vitro, and antiserum to RXRα specifically inhibits ARF6 activity in adipocyte nuclear extracts. Moreover, forced expression of mPPARγ2 and RXRα activates the adipocyte-specific aP2 enhancer in cultured fibroblasts, and this activation is potentiated by peroxisome proliferators, fatty acids, and 9-*cis* retinoic acid. These results identify mPPARγ2 as the first adipocyte-specific transcription factor and suggest mechanisms whereby fatty acids, peroxisome proliferators, 9-*cis* retinoic acid, and other lipids may regulate adipocyte gene expression and differentiation.

[*Key Words*: Adipocyte transcription factor; adipocyte P2 enhancer; ARF6; peroxisome proliferator-activated receptor; retinoid X receptor; adipocyte differentiation]

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The development of functionally and morphologically distinct cell types in higher eukaryotes results from the execution of an elaborate program of differential gene expression. In contrast with certain other cell types, relatively little is known about the regulatory mechanisms that direct adipocyte-specific gene expression. Although several factors, including CCAAT/enhancer-binding protein (C/EBPα) (Christy et al. 1989; Herrera et al. 1989) and the recently described basic helix–loop–helix (bHLH)–leucine zipper protein ADD1 (Tontonoz et al. 1993), may play roles in the regulation of certain adipocyte genes, transcription factors exhibiting true specificity for adipose cells have not been described. Several lines of evidence indicate that C/EBPα may play a role in terminal adipocyte differentiation (Freytag and Geddes 1992; Umek et al. 1991). However, because C/EBPα mRNA is induced rather late in the time course of differentiation and is expressed in liver, lung, kidney, and intestine, as well as fat, it is unlikely to play a substantial role in either the initiation of the adipocyte program or the establishment of tissue specificity.

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The murine adipocyte P2 (aP2) gene encodes an intracellular lipid-binding protein and is expressed exclusively in adipose cells. We and others have been studying the transcriptional regulation of this gene in an effort to understand the molecular basis for its adipocyte-specific expression. Early studies demonstrated that the proximal promoter region (168 bp of the 5′ flank), which contains binding sites for transcription factors AP-1 and C/EBP, could direct low-level differentiation-dependent gene expression in cultured 3T3–F442A adipocytes (Christy et al. 1989; Herrera et al. 1989) but could not support adipose expression in transgenic mice (Ross et al. 1990). We subsequently searched farther upstream and identified a 518-bp DNA fragment extending from −5.4 to −4.9 kb that could direct high level adipose-specific expression of a chloramphenicol acetyl transferase (CAT) reporter gene in transgenic mice as well as cultured cells (Ross et al. 1990; Graves et al. 1991). Since its identification, this enhancer has been used in transgenic animals to direct adipose-specific expression of a number of genes that alter the biological function of this tissue, including SV40 large T antigen (Ross et al. 1992), diphtheria toxin A chain (Ross et al. 1993), and the insulin-sensitive glucose transporter GLUT4 (Shepherd et al. 1994).

Multiple *cis*-acting elements within the 518-bp enhancer have been shown to be important for its differentiation-dependent activity in cultured 3T3–F442A preadipocytes and adipocytes. Initial studies identified a binding site (ARE1) for a member of the NF-1 transcription factor family. This NF-1 site appears to contribute to the overall activity of the enhancer, but it is not functional as an isolated element, and the enhancer retains differentiation-dependent activity in the absence of this site (Graves et al. 1991). Further analysis established the existence of four additional *cis*-acting elements, each of which is also required for full enhancer activity in transient transfection assays (Graves et al. 1992). One pair of elements, ARE2 and ARE4, binds an apparently ubiquitous positive-acting factor termed ARF2. A second pair of elements, ARE6 and ARE7, binds a separate nuclear factor, termed ARF6, that is detected only in nuclear extracts derived from adipocytes. Multiple copies of either ARE6 or ARE7 activate transcription only in differentiated adipocytes. The observation that the ARF6-binding sites are both necessary and sufficient for adipocyte-specific expression suggests that ARF6 functions as a differentiation-dependent and tissue-specific switch for the aP2 enhancer.

In this paper we report the identification and cloning of a nuclear hormone receptor, mPPARγ2, that appears to be an important component of the ARF6 complex. Expression of mPPARγ2 is highly specific for adipose tissue and is sufficient to activate the adipocyte-specific aP2 enhancer in heterologous cell types. We suggest that this novel peroxisome proliferator-activated receptor (PPAR) family member may function as a key regulator of adipogenic gene expression.

Results

ARF6-binding sequences in the aP2 enhancer are DR-1-type hormone response elements

We have used partially purified ARF6 derived from adipocyte nuclear extracts to characterize the DNA recognition sequence for this factor in detail. Initial comparison of the two binding sites for ARF6 in the aP2 enhancer (ARE6 and ARE7, Fig. 1A) identified a 10- out of 12-bp identity (Graves et al. 1992). Methylation interference analysis performed on the ARE6 and ARE7 sites indicated that the actual region of DNA–protein interaction extends significantly 5′ to the region of obvious homology. Guanosine residues identified as sites of DNA–protein contact are indicated by asterisks in Figure 1A. Extensive mutational analysis of the ARE7-binding site further defined the bases important for ARF6 binding. The effect of individual transversion mutations on ARF6 binding to the ARE7 site is diagramed schematically in Figure 1B.

This functional analysis of the ARE7 site reveals a homology between the ARF6 recognition sequence and the consensus nuclear hormone response element (HRE) half-site TGA$^{A}/_{C}$CT. Binding sites for members of the nuclear hormone receptor superfamily are typically composed of two HRE half-sites, arranged with a particular orientation and spacing. Various arrangements of HREs have been shown to preferentially recognize different nuclear hormone receptor combinations (Umesono et al. 1991; Kliewer et al. 1992b). The ARF6 recognition sequence can be interpreted as an imperfect version of a type of nuclear hormone receptor-binding site known as DR-1 (direct repeat with 1-nucleotide spacer). This motif has been shown to preferentially bind homodimers of retinoic acid X receptor (RXR) (Mangelsdorf et al. 1991) or the liver-restricted transcription factor HNF4 (Sladek et al. 1990), heterodimers of RXR and COUP-TF (Kliewer et al. 1992a), and heterodimers of RXR and the PPARs (Kliewer et al. 1992c). An alignment of the consensus DR-1 site with ARE6 and ARE7 is shown in Figure 2A. DNA mobility retardation experiments using various HRE sequences as competitor demonstrated that ARF6 preferentially recognizes DR-1 sites. As shown in Figure 2B, the consensus DR-1 sequence and the fatty acyl-CoA oxidase peroxisome proliferator response element (PPRE) are optimal competitors for ARF6 binding to the ARE7 site.

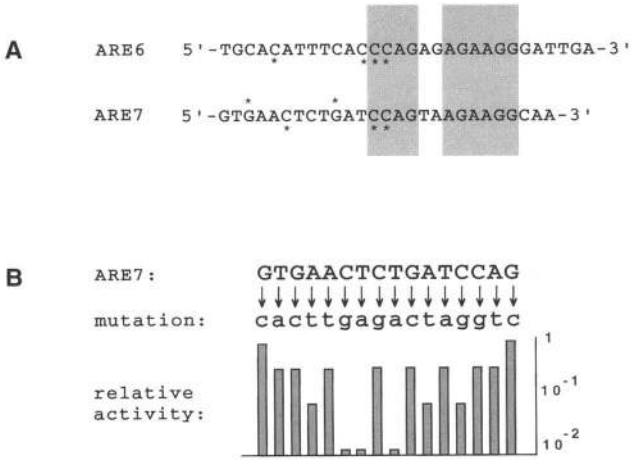

Figure 1. Definition of a functional ARF6-binding site. (*A*) Nucleotide sequence comparison of the ARE6 and ARE7 sites from the aP2 enhancer. Previously identified regions of homology are shaded. DNA–protein contact points identified by methylation interference analysis are indicated by asterisks (*). (*B*) Mutational analysis of the ARE7 site. The effect of individual transversion mutations on ARF6 binding is diagramed schematically.

RXRα is a component of the ARF6 complex

The above data strongly suggest that at least one component of ARF6 is a member of the nuclear hormone receptor superfamily. To determine whether the ARF6 complex contained proteins known to bind DR-1 sites, we obtained antisera to several such factors. DNA mobility retardation assays using labeled ARE7 oligonucleotide as probe were performed with nuclear extract and partially purified ARF6 in the presence of these antisera. As shown in Figure 3, antisera to the retinoic acid receptor α (RARα) and to murine PPARα have no effect on

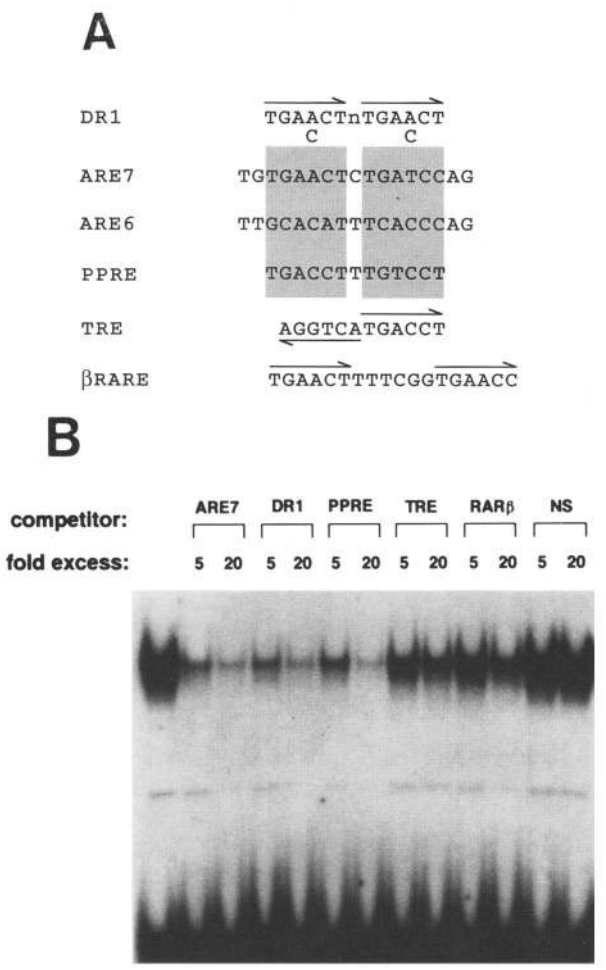

Figure 2. Relationship of ARF6-binding sites to HREs. (*A*) Sequence comparison of ARE6 and ARE7 with the consensus DR-1 HRE, fatty acyl-CoA oxidase PPRE, palindromic thyroid hormone response element (TREp), and the retinoic acid response element from RARβ (βRARE). (*B*) ARF6 preferentially binds DR-1-type HREs. Double-stranded radiolabeled ARE7 oligonucleotide was used as probe in a DNA mobility retardation assay of partially purified ARF6 from adipocyte nuclear extract. DNA protein complexes were resolved from free DNA on a 5% polyacrylamide gel. The gel was dried and exposed to film for 2 hr at −70°C. The HREs shown in *A* were used as competitors in the binding reaction in 5- and 20-fold molar excess as indicated. The ARE2 site from the aP2 enhancer was used a nonspecific competitor (NS). (See Materials and methods for complete sequence of the oligonucleotides used.)

ARF6 binding. Antisera to RXRα, however, specifically inhibit ARF6 complex formation and give a characteristic "supershift" when incubated with either nuclear extract or partially purified ARF6. This antisera were raised to a single peptide specific to RXRα (Kliewer et al. 1992c). Thus, at least one of the polypeptides comprising ARF6 is RXRα itself or a very closely related molecule.

Cloning of a novel PPAR from adipose tissue

The ARF6 complex is unlikely to be a homodimer of RXRα, because such dimers bind to DNA relatively poorly and bind only in the presence of the ligand 9-*cis*-acting retinoic acid (Zhang et al. 1992). Although the antisera supershift data above indicated that mPPARα was not present in the ARF6 complex, three distinct PPAR family members with different tissue distributions have been described in *Xenopus*, including one (xPPARγ) that is expressed at high levels in the fat body (Dreyer et al. 1992). We hypothesized that the partner for RXRα in the ARF6 complex might be an adipose-expressed member of the PPAR family. We therefore designed primers for the polymerase chain reaction (PCR) to specifically amplify sequences encoding RXR- and PPAR-related receptors from adipocyte cDNA (see Materials and methods). Products from these PCR reactions were subcloned and sequenced. Of 20 clones isolated, 2 encoded mPPARα, three encoded the murine homolog of the PPAR family member NUC-1, and 15 were found to encode a novel member of the PPAR family subsequently named PPARγ2. Full-length mPPARγ2 cDNA clones were subsequently isolated from an adipocyte λZapII cDNA library using the PCR-amplified fragment as probe.

The DNA and predicted amino acid sequence of the longest of these clones is shown in Figure 4. It potentially encodes a 505-amino-acid protein with a predicted molecular mass of 55.6 kD. The 5′-most ATG codon in this sequence is in an appropriate context for translational initiation. Primer extension experiments indicated that there are only an additional 25 bp 5′ in the mRNA that are not present in this clone (data not shown). Data base searches revealed that the receptor encoded by this cDNA is a murine homolog of *Xenopus*

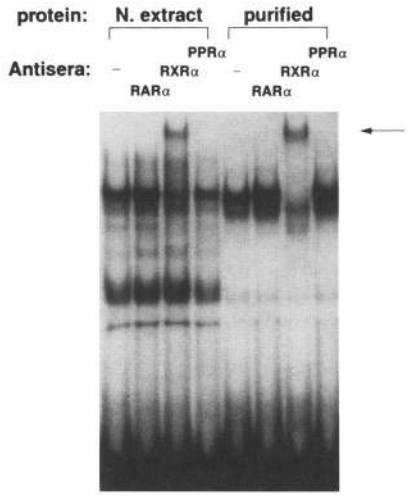

Figure 3. RXRα is a component of the ARF6 complex. Double-stranded radiolabeled ARE7 oligonucleotide was used as probe in a DNA mobility retardation assay of adipocyte nuclear extract and partially purified ARF6. Antisera to RARα, RXRα, or PPARα were included in the binding reaction as indicated. DNA protein complexes were resolved from free DNA on a 5% polyacrylamide gel. The gel was dried and exposed to film for 2 hr at −70°C.

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CCC AGT GTG AAT TAC AGC AAA TCT CTG TTT TAT GCT GTT ATG GGT GAA ACT CTG GGA GAT TCT CCT GTT GAC    72
                                                    M   G   E   T   L   G   D   S   P   V   D     11

CCA GAG CAT GGT GCC TTC GCT GAT GCA CTG CCT ATG AGC ACT TCA CAA GAA ATT ACC ATG GTT GAC ACA GAG   144
 P   E   H   G   A   F   A   D   A   L   P   M   S   T   S   Q   E   I   T   M   V   D   T   E    35

ATG CCA TTC TGG CCC ACC AAC TTC GGA ATC AGC TCT GTG GAC CTC TCC GTG ATG GAA GAC CAC TCG CAT TCC   216
 M   P   F   W   P   T   N   F   G   I   S   S   V   D   L   S   V   M   E   D   H   S   H   S    59

TTT GAC ATC AAG CCC TTT ACC ACA GTT GAT TTC TCC AGC ATT TCT GCT CCA CAC TAT GAA GAC ATT CCA TTC   288
 F   D   I   K   P   F   T   T   V   D   F   S   S   I   S   A   P   H   Y   E   D   I   P   F    83

ACA AGA GCT GAC CCA ATG GTT GCT GAT TAC AAA TAT GAC CTG AAG CTC CAA GAA TAC CAA AGT GCG ATC AAA   360
 T   R   A   D   P   M   V   A   D   Y   K   Y   D   L   K   L   Q   E   Y   Q   S   A   I   K   107

GTA GAA CCT GCA TCT CCA CCT TAT TAT TCT GAA AAG ACC CAG CTC TAC AAC AGG CCT CAT GAA GAA CCT TCT   432
 V   E   P   A   S   P   P   Y   Y   S   E   K   T   Q   L   Y   N   R   P   H   E   E   P   S   131

AAC TCC CTC ATG GCC ATT GAG TGC CGA GTC TGT GGG GAT AAA GCA TCA GGC TTC CAC TAT GGA GTT CAT GCT   504
 N   S   L   M   A   I   E   C   R   V   C   G   D   K   A   S   G   F   H   Y   G   V   H   A   155

TGT GAA GGA TGC AAG GGT TTT TTC CGA AGA ACC ATC CGA TTG AAG CTT ATT TAT GAT AGG TGT GAT CTT AAC   576
 C   E   G   C   K   G   F   F   R   R   T   I   R   L   K   L   I   Y   D   R   C   D   L   N   179

TGC CGG ATC CAC AAA AAA AGT AGA AAT AAA TGT CAG TAC TGT CGG TTT CAG AAG TGC CTT GCT GTG GGG ATG   648
 C   R   I   H   K   K   S   R   N   K   C   Q   Y   C   R   F   Q   K   C   L   A   V   G   M   203

TCT CAC AAT GCC ATC AGG TTT GGG CGG ATG CCA CAG GCC GAG AAG GAG AAG CTG TTG GCG GAG ATC TCC AGT   720
 S   H   N   A   I   R   F   G   R   M   P   Q   A   E   K   E   K   L   L   A   E   I   S   S   227

GAT ATC GAC CAG CTG AAC CCA GAG TCT GCT GAT CTG CGA GCC CTG GCA AAG CAT TTG TAT GAC TCA TAC ATA   792
 D   I   D   Q   L   N   P   E   S   A   D   L   R   A   L   A   K   H   L   Y   D   S   Y   I   251

AAG TCC TTC CCG CTG ACC AAA GCC AAG GCG AGG GCG ATC TTG ACA GGA AAG ACA ACG GAC AAA TCA CCA TTT   864
 K   S   F   P   L   T   K   A   K   A   R   A   I   L   T   G   K   T   T   D   K   S   P   F   275

GTC ATC TAC GAC ATG AAT TCC TTA ATG ATG GGA GAA GAT AAA ATC AAG TTC AAA CAT ATC ACC CCC CTG CAG   936
 V   I   Y   D   M   N   S   L   M   M   G   E   D   K   I   K   F   K   H   I   T   P   L   Q   299

GAG CAG AGC AAA GAG GTG GCC ATC CGA ATT TTT CAA GGG TGC CAG TTT CGA TCC GTA GAA GCC GTG CAA GAG  1008
 E   Q   S   K   E   V   A   I   R   I   F   Q   G   C   Q   F   R   S   V   E   A   V   Q   E   323

ATC ACA GAG TAT GCC AAA AAT ATC CCT GGT TTC ATT AAC CTT GAT TTG AAT GAC CAA GTG ACT CTG CTC AAG  1080
 I   T   E   Y   A   K   N   I   P   G   F   I   N   L   D   L   N   D   Q   V   T   L   L   K   347

TAT GGT GTC CAT GAG ATC ATC TAC ACG ATG CTG GCC TCC CTG ATG AAT AAA GAT GGA GTC CTC ATC TCA GAG  1152
 Y   G   V   H   E   I   I   Y   T   M   L   A   S   L   M   N   K   D   G   V   L   I   S   E   371

GGC CAA GGA TTC ATG ACC AGG GAG TTC CTC AAA AAC CTG CGG AAG CCC TTT GGT GAC TTT ATG GAG CCT AAG  1224
 G   Q   G   F   M   T   R   E   F   L   K   N   L   R   K   P   F   G   D   F   M   E   P   K   395

TTT GAG TTT GCT GTG AAG TTC AAT GCA CTG GAA TTA GAT GAC AGT GAC TTG GCT ATA TTT ATA GCT GTC ATT  1296
 F   E   F   A   V   K   F   N   A   L   E   L   D   D   S   D   L   A   I   F   I   A   V   I   419

ATT CTC AGT GGA GAC CGC CCA GGC TTG CTG AAC GTG AAG CCC ATC GAG GAC ATC CAA GAC AAC CTG CTG CAG  1368
 I   L   S   G   D   R   P   G   L   L   N   V   K   P   I   E   D   I   Q   D   N   L   L   Q   443

GCC CTG GAA CTG CAG CTC AAG CTG AAT CAC CCA GAG TCC TCT CAG CTG TTC GCC AAG GTG CTC CAG AAG ATG  1440
 A   L   E   L   Q   L   K   L   N   H   P   E   S   S   Q   L   F   A   K   V   L   Q   K   M   467

ACA GAC CTC AGG CAG ATC GTC ACA GAG CAC GTG CAG CTA CTG CAT GTG ATC AAG AAG ACA GAG ACA GAC ATG  1512
 T   D   L   R   Q   I   V   T   E   H   V   Q   L   L   H   V   I   K   K   T   E   T   D   M   491

AGC CTT CAC CCC CTG CTC CAG GAG ATC TAC AAG GAC TTG TAT TAG CAGGAAAGTCCCACCCGCTGACAACGTGTTCCTTC  1592
 S   L   H   P   L   L   Q   E   I   Y   K   D   L   Y   *                                       505

TATTGATTGCACTATTATTTTGAGGGAAAAAAATCTGACACCTAAGAAATTTACTGTGAAAAAGCATTTAAAAACAAAAGTTTTAGAAGATGATG  1687

ATCTATTTTATGCATATTGTTTATAAAGATACATTTACAATTTACTTTTAATATTAAAAATTACCACATTATAAAATTAAAAAAAAAAAAAAA  1780
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Figure 4. Nucleotide and corresponding amino acid sequence of the mPPARγ2 cDNA. The putative polyadenylation signal and initiation codons are shown in boldface type.

PPARγ (Dreyer et al. 1992). A cDNA encoding a different isoform of this PPAR was described while this manuscript was in preparation (Zhu et al. 1993). We have therefore designated this novel isoform mPPARγ2. The mPPARγ2 cDNA encodes an additional 30 amino acids amino-terminal to the first ATG codon of mPPARγ1 and has a different 5′-untranslated sequence (diagramed schematically in Fig. 5A). The ATG codon at position 31 represents the 5′-most ATG codon in the PPARγ1 cDNA. In vitro translation of the mPPARγ2 cDNA yields two major polypeptide species of relative molecular mass 56 and 52 kD (Fig. 5B, mPPARγ2), indicating that the ATG codons at positions 1 and 31 can both function as translational initiators. Translation of the PPARγ1 cDNA yields only the 52-kD species (mPPARγ1). We sequenced the 5′ ends of five separate cDNA clones, all of which encoded the mPPARγ2 isoform, suggesting it is the predominant form expressed in adipose tissue (see below).

mPPARγ mRNA is expressed at high levels specifically in adipose tissue

It has been demonstrated previously that ARF6-binding activity is differentiation-dependent in cultured adipocytes (Graves et al. 1992). Because RXRα mRNA is present in both preadipocytes and adipocytes (data not shown), it was likely that another component of the ARF6 complex would exhibit differentiation-dependent expression. We therefore examined the time course of mPPARγ expression during the differentiation of cultured 3T3–F442A and 3T3–L1 preadipocytes. 3T3–F442A cells differentiate spontaneously at confluence in presence of insulin and permissive serum, whereas differentiation of 3T3–L1 cells requires induction with methylisobutylxanthine and dexamethasone (see Materials and methods). Cultured adipocytes derived from these cell lines accumulate lipid and express most of the genes characteristic of adipose cells in vivo (Green and Ke-

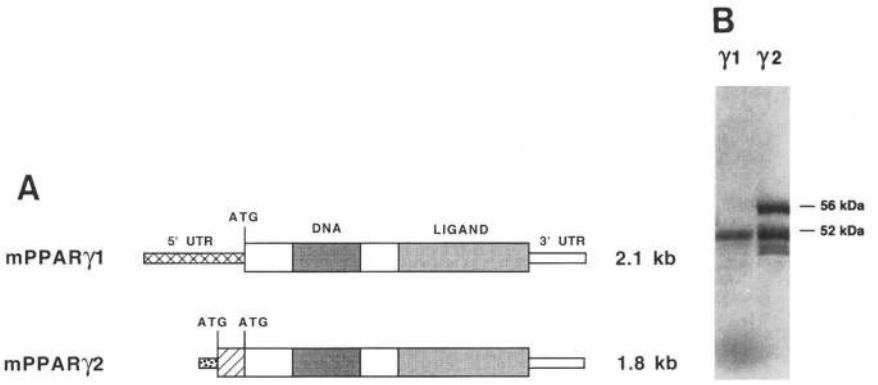

Figure 5. Structure of mPPARγ isoforms. (*A*) Schematic representation of the mPPARγ1 and mPPARγ2 cDNAs. Identical sequences are indicated by similar shading. (*B*) In vitro translation of the mPPARγ1 and mPPARγ2 cDNAs. mPPARγ1–SPORT and mPPARγ2–SPORT plasmids were translated by rabbit reticulocyte lysate in the presence of L[^{35}S]methionine. Translation products were resolved on an 9.0% SDS–polyacrylamide gel.

hinde 1974). As shown in Figure 6, the mPPARγ mRNA in adipocytes migrates as a single species of 2.0 kb. Although the mPPARγ cDNA probe used for Northern analysis hybridizes to both the mPPARγ1 and mPPARγ2 isoforms, S1 nuclease analysis indicates that the mPPARγ mRNA present in adipose cells encodes almost exclusively the mPPARγ2 isoform (data not shown). mPPARγ2 mRNA levels are induced very early (1–2 days postconfluence) in the course of differentiation of both 3T3–F442A and 3T3–L1 preadipocytes. This induction is coincident with the induction of lipoprotein lipase (LPL), one of the earliest markers of adipocyte differentiation, and clearly precedes the induction of aP2.

The tissue distribution of mPPARγ and RXRα was examined using RNA prepared from a variety of mouse tissues. RXRα mRNA has been demonstrated previously to be expressed in a number of tissues (Mangelsdorf et al. 1992). Figure 7A (bottom) shows that that RXRα expression is especially prominent in adipose tissue and liver. In contrast, mPPARγ is expressed at very high levels only in adipose tissue [(F) fat, Fig. 7A, (top)]. As noted above, this mRNA encodes predominently the PPARγ2 isoform. Expression is at least 20-fold higher in adipose tissue than all other tissues examined. A prolonged exposure of this blot also reveals minimal expression in heart, skeletal muscle, kidney, and liver (Fig. 7B, top). At this level of signal, however, one must be concerned about unavoidable contamination of these tissues with adipose cells. To address this issue, this same blot was probed with the cDNA for aP2, which has been shown previously to be expressed exclusively in fat (Bernlohr et al. 1984). Figure 7B (bottom) demonstrates that the heart, kidney, and skeletal muscle samples do contain significant amounts of aP2 mRNA, suggesting contamination with small numbers of adipose cells. It is therefore likely that even some of the minimal mPPARγ expression observed in nonadipose tissues actually results from fat cells present in these tissues. Northern analysis of rat tissue RNA indicates that PPARγ2, like aP2, is expressed in both white and brown adipose tissue (data not shown).

mPPARγ2/RXRα heterodimers bind to ARF6-binding sites in the aP2 enhancer

To determine whether PPARγ2 could bind to ARF6-

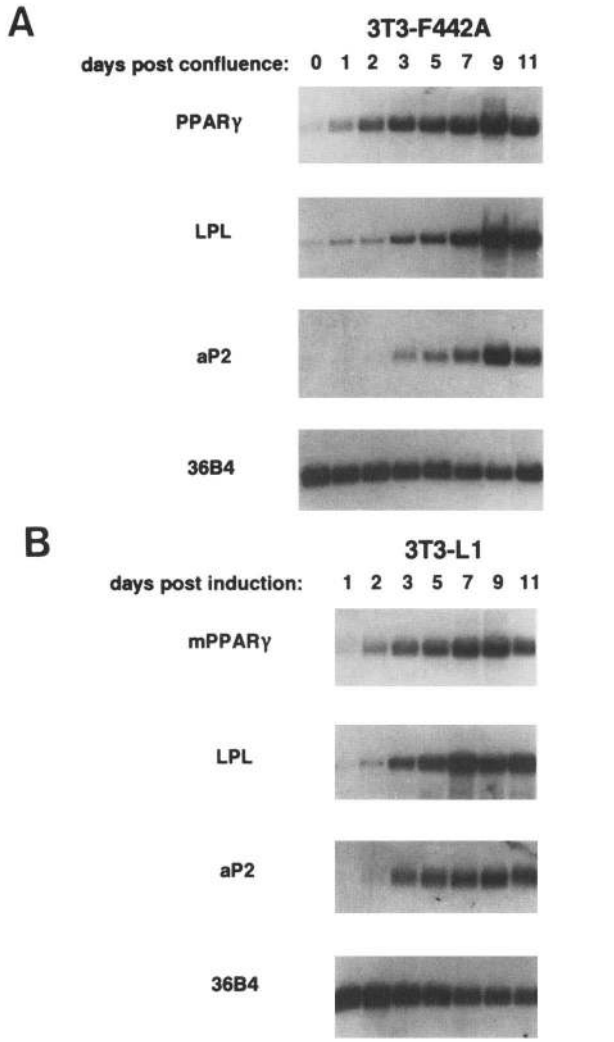

Figure 6. Regulation of mPPARγ mRNA in two cell culture models of adipocyte differentiation. Total RNA (10 μg per lane) was isolated, blotted to nylon, and hybridized with ^{32}P-labeled mPPARγ2 cDNA as described in Materials and methods. Equivalent amounts of intact RNA were run in each lane as indicated by hybridization to a 36B4 cDNA probe. (*A*) Time course of differentiation of 3T3–F442A preadipocytes; (*B*) Time course of differentiation of 3T3–L1 preadipocytes.

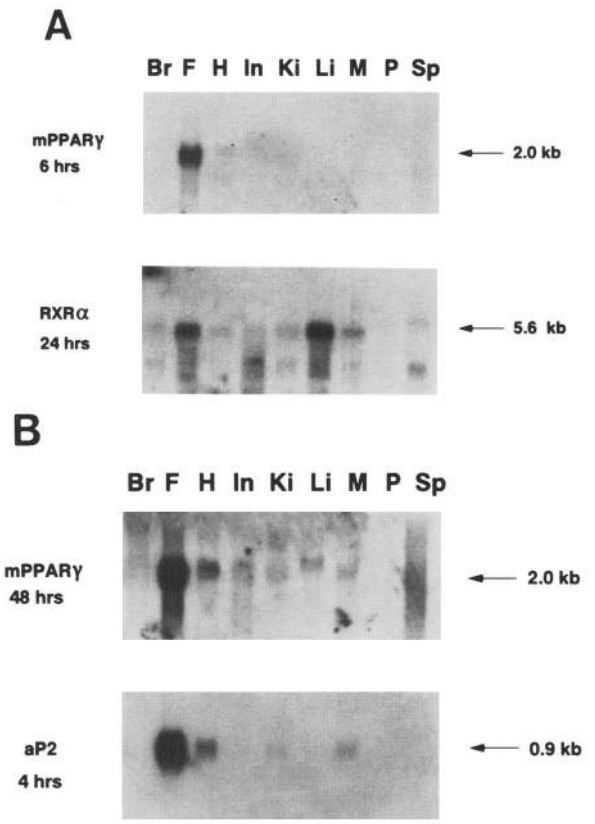

Figure 7. mPPARγ is expressed with adipose specificity. (*A*) mPPARγ, 6-hr exposure; RXRα, 24-hr exposure. (*B*) mPPARγ, 48-hr exposure; aP2, 4-hr exposure. Total RNA (10 μg per lane) was isolated from adult mouse tissues, blotted to nylon, and hybridized with ^{32}P-labeled mPPARγ2 or RXRα cDNA as described in Materials and methods. Note: The mPPARγ2 probe recognizes both the mPPARγ1 and mPPARγ2 isoforms. (B) Brain; (F) epidymal white fat; (H) heart; (In) intestine; (Ki) kidney; (Li) liver; (M) skeletal muscle; (P) pancreas; (Sp) spleen. Equivalent amounts of intact RNA were run in each lane as indicated by ethidium bromide staining of the membrane after transfer and hybridization to a 36B4 cDNA probe (not shown).

binding sequences from the aP2 enhancer, DNA mobility retardation assays were performed with in vitro-translated protein and labeled ARE7 oligonucleotide. Because RXRs have been demonstrated to be required cofactors for binding of PPARs to DNA (Keller et al. 1993; Kliewer et al. 1992c), RXRα was analyzed in parallel. As shown in Figure 8, neither RXRα nor mPPARγ2 binds independently to ARE7 (lanes 3,4); however, a prominent complex is formed when both mPPARγ2 and RXRα are present in the binding reaction (lane 5). This complex migrates with precisely the same mobility as the ARF6 complex from adipocyte nuclear extract (lane 1), and, as expected, complex formation can be inhibited by antisera to RXRα but not by antisera to mPPARα (lanes 6,7). As has been demonstrated previously (Graves et al. 1992), ARF6-binding activity is not present in preadipocyte nuclear extracts (lane 8). To determine whether RXRα or mPPARγ2 is the limiting component of the complex before differentiation, in vitro-translated proteins were added to preadipocyte nuclear extract. No complex is formed on ARE7 with preadipocyte nuclear extract plus in vitro-translated RXRα (lane 9). However, the addition of in vitro-translated mPPARγ2 to preadipocyte nuclear extract generates an ARF6-like complex (lane 10), and this complex is disrupted by antisera to RXRα (lane 11). These data strongly suggest that mPPARγ2 is the stoichiometrically limiting component of the ARF6 complex in preadipocytes.

PPARγ2 activates the aP2 enhancer through ARF6-binding sites

The above data demonstrate that the DNA-binding activity of the PPARγ2/RXRα heterodimer is virtually identical to that of the adipocyte factor ARF6. Because ARF6 has been suggested previously to be the key regulator of the aP2 enhancer (Graves et al. 1992), we investigated whether PPARγ2 and RXRα could activate the aP2 enhancer in nonadipose cells. Each of these cDNAs was introduced into pSV-SPORT, an expression vector driven by the SV40 enhancer/promoter (see Materials and methods), and transient transfection experiments were performed in NIH–3T3 cells. As shown in Figure 9, cotransfection of either the RXRα expression vector (3.5-fold activation above basal, lane 2) or the mPPARγ2 expression vector (4-fold activation above basal, lane 3) stimulated transcription of a CAT reporter construct containing the 518-bp aP2 enhancer linked to the SV40 enhancerless promoter (Graves et al. 1992). Transfection

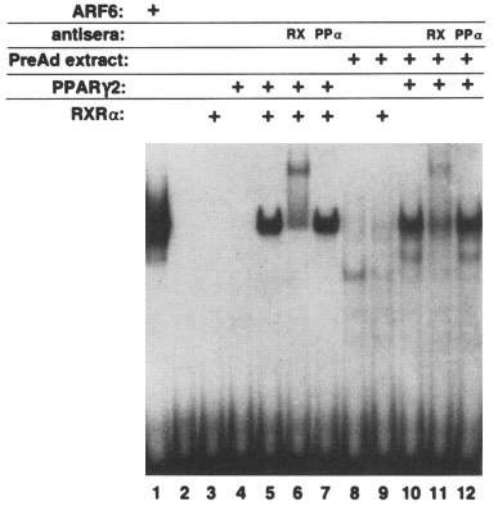

Figure 8. mPPARγ2 and RXRα bind to the ARE7 site from the aP2 enhancer as a heterodimer. Double-stranded ^{32}P-labeled ARE7 oligonucleotide was used as probe in a DNA mobility retardation assay with partially purified ARF6, preadipocyte nuclear extract, and in vitro-translated mPPARγ2 and RXRα. Antisera to RXRα (RXα) or mPPARα (PPα) were included in the binding reaction where indicated. DNA protein complexes were resolved from free DNA on a 5% polyacrylamide gel. The gel was dried and exposed to film for 1 hr at −70°C.

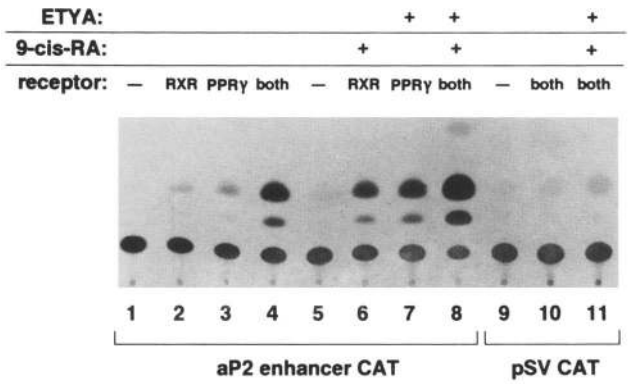

Figure 9. mPPARγ2 and RXRα activate the aP2 enhancer in nonadipose cells. NIH–3T3 cells were cotransfected with either the aP2 enhancer CAT contruct or the parental pSVKS1 CAT construct (2 μg) and mPPARγ2 and/or RXRα expression vector (2 μg) as described in Materials and methods. After transfection, cells were treated for 24 hr with 1 μM 9-*cis* retinoic acid or 1 μM ETYA as indicated. The level of CAT gene expression resulting from each transfection was determined by measuring CAT enzyme activity.

of both constructs simultaneously had an apparently synergistic effect (15-fold activation above basal, lane 4). It has been shown previously that the transcriptional activating function of both RXRs and PPARs is strongly dependent on the presence of specific ligands and/or activators (Keller et al. 1993, Kliewer et al. 1992c). Transcriptional activation of the aP2 enhancer by RXRα is enhanced when the cells are treated with its high-affinity ligand 9-*cis* retinoic acid (lane 6); activation by PPARγ2 is enhanced when cells are treated with 5,8,11,14-eicosatetraynoic acid (ETYA, lane 7), a synthetic arachadonic acid analog and potent activator of PPAR family members. Simultaneous addition of both receptors and their activators has a maximal effect (lane 8). No significant activation of the basal SV40 promoter CAT construct (pSV CAT) is observed even in the presence of both activators (lanes 9–11).

The 518-bp aP2 enhancer is a complex regulator containing multiple *cis*-acting elements. The above data demonstrate that mPPARγ2 and RXRα can dominantly activate the aP2 enhancer but do not establish that this activation is mediated by the ARF6-binding sites. We therefore tested the ability of mPPARγ2 and RXRα to activate transcription of a CAT construct containing multiple copies of an isolated ARF6-binding sequence. We have shown previously that mutiple copies of these sequences (ARE6 and ARE7) stimulate adipocyte-specific expression of a CAT reporter gene in cultured cells (Graves et al. 1992). Figure 10 demonstrates that RXRα and mPPARγ2 can each independently stimulate transcription of the ARE7/CAT reporter construct in the presence of their appropriate activators (lanes 2,5). Again, simultaneous expression of both receptors yields a maximal effect (lane 6). Previous studies have suggested that fatty acids may stimulate transcription of aP2 in adipocytes (Amri et al. 1991). Because other PPAR family members have been shown to be activated by fatty acids (Keller et al. 1993), we investigated whether mPPARγ2 could be similarly activated. Figure 10 demonstrates that mPPARγ2 can be effectively activated by linoleic acid (lane 3) and by the peroxisome proliferator clofibric acid (lane 4), as well as by ETYA. No significant activation is observed when the basal pSVKS1 CAT construct is used as the reporter (lanes 8,9). These data demonstrate that mPPARγ2 can function as a dominant, activator-dependent regulator of an adipose-specific enhancer, presumably through formation of a heterodimeric DNA-binding complex with RXRα.

Discussion

The PPARs comprise a subfamily of nuclear hormone receptors first identified by virtue of their ability to be activated by certain hypolipidemic drugs, plasticizers, and herbicides that cause proliferation of peroxisomes in rodent liver (Isseman and Green 1990). Such compounds are also known to induce enzymes of the peroxisomal fatty acid β-oxidation system. PPARs have subsequently been shown to directly activate transcription of the acyl-CoA oxidase gene, which catalyzes the rate-limiting step in the β-oxidation pathway (Dreyer et al. 1992). In addition to the original murine PPAR (mPPARα), three PPARs have been identified in *Xenopus* (xPPAR α,β, and γ), one in rat (rPPAR, an α homolog), and two in human (hPPARα and hPPAR/NUC-1, a β homolog). We have identified a novel murine PPAR termed mPPARγ2 that is expressed at high levels in adipose tissue. A different isoform of mPPARγ, which we propose be referred to as mPPARγ1, has recently been described independently by Zhu et al. (1993). It remains to be determined whether

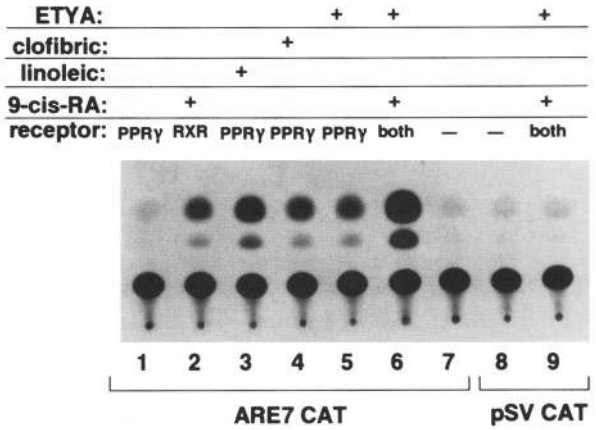

Figure 10. mPPARγ2 and RXRα activate transcription through the ARE7 site from the aP2 enhancer. NIH–3T3 cells were cotransfected with either the ARE7 CAT contruct or the parental pSVKS1 CAT construct (2 μg) and mPPARγ2 and/or RXRα expression vector (2 μg). After transfection, cells were treated for 24 hr with 1 μM 9-*cis* retinoic acid, 50 μM linoleic acid, 50 μM clofibric acid, or 1 μM ETYA as indicated. The level of CAT gene expression resulting from each transfection was determined by measuring CAT enzyme activity.

each isoform has a specific biological role. Multiple isoforms resulting from alternative promoter usage and differential splicing have been described for numerous other nuclear hormone receptors, including the progesterone receptor and RARα, RARβ, and RARγ (Kastner et al. 1990; Chambon et al. 1991). Because mPPARγ1 and mPPARγ2 have different 5′-untranslated sequences, their mRNAs are most likely transcribed from different promoters. While mPPARγ1 and mPPARγ2 represent the first examples of isoforms in the PPAR family, it is likely that multiple isoforms will be identified for other PPARs as well.

Previously, we identified a *trans*-acting factor termed ARF6 as the key factor controlling the fat-specific expression of the aP2 gene (Graves et al. 1992). In the present work we demonstrate that mPPARγ2 forms heterodimers with RXRα and that the DNA-binding and transcriptional activation properties of the mPPARγ2/RXRα complex are identical to those of the adipocyte transcription factor ARF6. The mPPARγ2/RXRα heterodimer binds to ARF6 target sequences in vitro and can activate expression of the fat-specific aP2 enhancer in nonadipose cells in transient transfections. Maximal activation is observed when both receptors and their activators are present, suggesting that the mPPARγ2/RXRα heterodimer is the functional ARF6 complex in vivo.

While all members of the PPAR family described thus far have identical DNA recognition sequences, and thus would all be expected to bind to ARF6 sites in vitro, several lines of evidence strongly suggest that mPPARγ2 in particular is a major regulator, if not the dominant regulator of the aP2 enhancer in adipocytes. First, mPPARγ mRNA is very abundant as determined by both Northern blotting and the screening of cDNA libraries. Second, it exhibits adipose cell specificity consistent with the expression of ARF6 and the aP2 mRNA itself. mPPARγ mRNA is at least 20 times more abundant in white and brown fat than any other tissue. Third, the mPPARγ mRNA expressed in adipose tissue encodes almost exclusively the mPPARγ2 isoform. Fourth, mPPARγ2 forms a complex with RXRα in vitro that migrates with precisely the same mobility in electrophoretic DNA-binding assays as ARF6 from cultured adipocytes. Finally, the other two known murine PPARs, mPPARα and mNUC-1, are expressed at much lower levels in adipose tissue and are not tissue-specific (Isseman and Green 1990; E. Hu, unpubl.). While the existence of additional adipose-specific PPAR family members cannot be ruled out, extensive PCR analysis of fat cell mRNA with oligonucleotides designed to amplify such molecules has failed to identify additional PPARs. Preliminary analysis of peptide sequences derived from affinity-purified ARF6 preparations indicates that a major polypeptide present in this material is PPARγ (P. Tontonoz, unpubl.).

If mPPARγ2 plays a broad role in the regulation of adipogenesis, it will likely be implicated in the regulation of other adipocyte-specific genes in addition to aP2. We have recently identified an ARF6-binding site in the 5′-flanking region of the phosphoenolpyruvate carboxykinase (PEPCK) gene, which is expressed in a differentiation-dependent manner in adipose cells (P. Tontonoz, E. Beale, and B.M. Spiegelman, unpubl.). This binding site is located within a region demonstrated previously to be important for adipose expression in transgenic mice (Short et al. 1992). Our definition of the ARF6 recognition sequence as a DR-1-type HRE should facilitate the identification of ARF6-binding sites in regulatory regions of other adipocyte genes as well.

Identification of the endogenous ligand(s) for mPPARγ2 will be an important goal of future studies. Several groups have demonstrated previously that polyunsaturated fatty acids and arachadonic acid are endogenous activators of PPARs; however, it is not yet clear whether such molecules actually bind to PPARs directly (Gottlicher et al. 1992; Keller et al. 1993). Previous observations that the ARE6 and ARE7 sites of the aP2 enhancer are constitutively active in adipocytes suggest that either mPPARγ2 is capable of ligand-independent activation in vivo or that the endogenous ligand for mPPARγ2 is normally present at significant levels in differentiated adipocytes. Studies have shown that fatty acids can induce the expression of aP2 by both transcriptional and post-transcriptional mechanisms (Amri et al. 1991, Distel et al. 1992). Our demonstration here that fatty acids can activate mPPARγ2 suggests that mPPARγ2 is likely to mediate the transcriptional activation of aP2 by fatty acids in vivo.

The observation that liver- and kidney-expressed PPARs can be activated by fatty acids correlated well with the finding that high fat diets can induce the peroxisomal β-oxidation system (Osmundsen et al. 1991). PPARs have therefore been postulated to be responsible for activation of a safeguard system of non-energy-generating fatty acid oxidation in response to physiologic lipid overload (Keller and Wahli 1993). Our data suggest that certain members of the PPAR family may play a more central physiologic role as key regulators of gene expression in adipocytes, an important cell type responsible for lipid storage and the maintenance of energy balance. The identification of a PPAR family member that exhibits specificity for adipose cells and functions as a key regulator of an adipocyte enhancer suggests a molecular link between lipid homeostasis and adipose development. Obviously, lipid metabolism must be linked to fat cell differentiation in that marked overfeeding leads to increased adiposity in vertebrate animals. This increase in adipose tissue mass results largely from de novo differentiation of new adipocytes from preadipocytes (Aihaud et al. 1992). If fatty acids or other lipids derived from the diet can serve to activate mPPARγ2, this may stimulate adipogenic gene expression or even fat cell differentiation per se. Although this paper presents no data that directly implicate mPPARγ2 in the regulation of the differentiation process, PPAR activators such as the fibrate hypolidipemic drugs have been reported previously to induce and/or potentiate the differentiation of certain preadipocyte cell lines (Brandes et al. 1987; Gharbi-Chihi et al. 1993). It is reasonable to hypothesize that this effect may be mediated by an adi-

pocyte-specific family member such as mPPARγ2. Further studies, including the experimental alteration of mPPARγ2 expression in various adipose and nonadipose cells, will be necessary to determine whether this factor plays such a central role in adipocyte development.

Materials and methods

Cloning of the mPPARγ2 cDNA

First-strand cDNA was prepared from 3T3–F442A adipocyte total RNA (10 μg) using the cDNA Cycle Kit (Invitrogen). The sequences of the PCR primers used were 5′-GAT/CAAA/GGCITCIGGCTTT/CCA-3′ and 5′-CGGATIGCA/GTTA/GTGIGACAT-3′. PCR was performed for 35 cycles of 94°C/2 min, 60°C/1 min, and 72°C/2 min. PCR products were isolated following electrophoresis through an agarose gel, subcloned into pCRII using the TA Cloning Kit (Invitrogen) and sequenced using the Sequenase Kit (U.S. Biochemical). Full-length cDNA clones were isolated from a λZAPII 3T3–F442A adipocyte cDNA library (custom made by Stratagene), by high stringency hybridization using the subcloned PCR fragment as probe (Maniatis et al. 1982). cDNA sequences were subcloned into pBluescript SK (Stratagene) by in vivo excision from the λZAPII phage as described by the manufacturer and sequenced using a series of internal oligonucleotide primers.

RNA analysis

Total RNA was isolated from cultured cells and tissues of adult mice by guanidine isothiocyanate extraction as described (Chirgwin et al. 1979). Ten micrograms of RNA was denatured in formamide and formaldehyde at 55°C for 15 min and electrophoresed through formaldehyde-containing agarose gels as described (Maniatis et al. 1982). RNA was blotted to BioTrans nylon membranes (ICN), and membranes were cross-linked, hybridized, and washed as directed by the manufacturer. cDNA probes were labeled with [α-^{32}P]dCTP (6000 Ci/mmole) by the random priming method (Fineberg and Vogelstein 1984) to a specific activity of at least 10^9 cpm/μg. Autoradiographs were digitally scanned and quantitated using Digital Darkroom and Image software for Apple Macintosh computers.

DNA-binding assays

DNA mobility retardation assays were performed as described (Graves et al. 1991) except that the reaction buffer consisted of 20 mM HEPES (pH 7.9), 150 mM NaCl, 5% glycerol, and 0.1% NP-40. 0.5–1.0 μg of poly[d(I-C)] (Pharmacia) was included in each reaction as nonspecific competitor. When antisera were used, binding reactions were incubated with antisera for 15 min at room temperature before the addition of the probe. RXRα, mPPARα, and RARα antisera were obtained from R. Evans (Salk Institute, La Jolla, CA). The methylation interference footprinting assay was performed under standard DNA mobility retardation conditions using methylated DNA probes as described (Siebenlist and Gilbert 1980). In vitro translation of RXRα–SPORT, mPPARγ1–SPORT and PPARγ2–SPORT plasmids were performed using the TNT SP6-coupled reticulocyte lysate system (Promega) as recommended by the manufacturer. Five microliters of the 50 μl translation product was used in each binding reaction. For ^{35}S-labeling, in vitro translations were carried out in the presence of 50 μCi of translation grade L-[^{35}S]methionine (1100 Ci/mmole), and 5 μl of each reaction was resolved on a 9.0% SDS–polyacrylamide gel. Preadipocyte nuclear extracts were prepared as described (Dignam et al. 1983), except that extracts were not dialyzed but added directly to the binding reaction. The purification of ARF6 from adipocyte nuclear extract will be described elsewhere (P. Tontonoz and B.M. Spiegelman, unpubl.).

Oligonucleotides

The sequences of double-stranded oligonucleotides used were as follows (only one strand shown): ARE7, 5′-GATCTGTGAACTCTGATCCAGTAAG-3′; TREp, 5′-GATCTCTCAGGTCATGACCTGAATG-3′; PPRE, 5′-GATCTGTGACCTTTGTCCTAGTAAG-3′; DR-1, 5′-GATCTGTGACCTCTGACCTAGTAAG-3′; βRARE, 5′-GATCTGGGTTCACCGAAAGTTCACG-3′. The nonspecific oligonucleotide was the ARE2 oligonucleotide described in Graves et al. (1992).

Plasmids, cell culture, and transfections

The mPPARγ2 expression vector was contructed by ligating the entire 1.8-kb cDNA as an *Xba*I–*Xho*I fragment into the *Xba*I–*Hin*dIII sites of pSV-SPORT (GIBCO-BRL). The RXRα expression vector was constructed by ligating the 1.8-kb *Eco*RI fragment from the RXRα λXR3-1 clone (Mangelsdorf et al. 1990) into the *Eco*RI site of pSV-SPORT. 3T3–F442A and 3T3–L1 preadipocytes were cultured and differentiated as described (Green and Kehinde 1974; Graves et al. 1991). The 518-bp aP2 enhancer CAT construct was described previously (Graves et al. 1992). The ARE7 CAT construct was constructed by ligating three copies of the ARE7 oligonucleotide (see above) into the CAT reporter vector pSVKS1 (Graves et al. 1992). NIH-3T3 cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% bovine calf serum (HyClone) and transfected 1 day postconfluence by the modified calcium phosphate method described previously (Graves et al. 1991). Each 90-mm dish received 2 μg of pSVKS1-derived reporter, 1 μg of pSV-SPORT-derived expression vector, 1 μg of β-actin–LacZ plasmid as an internal control (Oliviero et al. 1992), and 15 μg of sonicated salmon sperm DNA (Sigma). Twelve hours after transfection, cells were refed with DMEM containing 10% charcoal-stripped calf serum and activators as indicated. ETYA, clofibric acid, and linoleic acid were from Sigma; 9-*cis* retinoic acid was obtained from A. Levin (Hoffmann-La Roche, Nutley, NJ). Activators were dissolved in dimethylsulfoxide and applied to the cells in a volume of 10 μl. Transfections were performed in duplicate and repeated a minimum of four times with quantitatively and qualitatively similar results. CAT activity was assayed as described (Graves et al. 1991) and quantitated using a PhosphorImaging device (Molecular Dynamics).

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Note added in proof

The nucleotide sequence data for PPARγ2 DNA has been submitted to the EMBL data library under accession number U09138.

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