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PPARγ2 Regulates Adipose Expression of the Phosphoenolpyruvate Carboxykinase Gene

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Phosphoenolpyruvate carboxykinase (PEPCK) is expressed at high levels in liver, kidney, and adipose tissue. This enzyme catalyzes the rate-limiting step in hepatic and renal gluconeogenesis and adipose glyceroneogenesis. The regulatory factors important for adipose expression of the PEPCK gene are not well defined. Previous studies with transgenic mice established that the region between bp −2086 and −888 is required for expression in adipose tissue but not for expression in liver or kidney tissue. We show here that a DNA fragment containing this region can function as an enhancer and direct differentiation-dependent expression of a chloramphenicol acetyltransferase gene from a heterologous promoter in cultured 3T3-F442A preadipocytes and adipocytes. We further demonstrate that the adipocyte-specific transcription factor PPARγ2, previously identified as a regulator of the adipocyte P2 enhancer, binds in a heterodimeric complex with RXRα to the PEPCK 5′-flanking region at two sites, termed PCK1 (bp −451 to −439) and PCK2 (bp −999 to −987). Forced expression of PPARγ2 and RXRα activates the PEPCK enhancer in non-adipose cells. This activation is potentiated by peroxisome proliferators and fatty acids but not by 9-*cis* retinoic acid. Mutation of the PPARγ2 binding site (PCK2) abolishes both the activity of the enhancer in adipocytes and its ability to be activated by PPARγ2 and RXRα. These results establish a role for PPARγ2 in the adipose expression of the PEPCK gene and suggest that this factor functions as a coordinate regulator of multiple adipocyte-specific genes.

Phosphoenolpyruvate carboxykinase (PEPCK [EC 4.1.1.32]) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, the rate-limiting step in gluconeogenesis and glyceroneogenesis. The PEPCK gene is expressed at high levels in liver, kidney, and white and brown fat (37). Expression is regulated at the transcriptional level by multiple hormones and second messengers, including insulin, glucocorticoids, retinoic acid, thyroid hormone, and cyclic AMP (10, 21, 33). Hormonal and dietary regulation of PEPCK gene transcription is tissue specific. For example, PEPCK expression in the liver is linked to blood glucose concentration, whereas in the kidney it is primarily regulated by physiologic acid-base status. In the liver and kidney, expression is stimulated by glucocorticoids and cyclic AMP and inhibited by insulin (33). In adipose tissue, however, glucocorticoids are inhibitory (25).

The complexity of its transcriptional regulation makes PEPCK an attractive model for the study of hormone-linked and tissue-specific gene expression. The regions of the PEPCK promoter important for the hormonal and dietary regulation of this gene in liver have been analyzed in detail. A complex hormone response region extending from bp −460 to −349 from the transcriptional start site contains elements important for response to glucocorticoids, insulin and retinoic acid (14, 16, 24, 26). A thyroid hormone response element (10) and a cyclic AMP response element (31) are located at bp −332 to −308 and −87 to −74, respectively. Multiple binding sites for members of the C/EBP transcription factor family and a binding site for the liver/kidney-selective transcription factor HNF-1 have also been identified (28, 29).

Recently, several groups have analyzed regions of the PEPCK 5′ flank for their ability to direct appropriate expression of a human or bovine growth hormone reporter gene in transgenic mice (6, 23, 29, 34). These studies have provided in vivo evidence that distinct sequences are involved in transcriptional regulation of the PEPCK gene in liver, kidney, and adipose tissue. Constructs which contained the region of the PEPCK promoter extending from bp −888 to +69 were expressed in the liver, kidney, and small intestine and were appropriately regulated by diet and hormones. However, these constructs were not significantly expressed in white or brown adipose tissue, despite the presence of multiple C/EBP binding sites. Adipose expression appeared to be dependent upon sequences located between bp −2086 and −888. Since previous in vitro analyses of PEPCK transcriptional regulation have focused primarily on the sequences from bp −460 to the transcription start site (10, 14, 16, 21, 25, 26, 28, 31) and since most of these studies utilized cultured hepatoma cells as a model system, the regulatory factors important for adipose expression of the PEPCK gene have not been defined.

We have analyzed the PEPCK 5′-flanking region by transient transfection into cultured adipocytes and identified a differentiation-dependent enhancer in the region extending from bp −2086 to −464. We show that two functional binding sites for the adipocyte-specific transcription factor ARF6 are present in the PEPCK 5′ flank and that ARF6 binding is crucial for enhancer function. ARF6 has recently been purified from a cultured adipocyte cell line and identified as a heterodimeric complex of RXRα and a novel nuclear hormone receptor, PPARγ2 (38, 39). Our results suggest that the PPARγ2/RXRα heterodimer is an important regulator of PEPCK gene expression in adipose tissue. Moreover, these results establish that the regulatory targets of the PPARγ2/RXRα heterodimer are not limited to those specifically in-

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volved in lipid oxidation. Rather, this complex is likely to function as a coordinate regulator of multiple adipocyte-specific genes.

MATERIALS AND METHODS

RNA analysis. Total RNA was isolated from cultured 3T3-F422A cells by guanidine isothiocyanate extraction (2). 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 previously (22). RNA was blotted to BioTrans nylon membranes (ICN), and membranes were cross-linked, hybridized, and washed as directed by the manufacturer. A mouse PEPCK cDNA was cloned by reverse transcriptase PCR and inserted into pBluescript (Stratagene). The 326-nucleotide cDNA in this plasmid (referred to as pmPCR10) is complementary to most of the translated sequence of exon 10. A more complete description will be published elsewhere. cDNA probes were labeled with [α-^{32}P]dCTP (6,000 Ci/mmol) by the random priming method (7) to a specific activity of at least 10^9 cpm/μg.

DNA binding assays. DNA mobility shift assays were performed as described in reference 11, except that the reaction buffer consisted of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 150 mM NaCl, 5% glycerol, and 0.1% Nonidet P-40. poly(dI-dC) (0.5 to 1.0 μg) (Pharmacia) was included in each reaction mixture as a nonspecific competitor. When antiserum was used, binding reactions were incubated with antiserum for 15 min at room temperature prior to the addition of the probe. RXRα and mouse PPARα (mPPARα) antisera were obtained from R. Evans, Salk Institute. In vitro translation of RXRα-SPORT and PPARγ2-SPORT plasmids was performed with the TNT SP6-coupled reticulocyte lysate system (Promega) as recommended by the manufacturer. One microliter of the 50-μl translation product was used in each binding reaction. 3T3-F442A preadipocyte and adipocyte nuclear extracts were prepared as described previously (4), except that the extracts were not dialyzed but were added directly to the binding reaction mixture.

Oligonucleotides. The sequences of double-stranded oligonucleotides (including a *Bam*HI 5′ overhang) were as follows (only one strand shown): PCK1 (bp −453 to −433), 5′-GATCCATGACCTTTGGCCGTGGGAG-3′; and PCK2 (bp −1005 to −985), 5′-GATCCGAGACCTTTATCCCAGTTGT3′. The ARE6 (12) and ARE7 oligonucleotides (39) were described previously. The nonspecific oligonucleotide was the ARE2 oligonucleotide described in reference 12.

Plasmids, cell culture, and transfections. The PEPCK-2.0 kb chloramphenicol acetyltransferase (CAT) reporter (−2.0 PEPCK/CAT) was constructed by ligating a *Sac*I-*Bgl*II fragment (bp −2086 to +69) from the rat genomic PEPCK clone λPC112 (1) into the *Sal*I-*Bam*HI site of pOCAT (30). The PEPCK enhancer/CAT reporter was constructed by ligating the 1.6-kb *Sal*I-*Eco*RI fragment (bp −2086 to −460) from −2.0 PEPCK/CAT as a blunt fragment into the *Sma*I site of pSVKS1 (10). The PCK2 site in the PEPCK enhancer (5′-AGACCTTTA TCCC-3′) was mutated to the sequence 5′-AGACCAGGATCCC-3′ by using the Amersham site-directed mutagenesis kit. NIH 3T3 and 3T3-F442A cells were cultured as described previously (12, 13, 39). The 518-bp aP2 enhancer/CAT construct and ARE2/CAT construct were described previously (12). Transfections were performed by the modified calcium phosphate method described in reference 11. Each 90 mM dish received 2 to 5 μg of pSVKS1-derived reporter, 1 μg of pSV-SPORT-derived expression vector, and 15 μg of sonicated salmon sperm DNA (Sigma). In NIH 3T3 transfections, 1 μg of β-actin–LacZ plasmid (27) was included as an internal control. In adipocyte transfections, 1 μg of pXGH5 human growth hormone expression vector was included as an internal control (12). For transactivation experiments, cells were refed 12 h after transfection with Dulbecco's modified Eagle's medium containing 10% delipidized calf serum and 5, 8, 11, 14-eicosatetraynoic acid (ETYA [Sigma]), linoleic acid (Sigma), or 9-*cis* retinoic acid (obtained from A. Levin, Hoffmann-LaRoche, Nutley, N.J.). Activators were dissolved in dimethyl sulfoxide 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 previously (11) and quantitated with a phosphorimaging device (Molecular Dynamics).

RESULTS

The 3T3-F442A cell culture system has been useful for analyzing gene expression related to adipocyte differentiation. Differentiation of 3T3-F442A preadipocytes in vitro leads to activation of most of the genes characteristic of adipose tissue in vivo (13, 36). To confirm that PEPCK was regulated appropriately in this system, we performed Northern (RNA) analysis of PEPCK mRNA levels during the time course of 3T3-F442A preadipocyte differentiation. As shown in Fig. 1, PEPCK mRNA is induced during differentiation, and this induction is coincident with the induction of aP2. The induction of lipoprotein lipase, an early marker, is shown for comparison.

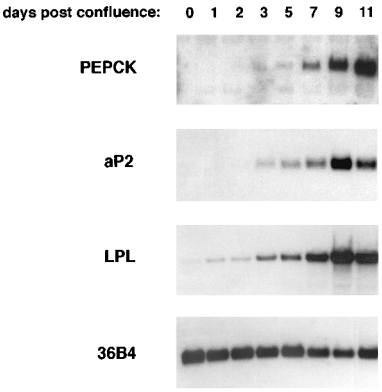


FIG. 1. PEPCK mRNA is induced during the differentiation of 3T3-F442A adipocytes. Total RNA (10 μg per lane) was isolated, blotted to nylon, and hybridized with ^{32}P-labeled murine PEPCK, aP2, and lipoprotein lipase (LPL) cDNA fragments as described in Materials and Methods. An equivalent amount of intact RNA was run in each lane as indicated by hybridization to a 36B4 cDNA probe.

Analysis of the PEPCK 5′-flanking region in transgenic mice established that the sequence extending from bp −2086 to +69 relative to the start of transcription was sufficient to direct adipose expression of a growth hormone reporter gene (6, 23, 34). In order to determine whether this region could also direct differentiation-dependent gene expression in cultured adipocytes, we transiently transfected a PEPCK (bp −2086 to +69)/CAT construct into 3T3-F442A preadipocytes and adipocytes. Maps of this construct and of constructs that we will describe subsequently are shown in Fig. 2. Figure 3 demonstrates that the sequences extending from bp −2086 to +69 can stimulate transcription of the CAT reporter gene in adipocytes but not in preadipocytes. These data establish that this region can direct differentiation-dependent expression of a CAT reporter gene in cultured cells.

Previous studies in transgenic mice suggested that sequences important for adipose expression were present in the region between bp −2086 and −888 of the PEPCK 5′ flank (6, 23, 34). We analyzed the ability of this region to function as an enhancer in cultured 3T3-F442A adipocytes. A fragment extending from bp −2086 to −460 was cloned into pSVKS1, a CAT reporter plasmid driven by the enhancerless simian virus 40 promoter (12). The level of CAT activity resulting from transient transfection of this construct into preadipocytes and adipocytes was quantitated. As shown in Fig. 4, the bp −2086 to

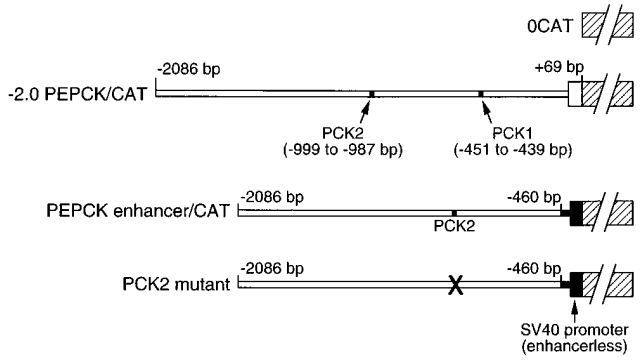


FIG. 2. Schematic represention of PEPCK/CAT chimeric reporter constructs. See text for description. SV40, simian virus 40.

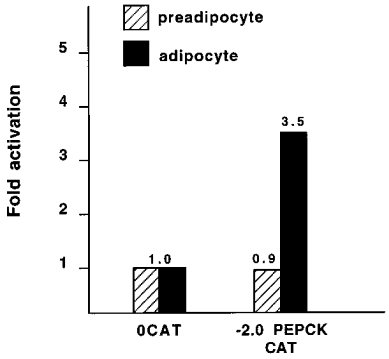


FIG. 3. The PEPCK 5′-flanking region directs differentiation-dependent expression in cultured adipocytes. 3T3-F442A preadipocytes and adipocytes were transfected with 5 μg of either the −2.0 PEPCK/CAT construct or the parental p0CAT construct as described in Materials and Methods. The level of CAT gene expression resulting from each transfection was determined by measuring CAT enzyme activity. The data shown represent numerical averages of duplicate experiments, quantitated with a phosphorimager. CAT activity is presented as fold activation above basal p0CAT activity. The experiment was repeated four times with qualitatively and quantitatively similar results.

−460 PEPCK gene fragment stimulates expression from the enhancerless simian virus 40 promoter only in differentiated adipocytes. As controls, we also transfected an aP2 enhancer/CAT construct, which is active only in adipocytes, and a CAT construct containing multiple copies of the ARE2 site from the aP2 gene, which is active in both cell types (12). These results indicate that the bp −2086 to −460 region can function as a differentiation-dependent enhancer in the absence of proximal PEPCK promoter sequences. This region will be referred to subsequently as the PEPCK adipocyte enhancer.

The DNA sequence of the PEPCK 5′-flanking region was analyzed for similarities to known adipocyte transcription factor binding sites, including C/EBP (3, 15) and ARF6 (12).

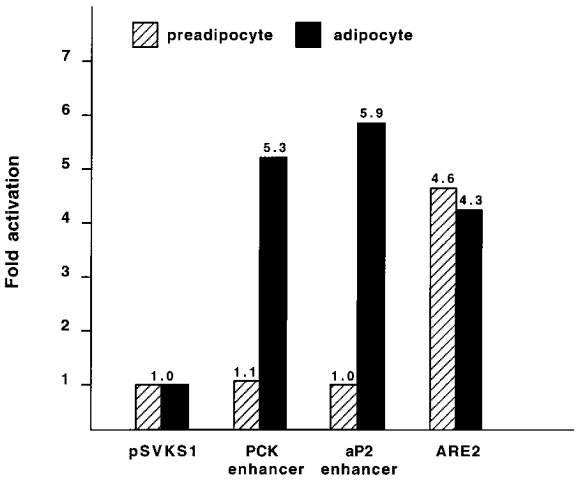


FIG. 4. Identification of a differentiation-dependent enhancer in the PEPCK 5′ flank. 3T3-F442A preadipocytes and adipocytes were transfected with 5 μg of either the PEPCK (bp −2086 to −464)/CAT construct (PEPCK enhancer), the aP2 enhancer/CAT construct, the ARE2/CAT construct, or the parental pSVKS1/CAT construct as described in Materials and Methods. The level of CAT gene expression resulting from each transfection was determined by measuring CAT enzyme activity. The data shown represent numerical averages of duplicate experiments, quantitated with a phosphorimager. CAT activity is presented as fold activation above basal pSVKS1 activity. The experiment was repeated four times with qualitatively and quantitatively similar results.

Multiple C/EBP binding sequences have been identified previously within the region extending from bp −400 to the start of transcription (28). We identified two regions of the PEPCK 5′ flank as potential binding sites for the adipocyte-specific transcription factor ARF6, a heterodimeric complex of PPARγ2 and RXRα (38, 39). These regions, termed PCK1 (bp −451 to −439) and PCK2 (bp −999 to −987), like the ARF6 sites in the aP2 enhancer, are imperfect versions of the DR-1 hormone response element (direct repeat with one nucleotide spacer) (20, 42). Interestingly, the PCK1 site corresponds to the AF-1 element, defined previously as a sequence important for induction of the PEPCK gene in liver cells by glucocorticoids and retinoic acid (16, 21, 24). In the liver, this site appears to be a target for the liver transcription factor HNF4 (35) and the RAR/RXRα heterodimer (14). An alignment of the PCK1 and PCK2 sites with other known ARF6 binding sequences is shown in Fig. 5A. Synthetic double-stranded oligonucleotides containing either PCK1 or PCK2 were analyzed for their ability to compete for PPARγ2/RXRα binding to the ARE7 site from the aP2 enhancer (39). DNA mobility shift assays were performed with in vitro-translated PPARγ2 and RXRα and labeled double-stranded ARE7 oligonucleotide. As shown in Fig. 5B, both PCK1 and PCK2 are effective competitors for PPARγ2/RXRα binding to ARE7. Moreover, the relative affinities of the PCK1 and PCK2 sequences for PPARγ2/RXRα are comparable to that of ARE7. Figure 5C demonstrates that in vitro-translated PPARγ2 and RXRα can also bind directly to labeled PCK1 and PCK2 oligonucleotides. No binding to labeled PCK1 and PCK2 oligonucleotides is observed when unprogrammed reticulocyte lysate is used in the binding reaction mixture (data not shown).

To determine whether the PCK2 site was recognized by the endogenous ARF6 complex, DNA mobility shift assays were performed with labeled ARE7 and PCK2 oligonucleotides and nuclear extract from 3T3-F442A preadipocytes and adipocytes. By definition, the differentiation-dependent nuclear factor which binds to the ARE7 site is ARF6 (12). As shown in Fig. 6, the ARE7 oligonucleotide (lanes 1 to 7) and the PCK2 oligonucleotide (lanes 8 to 14) each bind a nuclear factor present only in adipocyte nuclear extract. The ARE7 and PCK2 oligonucleotides can effectively compete for formation of the complexes on both ARE7 and PCK2; however, a mutant ARE7 oligonucleotide cannot. Moreover, formation of both complexes can be inhibited by antisera to RXRα (lanes 6 and 13) but not by those to PPARα (lanes 7 and 14). Taken together, these data strongly suggest that the same differentiation-dependent adipocyte nuclear factor, ARF6 (PPARγ2/RXRα), binds to both the ARE7 and PCK2 sites.

Forced expression of the PPARγ2/RXRα heterodimer is sufficient to activate the adipocyte-specific aP2 enhancer in cultured fibroblast cell lines (39). To determine whether the PEPCK enhancer could also be activated by PPARγ2/RXRα, transient transfections were performed in NIH 3T3 cells. As shown in Fig. 7A, the heterologous PEPCK enhancer (bp −2086 to −460)/CAT construct (PEPCK enhancer), which contains only the PCK2 site, is effectively activated by cotransfection of PPARγ2 and RXRα (lane 2). We compared the abilities of the synthetic PPAR activator ETYA (17), the polyunsaturated fatty acid linoleic acid, and the RXR ligand 9-*cis* retinoic acid to activate the PEPCK enhancer through PPARγ2/RXRα. As shown in Fig. 7A, activation of the enhancer by PPARγ2/RXRα is potentiated in a dose-dependent manner by ETYA and linoleic acid but not by 9-*cis* retinoic acid. The relative potency of ETYA and linoleic acid in activating the PEPCK enhancer through PPARγ2/RXRα is similar to that observed previously for the aP2 enhancer (37). No

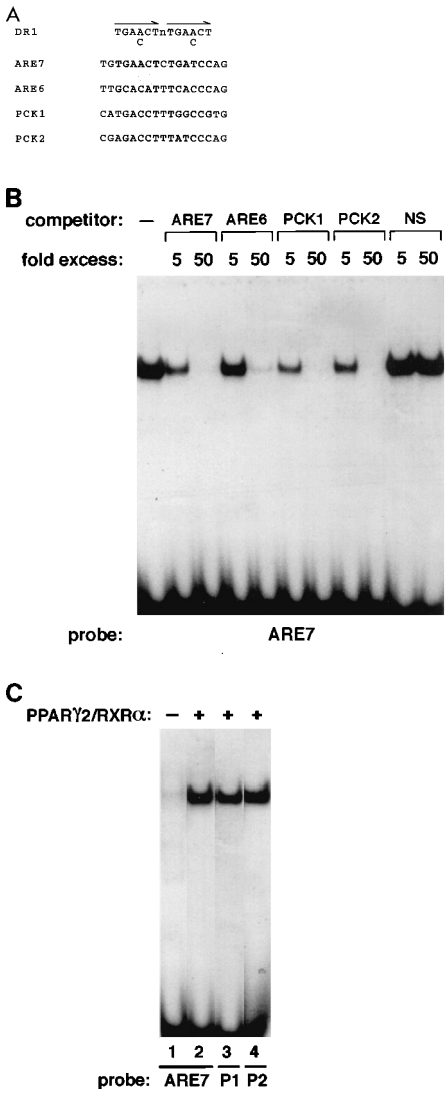


FIG. 5. (A) Sequence comparison of known ARF6 binding sites. (B) The PPARγ2/RXRα heterodimer binds to the PCK1 and PCK2 sites from the PEPCK 5′ flank. Double-stranded ^{32}P-labeled ARE7 oligonucleotide was used as a probe in a DNA mobility shift assay with in vitro-translated PPARγ2 and RXRα. The oligonucleotides shown in panel A were used as competitors in the binding reaction in 5- and 50-fold molar excesses as indicated. The ARE2 site from the aP2 enhancer was used as a nonspecific competitor (NS). (See Materials and Methods for complete sequences of the oligonucleotides used.) DNA protein complexes were resolved from free DNA on a 5% polyacrylamide gel. The gel was dried and exposed to film for 1 h at −70°C. (C) Direct binding of in vitro-translated PPARγ2 and RXRα to ^{32}P-labeled ARE7, PCK1, and PCK2 oligonucleotides.

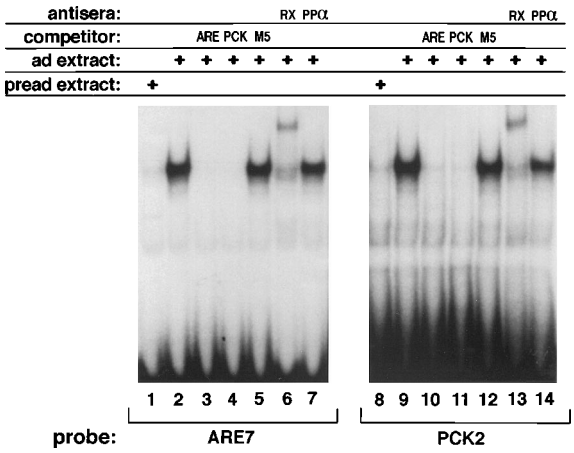


FIG. 6. ARF6 activity in adipocyte nuclear extract binds to both ARE7 and PCK2 sites. Double-stranded ^{32}P-labeled ARE7 and PCK2 oligonucleotides were used as probe in a DNA mobility shift assay with preadipocyte (pread) and adipocyte (ad) nuclear extracts. Antiserum to RXRα (RX) or PPARα (PPα) was included in the binding reaction mixture as indicated. Competitor oligonucleotides were added in 30-fold molar excess. DNA-protein complexes were resolved from free DNA on a 5% polyacrylamide gel. The gel was dried and exposed to film for 1 h at −70°C.

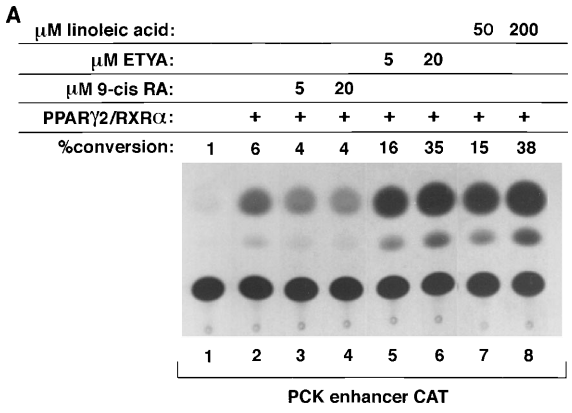
significant activation of the PEPCK enhancer by these compounds is observed in the absence of transfected PPARγ2/RXRα (not shown). The PEPCK (bp −2086 to +69)/CAT construct (−2.0 PEPCK), which contains both the PCK1 and PCK2 sites, is activated approximately 20-fold by cotransfection of PPARγ2 and RXRα expression vectors in the presence of 5 μM ETYA (Fig. 7B, lane 4). No significant activation is observed when the enhancerless parental pOCAT2 construct is used as the reporter (lane 2).

In order to demonstrate that activation of the PEPCK enhancer was mediated by the PCK2 binding site, a 3-bp mutation was introduced into this sequence by site-directed mutagenesis (see Materials and Methods). As expected, this mutation completely abolishes the ability of the sequence to bind the PPARγ2/RXRα heterodimer in DNA mobility shift assays (data not shown). A PEPCK enhancer/CAT construct containing the PCK2 site mutation (PCK2 mutant) cannot be activated by PPARγ2 and RXRα in transient transfection assays (Fig. 7B, lanes 7 and 8).

A crucial question is whether the PPARγ2/RXRα binding site identified here plays a role in the endogenous function of the PEPCK enhancer in adipocytes. Wild-type and mutant enhancer CAT constructs were transiently transfected into 3T3-F442A adipocytes, and levels of CAT activity were determined. As shown in Fig. 8, mutation of the PCK2 site completely abolishes enhancer activity. The mutation had no detectable effect on the minimal level of enhancer activity in preadipocytes (data not shown). Although this region is likely to contain additional transcription factor binding sites that may contribute to its activity, these data clearly demonstrate that the PCK2 site, the binding site for the PPARγ2/RXRα heterodimer, is critical for PEPCK enhancer activity in cultured adipocytes.

DISCUSSION

The molecular mechanisms that control adipocyte gene expression and differentiation are not well understood. In recent years, several transcription factors that may play a role in adipocyte gene regulation have been identified. The best characterized of these is C/EBPα, a basic leucine zipper protein that binds to the promoter regions of several fat cell genes, including aP2 and steroyl-coenzyme A desaturase (3, 15). Multiple lines of evidence indicate that C/EBPα may play a role in terminal adipocyte differentiation (8, 32, 41), and overexpression of this factor appears to promote the adipogenic program in several fibroblast cell lines (9). However, because C/EBPα mRNA is induced rather late in the time course of differentiation, it is unlikely to be the initiator of the program during normal development. Furthermore, because C/EBPα mRNA is expressed at high levels in liver as well as fat, it cannot by itself account for the tissue-specific expression of many fat cell



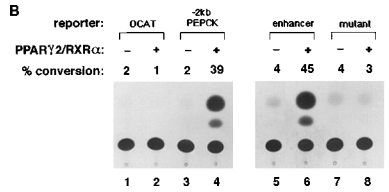


FIG. 7. PPARγ2 and RXRα activate the PEPCK enhancer in an activator-dependent manner in nonadipose cells. NIH 3T3 cells were cotransfected with either the −2.0 PEPCK/CAT construct, the parental p0CAT construct, the PEPCK enhancer/CAT construct or a PEPCK enhancer/CAT construct carrying a mutation in the PCK2 site (2 μg), and PPARγ2 and RXRα expression vectors (2 μg) as described in Materials and Methods. The level of CAT enzyme activity resulting from each transfection was quantitated with a phosphorimager. Data are presented as the percentage of conversion of the chloramphenicol to acetylated forms. (A) PPARγ2/RXRα mediates activation of the PEPCK enhancer by ETYA and fatty acid. After transfection, cells were treated for 24 h with different activators at the concentrations indicated. RA, retinoic acid. (B) PCK2 mutant PEPCK enhancer cannot be activated by PPARγ2/RXRα. After transfection, cells were treated for 24 h with 5 μM ETYA.

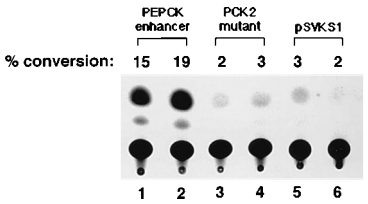


FIG. 8. ARF6 binding is required for the activity of the PEPCK enhancer. 3T3-F442A adipocytes were transfected with 5 μg of either the PEPCK enhancer/CAT construct, the PEPCK enhancer/CAT construct carrying a mutation in the PCK2 site, or the parental pSVKS1/CAT construct as described in Materials and Methods. The level of CAT enzyme activity resulting from each transfection was quantitated with a phosphorimager. Data are presented as the percentage of conversion of the chloramphenicol to acetylated forms. The experiment was repeated four times with qualitatively and quantitatively similar results.

genes. A second putative adipocyte regulatory factor is ADD1, an unusual basic helix-loop-helix-leucine zipper protein that is most highly expressed in adipose tissue and liver (40). The human homolog of ADD1 has been isolated independently by Yokoyama et al. as SREBP1 (43), a protein involved in sterol-dependent transcriptional regulation of the low-density lipoprotein receptor gene. Basic helix-loop-helix proteins are known to be involved in the differentiation of several other tissues, including skeletal muscle and the central nervous system. The precise role of ADD1 in adipose tissue development is not yet clear.

To date, the only transcription factor known to exhibit a high degree of specificity for adipose cells is the murine peroxisome proliferator-activated receptor γ (39, 44). Of the two isoforms thus far described (mPPARγ1 and mPPARγ2), PPARγ2 is the predominant form expressed in fat (38). In vitro and in vivo, PPARγ forms a heterodimeric complex with RXRα, and together these factors compose the adipocyte transcription factor originally termed ARF6 (38, 39). ARF6 was identified as a transcription factor that binds two critical *cis*-acting elements in the adipocyte-specific aP2 enhancer (12). ARF6 binding appears to be both necessary and sufficient to direct adipocyte-specific expression in cultured cells.

We have demonstrated here that the PPARγ2/RXRα complex is likely to be an important transcriptional regulator of the PEPCK gene. The region of the PEPCK 5′-flanking region extending from bp −2086 to −460 can function as a differentiation-dependent transcriptional enhancer in cultured adipocytes. The same region has previously been demonstrated to be required for adipose expression in vivo (6, 23, 34). Two ARF6-binding sequences, termed PCK1 and PCK2, are present in the PEPCK 5′-flanking region. The PCK2 site is required both for the activity of the PEPCK enhancer in cultured adipocytes and for the ability of the enhancer to be bound and activated by the PPARγ2/RXRα heterodimer. Although additional members of the PPAR and RXR families are expressed at very low levels in adipose tissue and would therefore be expected to bind the PCK2 site, PPARγ2 and RXRα are by far the most abundant DR-1-binding factors in adipose tissue (38, 39). Moreover, only PPARγ2 displays significant adipose specificity. In transgenic mice, the sequences extending from bp −888 to +69, which include the PCK1 site, are not sufficient for adipose expression (6, 23, 34). One interpretation of this observation is that multiple ARF6 binding sites are required for adipose expression in vivo. Alternatively, the PCK2 site in particular and/or additional sites within the bp −2086 to −460 region may be required.

Glucocorticoids have opposite effects on PEPCK gene expression in liver and adipose tissue (25, 37). One of the ARF6 binding sequences we have identified, PCK1, lies within the complex glucocorticoid response unit defined by Granner and colleagues in liver cells (16). The PCK1 site is expected to be principally occupied by the hepatic transcription factor HNF4 in liver and by PPARγ2/RXRα in adipose tissue. Although it is not yet clear whether this site is involved in the glucocorticoid response in adipose cells, an interesting possibility is that differential occupancy of the PCK1 site plays a role in the tissue-specific response of the PEPCK gene to glucocorticoids. Further experiments will be necessary to determine whether PPARγ2/RXRα contributes to the regulation of PEPCK by glucocorticoids or other hormones.

The PPARs comprise a subfamily of nuclear receptors that are activated in experimental systems by clofibrate hypolipidemic drugs, arachidonate analogs, and polyunsaturated fatty acids (17, 18). However, there is no evidence that any of these molecules actually bind to PPARs directly. The endogenous ligand for the PPARs, if one exists, remains unknown. We have demonstrated here that PPARγ2/RXRα can mediate activation of the PEPCK enhancer by the synthetic PPAR activator ETYA, as well as the dietary polyunsaturated fatty acid linoleic acid. From a physiologic perspective, it would make sense that lipids derived from the diet might stimulate adipose expression of the PEPCK gene in order to provide glycerol for triglyceride synthesis. The RXR ligand 9-*cis* retinoic acid has been shown previously to activate the heterodimeric complex of PPARα/RXRα in transfection assays (18, 20). Activation of the PEPCK enhancer by the PPARγ2/RXRα complex, however, is not potentiated by 9-*cis* retinoic acid. One explanation for

these observations is that the PPARγ2/RXRα heterodimer, unlike the PPARα/RXRα heterodimer, may be unable to bind 9-*cis* retinoic acid. Alternatively, other factors binding to the PEPCK enhancer may function to prevent transcriptional activation by retinoids. Further studies will be necessary to differentiate between these possibilities.

In tissues such as liver and kidney, PPARs have been implicated in the transcriptional regulation of a specific set of genes involved in peroxisomal lipid oxidation (5, 18, 19). The adipocyte-specific family member PPARγ2 is likely to have a biological role distinct from other PPARs. The observation that the PPARγ2/RXRα heterodimer regulates PEPCK as well as aP2 suggests that the regulatory targets of this factor are not limited to those directly involved in lipid oxidation. Rather, it now appears that PPARγ2 is likely to function as a coordinate regulator of multiple adipocyte-specific genes. It is interesting to note that the only two DNA sequences known to be capable of directing adipose-specific expression in vivo—the aP2 and PEPCK enhancers—both contain functional binding sites for ARF6. Transgenic experiments are currently in progress to address whether the ARF6 binding sites identified here are required for the adipose expression of the PEPCK gene in vivo. Further experiments, including the creation of mouse strains lacking PPARγ2, will be necessary to determine whether this factor plays a broad role in the development of the adipose lineage.

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ADDENDUM IN PROOF

We have now demonstrated that forced expression of PPARγ2 activates the entire program of adipose differentiation in cultured fibroblasts (P. Tontonoz, E. Hu, and B. M. Spiegelman, Cell, in press).

REFERENCES

1. **Beale, E. G., N. B. Chrapkiewicz, H. A. Scoble, R. J. Metz, D. P. Quick, R. L. Noble, S. E. Donelson, K. Biemann, and D. Granner.** 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP). Structures of the protein, messenger RNA and gene. J. Biol. Chem. **260:**10748–10760.
2. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
3. **Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Geiman, W. M. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane.** 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev. **3:**1323–1335.
4. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
5. **Dreyer, C., G. Krey, H. Keller, F. Givel, G. Helftenbein, and W. Wahli.** 1992. Control of the peroxisomal b-oxidation pathway by a novel family of nuclear hormone receptors. Cell **68:**879–887.
6. **Eisenberger, C. L., H. Nechushtan, H. Cohen, M. Shani, and L. Reshef.** 1992. Differential regulation of the rat phosphoenolpyruvate carboxykinase gene expression in several tissues of transgenic mice. Mol. Cell. Biol. **12:**1396–1403.
7. **Fineberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **137:**266–267.
8. **Freytag, S. O., and T. J. Geddes.** 1992. Reciprocal regulation of adipogenesis by Myc and C/EBP alpha. Science **256:**379–382.
9. **Freytag, S. O., D. L. Paielli, and J. D. Gilbert.** 1994. Ectopic expression of the CCAAT/enhancer-binding protein a promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes Dev. **8:**1654–1663.
10. **Giralt, M., E. A. Park, A. L. Gurney, J. S. Liu, P. Hakimi, and R. W. Hanson.** 1991. Identification of a thyroid hormone response element in the phosphoenolpyruvate carboxykinase (GTP) gene. Evidence for synergistic interaction between thyroid hormone and cAMP cis-regulatory elements. J. Biol. Chem. **266:**21991–21996.
11. **Graves, R. A., P. Tontonoz, S. R. Ross, and B. M. Spiegelman.** 1991. Identification of a potent adipocyte-specific enhancer: involvement of an NF-1-like factor. Genes Dev. **5:**428–437.
12. **Graves, R. A., P. Tontonoz, and B. M. Spiegelman.** 1992. Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. Mol. Cell. Biol. **12:**1202–1208.
13. **Green, H., and O. Kehinde.** 1974. Sublines of mouse 3T3 cells that accumulate lipid. Cell **1:**113–116.
14. **Hall, R. K., D. K. Scott, E. L. Noisin, P. C. Lucas, and D. K. Granner.** 1992. Activation of the phosphoenolpyruvate carboxykinase gene retinoic acid response element is dependent on a retinoic acid receptor/coregulator complex. Mol. Cell. Biol. **12:**5527–5535.
15. **Herrera, R., H. S. Ro, G. S. Robinson, K. G. Xanthopoulos, and B. M. Spiegelman.** 1989. A direct role for C/EBP and the AP-I-binding site in gene expression linked to adipocyte differentiation. Mol. Cell. Biol. **9:**5331–5339.
16. **Imai, E., P.-E. Stromstedt, P. G. Quinn, J. Carlstedt-Duke, J.-Å. Gustafsson, and D. K. Granner.** 1990. Characterization of a complex glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. **10:**4712–4719.
17. **Isseman, I., and S. Green.** 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature (London) **347:**645–649.
18. **Keller, H., C. Dreyer, J. Medin, A. Mahfoudi, K. Ozato, and W. Wahli.** 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proc. Natl. Acad. Sci. USA **90:**2160–2164.
19. **Keller, H., and W. Wahli.** 1993. Peroxisome proliferator-activated receptors: a link between endocrinology and nutrition? Trends Endocrinol. Metab. **4:**291–296.
20. **Kliewer, S. A., K. Umesono, D. Noonan, R. Heyman, and R. M. Evans.** 1992. Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature (London) **358:**771–774.
21. **Lucas, P. C., R. M. O'Brien, J. A. Mitchell, C. M. Davis, E. Imai, B. M. Forman, H. H. Samuels, and D. K. Granner.** 1991. A retinoic acid response element is part of a pleiotropic domain in the phosphoenolpyruvate carboxykinase gene. Proc. Natl. Acad. Sci. USA **88:**2184–2188.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **McGrane, M. M., J. S. Yun, A. F. Moorman, W. H. Lamers, G. K. Hendrick, B. M. Arafah, E. A. Park, T. E. Wagner, and R. W. Hanson.** 1990. Metabolic effects of developmental, tissue-, and cell-specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice. J. Biol. Chem. **265:**22371–22379.
24. **Mitchell, J., E. Noisin, R. Hall, R. O'Brien, E. Imai, and D. Granner.** 1994. Integration of multiple signals through a complex hormone response unit in the phosphoenolpyruvate carboxykinase gene promoter. Mol. Endocrinol. **8:**585–594.
25. **Nechustan, H., N. Benvenisty, R. Brandeis, and L. Reshef.** 1987. Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue specific manner. Nucleic Acids Res. **15:**6405–6411.
26. **O'Brien, R. M., P. C. Lucas, C. D. Forest, M. A. Magnuson, and D. K. Granner.** 1990. Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. Science **249:**533–537.
27. **Oliviero, S., G. S. Robinson, K. Struhl, and B. M. Spiegelman.** 1992. Yeast GCN4 as a probe for oncogenesis by AP-1 transcription factors: transcriptional activation through AP-1 sites is not sufficient for cellular transformation. Genes Dev. **6:**1799–1809.
28. **Park, E. A., W. J. Roesler, J. Liu, D. J. Klemm, A. L. Gurney, J. D. Thatcher, J. Shuman, A. Friedman, and R. W. Hanson.** 1990. The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). Mol. Cell. Biol. **10:**6264–6272.
29. **Patel, Y. M., J. S. Yun, J. Liu, M. M. McGrane, and R. W. Hanson.** 1994. An analysis of regulatory elements in the phosphoenolpyruvate carboxykinase (GTP) gene which are responsible for its tissue-specific expression and metabolic control in transgenic mice. J. Biol. Chem. **269:**5619–5628.
30. **Prost, E., and D. D. Moore.** 1986. CAT vectors for analysis of eukaryotic promoters and enhancers. Gene **45:**107–111.
31. **Quinn, P. G., T. W. Wong, M. A. Magnuson, J. B. Shabb, and D. K. Granner.** 1988. Identification of basal and cyclic AMP regulatory elements in the promoter of the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. **8:**3467–3475.
32. **Samuelsson, L., K. Stromberg, K. Vikman, G. Bjursell, and S. Enerback.**

1991. The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development. EMBO J. **10:**3787–3793.

33. **Sasaki, K., T. P. Cripe, S. R. Koch, T. L. Andreone, D. D. Petersen, E. G. Beale, and D. K. Granner.** 1984. Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. J. Biol. Chem. **259:**15242–15251.
34. **Short, M. K., D. E. Clouthier, I. M. Schaefer, R. E. Hammer, M. A. Magnuson, and E. G. Beale.** 1992. Tissue-specific, developmental, hormonal, and dietary regulation of a rat phosphoenolpyruvate carboxykinase-human growth hormone fusion gene in transgenic mice. Mol. Cell. Biol. **12:**1007–1020.
35. **Sladek, F. M., W. Zhong, E. Lai, and J. E. Darnell.** 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. **4:**2353–2365.
36. **Spiegelman, B., and H. Green.** 1980. Control of specific protein biosynthesis during the adipose conversion of 3T3 cells. J. Biol. Chem. **255:**8811–8818.
37. **Tilghman, S. M., F. J. Ballard, and R. W. Hanson.** 1976. Hormonal regulation of phosphoenolpyruvate carboxykinase (GTP) in mammalian tissues, p. 47–91. *In* R. W. Hanson and M. A. Mehlman (ed.), Gluconeogenesis: its regulation in mammalian species. Wiley and Sons, New York.
38. **Tontonoz, P., R. A. Graves, A. I. Budavari, H. Erdjument-Bromage, M. Lui, E. Hu, P. Tempst, and B. M. Spiegelman.** Nucleic Acids Res., in press.
39. **Tontonoz, P., E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman.** 1994. mPPARg2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. **8:**1224–1234.
40. **Tontonoz, P., J. B. Kim, R. A. Graves, and B. M. Spiegelman.** 1993. ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. Mol. Cell. Biol. **13:**4753–4759.
41. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT/enhancer binding protein: a component of a differentiation switch. Science **251:**288–292.
42. **Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65:**1255–1266.
43. **Yokoyama, C., X. Wang, M. Briggs, A. Admon, J. Wu, X. Hua, J. L. Goldstein, and M. S. Brown.** 1993. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. Cell **75:**187–197.
44. **Zhu, Y., K. Alvares, Q. Huang, M. S. Rao, and J. K. Reddy.** 1993. Cloning of a new member of the peroxisome proliferator-activated receptor gene family from mouse liver. J. Biol. Chem. **268:**26817–26820.