

Differentiation-Dependent Expression of the Brown Adipocyte Uncoupling Protein Gene: Regulation by Peroxisome Proliferator-Activated Receptor γ

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Received 20 October 1995/Returned for modification 5 December 1995/Accepted 4 April 1996

Uncoupling protein (UCP) is expressed only in brown adipocytes and is responsible for the unique thermogenic properties of this cell type. The novel brown preadipocyte cell line, HIB-1B, expresses UCP in a strictly differentiation-dependent manner. Transgenic mice studies have shown that a region from kb -2.8 to -1.0 of the murine UCP gene is required for brown adipocyte-specific expression. Subsequent analysis identified a potent 220-bp enhancer from kb -2.5 to -2.3 . We show that this enhancer is active only in differentiated HIB-1B adipocytes, and we identify a peroxisome proliferator-activated receptor γ (PPAR γ) response element, referred to as UCP regulatory element 1 (URE1), within the enhancer. URE1 has differentiation-dependent enhancing activity in HIB-1B cells and is required for enhancer action, since mutations of URE1 that block protein binding abolish enhancer activity. We also show that PPAR γ antibodies block binding to URE1 of nuclear extracts from cultured brown adipocytes and from the brown adipose tissue of cold-exposed mice. Protein binding to URE1 increases substantially during differentiation of HIB-1B preadipocytes, and PPAR γ mRNA levels increase correspondingly. Although forced expression of PPAR γ and retinoid X receptor α activates the enhancer in HIB-1B preadipocytes, these receptors are not capable of activating the enhancer in NIH 3T3 fibroblasts. Our results show that PPAR γ is a regulator of the differentiation-dependent expression of UCP and suggest that there are additional factors in HIB-1B cells required for brown adipocyte-specific UCP expression.

Vertebrates possess two distinct types of adipose tissue, brown and white. White adipose tissue stores and releases fat according to the nutritional needs of the animal, whereas brown adipose tissue (BAT) burns fat, releasing the energy as heat (i.e., nonshivering thermogenesis). The unique thermogenic properties of BAT reflect the activities of specialized mitochondria that contain the brown adipocyte-specific gene product, uncoupling protein (UCP). UCP, a mitochondrial proton carrier, uncouples respiration from oxidative phosphorylation by collapsing the proton gradient established from fatty acid oxidation without concomitant ATP synthesis (35). UCP expression is tightly regulated, primarily by sympathetic nervous systems, in response to physiological signals, such as cold exposure and excess caloric intake (12). Norepinephrine (NE) released from the local neurons interacts with β -adrenergic receptors on the brown adipocyte cell membrane, causing an increase in intracellular cyclic AMP (cAMP) levels. An increased level of transcription of the UCP gene is a critical component in the cascade of events leading to elevated BAT thermogenesis in response to increased cAMP (27, 38, 39).

BAT thermogenesis is used both (i) to maintain homeothermy by increasing thermogenesis in response to lower temperatures and (ii) to maintain energy balance by increasing energy expenditure in response to increases in caloric intake. Nearly all experimental rodent models of obesity are accompanied by diminished or defective BAT function, usually as the

first symptom in the progression of obesity (19, 20). Furthermore, ablation of BAT in transgenic mice by targeted expression of a toxin gene results in obesity (31). Thus, the growth and differentiation of brown adipocytes are key determinants of an animal's ability to maintain energy balance and prevent obesity.

Adipocyte differentiation can be studied with cultured cell lines that can be maintained and passaged as fibroblast-like preadipocytes (17, 18). Under appropriate culture conditions the cell can be induced to enter the terminal differentiation program. These cell lines have been useful in identifying transcription factors that initiate and regulate adipocyte differentiation (1, 2, 8). The results have implicated C/EBP α and peroxisome proliferator-activated receptor γ 2 (PPAR γ 2), a novel member of the nuclear hormone receptor superfamily, as key factors in regulating adipocyte differentiation (11, 48, 50). PPAR γ 2 is especially interesting, since it is expressed at high levels exclusively in adipose tissue and it is capable of inducing adipogenesis in fibroblasts (50, 51). PPAR γ 2 binding sites have been identified in the only known enhancers that are capable of directing adipose tissue-specific expression in transgenic mice (49, 50).

Although many cell culture models are available to study adipocyte differentiation, only one of these, the HIB-1B cell line, differentiates into adipocytes that can express UCP (40). HIB-1B cells can be maintained as fibroblast-like cells that express low levels of early markers of differentiation, e.g., lipoprotein lipase (LPL) and adipocyte lipid-binding protein (aP2). However, differentiated HIB-1B cells display the morphological appearance of adipocytes: they are spherical and contain lipid droplets, and they express high levels of adipo-

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cyte-specific genes, LPL, aP2, and adipsin (22). Most importantly, HIB-1B adipocytes are competent to express UCP. As in BAT of animals, UCP expression is induced in HIB-1B adipocytes by agents that increase intracellular cAMP levels, e.g., β -agonists or cAMP analogs (22, 40). Thus, UCP expression in HIB-1B cells requires both differentiation and β -adrenergic receptor activation or increased cAMP levels (22, 40).

Transgenic mice studies have shown that the regulatory elements needed for tissue-specific and cold-induced UCP expression are located within -2.8 (or -4.5) kb of the mouse (or rat) UCP gene (4, 5). An essential regulatory region located between kb -2.8 and -1.0 of the mouse UCP gene was required for transgene expression in BAT (4). Subsequent analysis of cultured brown fat tumor (hibernoma) cells identified a strong enhancer located from kb -2.5 to -2.3 (5, 28). A similar enhancer has been identified in the rat gene, and the sequence identity between the rat and mouse enhancers is remarkable (6). In these same studies, a silencer activity was identified from bp -900 to -272 ; it was proposed that BAT-specific expression resulted from the interaction of both the enhancer and the silencer regions (5, 28).

Several studies of factors regulating UCP expression have now been reported (3, 6, 28, 36, 53, 54). Many of these studies were done with nonbrown adipocytes that are not capable of expressing UCP and used transactivation studies to demonstrate the presence of *cis*-acting elements for the C/EBP, thyroid hormone receptor, and retinoic acid receptor (3, 36, 53). With hibernoma cells that express UCP in response to NE, a functional cAMP response element (CRE2) and two novel brown adipocyte regulatory elements (BRE1 and BRE2) have been identified in the 220-bp murine UCP enhancer (28).

In contrast to the hibernoma cells used in these prior studies, the HIB-1B hibernoma cell line expresses UCP only in the differentiated state (40). We have used this feature of the cell line to investigate the factors that control differentiation through an analysis of the element(s) needed for the differentiation-dependent activation of UCP expression. The 220-bp UCP enhancer (-2.5 to -2.3 kb) is shown to have differentiation-dependent enhancing activity that depends on a PPAR γ -retinoid X receptor α (RXR α) binding site referred to as UCP regulatory element 1 (URE1). We show that PPAR γ expression is regulated during HIB-1B-cell differentiation. Forced expression of PPAR γ 2 and RXR α is sufficient to activate the UCP enhancer in HIB-1B preadipocytes but not in NIH 3T3 fibroblasts. Our results provide further support for the notion that PPAR γ 2 is a key regulator of adipocyte differentiation (50, 51) and suggest that brown and white adipocytes have common pathways of differentiation.

MATERIALS AND METHODS

Cell culture, transfections, and CAT assays. HIB-1B cells were cultured and differentiated essentially as described previously (40) except that heat-inactivated (HI) serum and high-glucose Dulbecco's modified Eagle's medium (GIBCO catalog no. 12100-103) were used. Preadipocytes were cultured in 10% HI calf serum, while adipocytes were cultured in 5% HI fetal calf serum. Differentiation was induced as previously described (40). The subline HIB-1B clone 3 (C3), which differentiates more readily and reproducibly than the original HIB-1B cell line, was cultured for differentiation by plating 10^5 cells into 60-mm-diameter dishes in Dulbecco's modified Eagle's medium with 5% HI fetal calf serum, 17 nM insulin, and 1 nM triiodothyronine and refed every 2 to 3 days. The cells typically reach confluence 3 to 4 days after plating and are 90 to 95% differentiated (as determined by morphology) 5 to 7 days postconfluence. The differentiating C3 cells were transfected 3 to 5 days postconfluence, and the preadipocytes were transfected at confluence. All transfections were performed with a final DNA concentration of 8 μ g per 60-mm-diameter dish by the modified calcium phosphate precipitation method previously described (14, 15). 3T3F442A cells were cultured and transfected as described previously (15). NIH 3T3 cells were cultured in 10% calf serum–Dulbecco's modified Eagle's medium and transfected 1 day after confluence (50). In some experiments we cotrans-

ferred a plasmid encoding a β -galactosidase reporter gene driven by the β -actin promoter as an internal control (50). All transfections were repeated several times with quantitatively and qualitatively similar results. At least three different plasmid preparations of each construct were tested. Chloramphenicol acetyltransferase (CAT) assays were quantitated with a phosphor imaging device (Molecular Dynamics).

Nuclear extracts and DNA mobility shift assays. Nuclear extracts were prepared as described previously (43), except that the extracts were not dialyzed but frozen immediately after salt extraction of the nuclei and used directly for DNA binding assays. BAT nuclear extracts were prepared from the intrascapular BAT of 4-week-old mice (both sexes) that were placed in a cold room (4°C) for 18 h before sacrifice. Mobility shift DNA binding assays were performed as described previously (15), except that the reaction buffer consisted of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.7), 0.5 mM EDTA, 50 mM potassium acetate, 5% glycerol, and 0.1% Nonidet P-40. Salmon sperm DNA (5 μ g) and poly(dI-dC) (0.5 μ g) were included in each reaction mixture as nonspecific competitors. When antisera were used, binding reaction mixtures were incubated with antisera for 15 min at room temperature before addition of probe. PPAR γ antibody was a generous gift from Erding Hu, Peter Tontonoz, and Bruce Spiegelman (Dana-Farber Cancer Institute, Boston, Mass.). In vitro translation of RXR α and PPAR γ 2 was performed with the TNT SP6-coupled reticulocyte lysate system (Promega) as recommended by the manufacturer. Two microliters of the 50- μ l translation product was used in each binding reaction.

RNA analysis. Total RNA was isolated from cultured cells by guanidine hydrochloride extraction as previously described (46). Ten micrograms of RNA was denatured in formamide and formaldehyde at 70°C for 5 min and electrophoresed through a formaldehyde-containing agarose gel as described previously (32). RNA was blotted to a ZetaProbe GT nylon membrane (Bio-Rad), and the membrane was cross-linked, hybridized, and washed as directed by the manufacturer. cDNA probes were labeled with [α - 32 P]dATP (3,000 Ci/mmol) by the random priming method (9).

Plasmids and mutagenesis. The promoter plasmid, -73 CAT, was constructed by inserting an *Hae*III-*Pst*I fragment from the murine UCP gene (from bp -73 to $+120$ relative to the transcription start site) into the *Sma*I and *Pst*I sites of the pAHCAT plasmid. pAHCAT was constructed by inserting the *Apa*I-*Hind*III fragment from pSV2CAT (13), containing the CAT gene and simian virus 40 small t antigen RNA processing signals, into pBluescript II (Stratagene). 220/ -73 CAT and 120/ -73 CAT were constructed by ligating the *Hind*III-*Xba*I fragment (bp -2530 to -2310) or the *Hind*III-*Eco*RV fragment (bp -2530 to -2410) from the UCP gene, respectively, into the *Xba*I site of -73 CAT. 520/ -73 CAT was constructed by inserting the 520-bp aP2 gene enhancer (15) as an *Eco*RI-*Xba*I fragment into the *Xba*I site of -73 CAT. The plasmid 3PR1/ -73 CAT was created by ligating three tandem copies of the PR1 double-stranded oligonucleotide into the *Xba*I site of -73 CAT. The pSVSPORT (GIBCO) expression vectors for PPAR γ 2 and RXR α were previously described (50). The pAGGal plasmid was provided by Chiayang Wang, University of Illinois, Chicago, Ill. A Sculptor kit (Amersham) was used for site-directed mutagenesis. DNA sequencing (Sequenase; United States Biochemicals) was used to verify mutations and also to determine copy numbers and orientations of multimerized oligonucleotides.

Oligonucleotides. The sequences of the double-stranded oligonucleotides used are as follows (only the sense strands are shown): PR1, 5'-CTAGAGGGT CAGTCACCCCTTGACACACTGAAGTAGTCGTCAG-3'; CRE, 5'-AGAG ATTGCCTGACGTCAGAGAGCTAG-3' (Promega); ARE7, 5'-GATCTGTG AACTGTGATCCAGTAAG-3' (50); and DR-1, 5'-GATCTGTGACCTCTGA CCTAGTAAG-3' (50). The single-stranded oligonucleotides for in vitro mutagenesis (in boldface type) are as follows: M1, 5'-TGTGGTCAATGTGACT GAC-3'; and M2, 5'-TGTGGTCTAGGGTGACTGAC-3'.

RESULTS

In addition to requiring β -agonist activation, UCP expression in HIB-1B cells requires the differentiation of the cells into adipocytes. To identify the regulatory region(s) of the UCP gene that responds to the differentiation status of the cells, we evaluated the enhancing activities in adipocytes and preadipocytes of the recently identified 220-bp enhancer from the murine UCP gene (28). This enhancer was linked to a minimal UCP promoter (-73 to $+110$ bp) (Fig. 1) driving the reporter gene CAT. This construct, 220/ -73 CAT, and its parent, -73 CAT, were transiently transfected into HIB-1B preadipocytes or adipocytes. The cells were refed 12 to 16 h later and treated with phosphate-buffered saline (PBS), NE (adipocytes), or dibutyl cAMP (dbcAMP) (preadipocytes). The cells were collected 24 h later, and CAT activities were determined as measures of transcriptional enhancement. As shown in Fig. 2, the 220-bp enhancer had significant enhancing

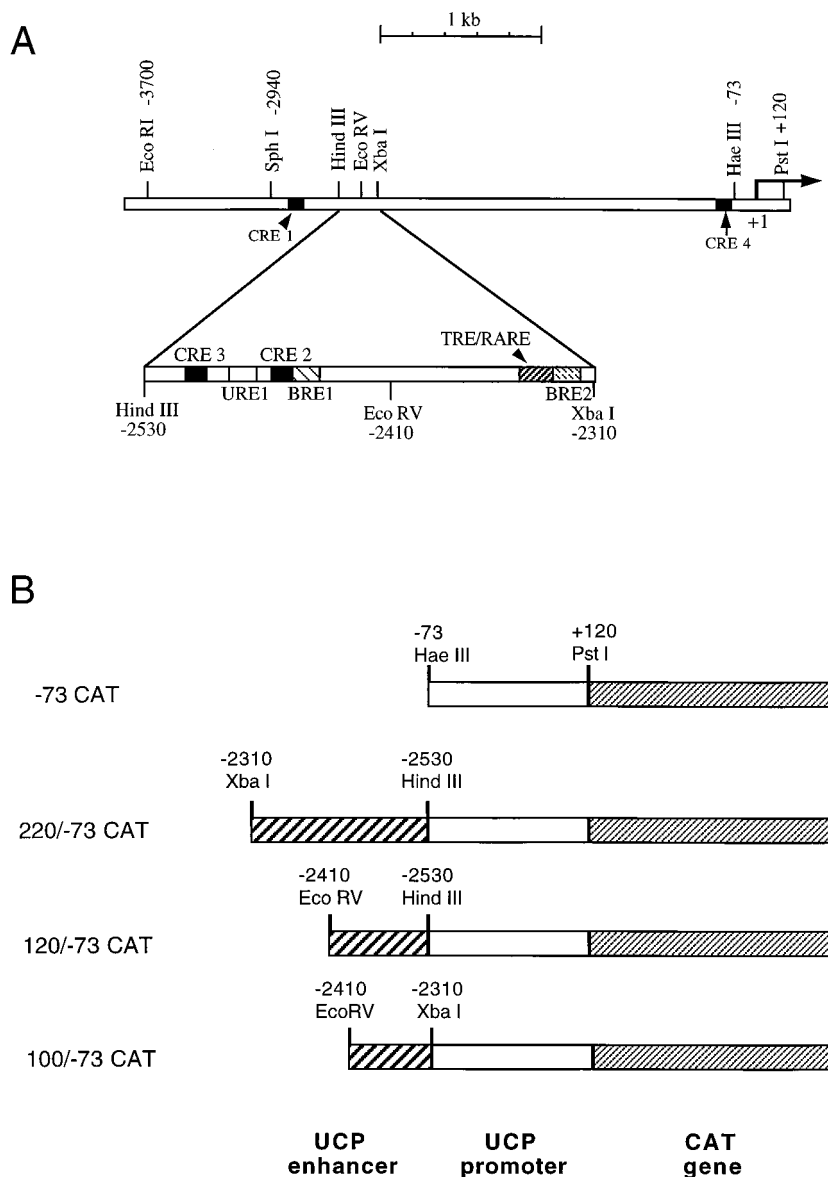


FIG. 1. Map of the murine UCP gene 5' flanking region and reporter constructs. (A) The region of the UCP gene from -3.7 kb to $+120$ bp is shown. Restriction sites and coordinates used in this study are shown; the coordinates are from the data of Kozak et al. (28). The start site of transcription is indicated by the bent arrow. The 220-bp enhancer region is enlarged, and the *cis*-acting elements that have been identified are indicated: CRE1 to -4 (28), BRE1 and -2 (28); TRE/RARE, the thyroid response element-retinoic acid response element (3, 6, 36); and URE1 (this study). Note that BRE1 overlaps the Ets1-NF-1 sites (6). (B) UCP reporter constructs. See Materials and Methods for construction details. Note that the minimal UCP promoter construct does not contain CRE4.

activity only in differentiated cells treated with NE (lanes 9 to 12) or with 1 mM dbcAMP (data not shown). The activity observed in preadipocytes (Fig. 2, lanes 1, 2, 5, and 6) results from a small amount of differentiation in our preadipocyte cultures and the greater transfection efficiencies of these cultures. For comparison of transfection efficiencies, we included a control construct, ARE3/SVCAT (47), containing three copies of a ubiquitously active enhancer element (Fig. 2, cf. lanes 7, 8, 13, and 14).

We then generated two smaller fragments from the 220-bp enhancer, a 5' 120-bp and a 3' 100-bp fragment, with *EcoRV* and inserted each into the -73 CAT vector (Fig. 1). The activities of these smaller fragments were tested by transfection into HIB-1B cells (Fig. 2). While the 100-bp fragment did not have autonomous enhancing activity (data not shown), the

120-bp fragment retained a level of differentiation-dependent enhancing activity similar to that of the whole enhancer (Fig. 2, lanes 11, 12, 17, and 18). To identify possible *cis*-acting elements involved in the differentiation-dependent activity, the 120-bp fragment was used as a probe in electrophoretic mobility shift assays (EMSA) with nuclear extracts from HIB-1B preadipocytes and adipocytes. We were able to identify a nucleoprotein complex that was more abundant in adipocyte than in preadipocyte nuclear extracts (see Fig. 5A). We used methylation interference analysis (45) to localize the binding site to a DR-1 element within the 120-bp fragment. This element, referred to as URE1, is shown in comparison with a consensus DR-1 sequence (the reverse complement is shown), and the methylation interference results are summarized in Fig. 3A. DR-1 elements are nuclear hormone response elements that

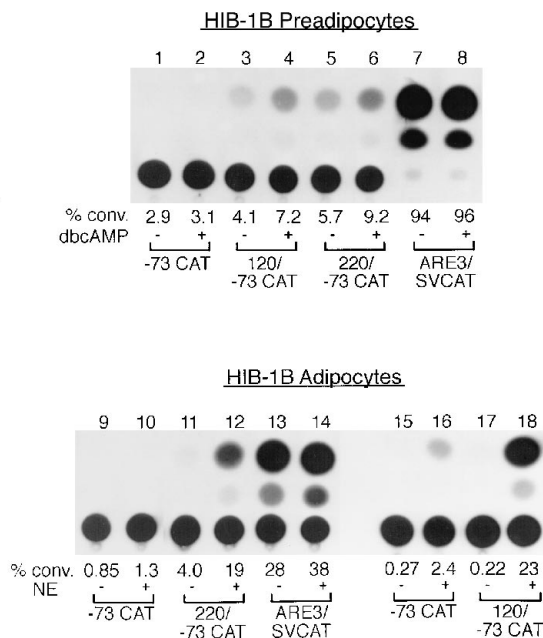


FIG. 2. Differentiation-dependent activation by UCP enhancer fragments. CAT activities resulting from transient transfection of HIB-1B preadipocytes (lanes 1 to 8) and adipocytes (lanes 9 to 18) with 8 μ g of reporter construct DNA (Fig. 1) by the calcium phosphate coprecipitation method (14) are presented. Following transfection, cells were treated with NE, dbcAMP, or PBS (-). ARE3/SVCAT contains three copies of a ubiquitously active enhancer element from the aP2 enhancer (47). The percent conversion of chloramphenicol to acetylated forms (% conv.) was quantitated with a PhosphorImager. Comparable results were obtained from five independent repetitions of this experiment with three different plasmid preparations.

contain two half-sites (AGGTCA) in a direct repeat separated by 1 bp; they can be recognized by homodimers of RXR or heterodimers of RXR with PPAR (25, 52).

Since PPAR family members regulate the expression of several genes involved in lipid metabolism (21) and the PPAR γ 2 isoform has been shown to be specifically expressed in white adipose tissue (BAT was not examined), we hypothesized that PPAR γ may be involved in regulating the activity of the UCP enhancer through the URE1 site. To determine if PPAR γ -RXR α heterodimers could bind to the 120-bp fragment, in vitro-translated PPAR γ and/or RXR α protein(s) were used in EMSA with the 120-bp enhancer fragment. As shown in Fig. 3B, neither PPAR γ nor RXR α alone is able to bind this fragment; however, if both proteins are included in the binding reaction, two nucleoprotein complexes are formed. Both of the complexes represent sequence-specific binding, since their formation is inhibited by a consensus DR-1 sequence but not by a consensus CRE. The significance of the more slowly migrating component of the complex seen with the in vitro translation products is unknown. It should be noted, however, that the PPAR γ 2 cDNA used for in vitro translation directs the synthesis of both the PPAR γ 1 and PPAR γ 2 isoforms, which differ by 30 amino acids at their amino termini (50). The faster-migrating component of the in vitro-translated 120-bp complexes has a mobility similar to that of the nucleoprotein complex formed by the interaction of HIB-1B adipocyte nuclear extract with the 120-bp fragment (Fig. 3B, cf. lanes 5 and 6). The binding of HIB-1B adipocyte nuclear extract to the 120-bp fragment is due to recognition of a DR-1 element, since binding is blocked by inclusion of a DR-1 sequence but not a CRE sequence (Fig. 3B, lanes 6 to 8). These data demonstrate that

PPAR γ -RXR α can bind to the 120-bp fragment and that a DR-1 element is responsible for the binding of HIB-1B adipocyte nuclear extracts to this fragment.

To explore nucleoprotein binding to this site, we performed EMSA using a synthetic double-stranded oligonucleotide, PR1, which contained URE1 and flanking sequences from the UCP enhancer; PR1 also includes the nonconsensus CRE2, which is a previously identified mediator of the NE response of the enhancer in brown adipocytes (28). The PR1 oligonucleotide was used to investigate the binding properties (Fig. 4) and the functional activity (see Fig. 7) of the URE1 element. A single major nucleoprotein complex is detected upon the binding of HIB-1B adipocyte nuclear extracts to the PR1 probe (Fig. 4A). This binding is prevented by inclusion of excess unlabeled PR1, ARE7 (a PPAR γ -RXR α binding site from the aP2 enhancer [50]), or a consensus DR-1 sequence in the binding reaction (Fig. 4A). Inclusion of a consensus CRE sequence had no effect on the major complex, although it does appear to compete with the more slowly migrating complexes that may represent binding of CREB; however, this possibility was not further investigated. To demonstrate that an intact

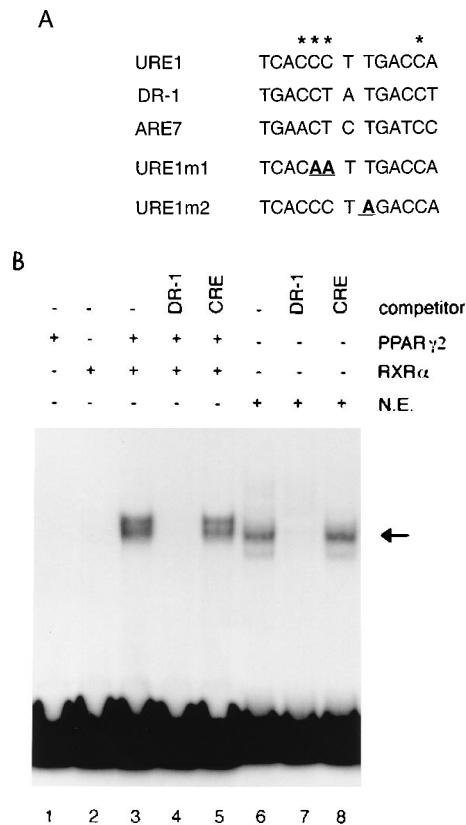


FIG. 3. Identification of a PPAR γ binding site in the UCP enhancer. (A) The DNA sequence of the UCP gene from -2490 to -2478 (URE1) is shown, and the methylation interference results are indicated by the asterisks. The sense strand of the UCP gene is shown and represents the reverse complement of the generally reported consensus half-site, AGGTCA. URE1 is compared with a reverse complement of a consensus DR-1 site (26, 52) and with ARE7, a PPAR γ binding site from the aP2 enhancer (50). Also shown are mutations of either the 5' half-site (URE1m1) or the 3' half-site (URE1m2). The mutated nucleotides are indicated by boldface underlined text. (B) EMSA of protein binding to the 120-bp UCP enhancer region (-2530 to -2410). The source of protein(s) is indicated: PPAR γ 2, in vitro-translated PPAR γ 2 cDNA; RXR α , in vitro-translated RXR α cDNA; and N.E., nuclear extracts from HIB-1B C3 adipocytes. Competitors were included at a 20-fold molar excess over the amount of the probe. CRE, consensus CRE oligonucleotide.

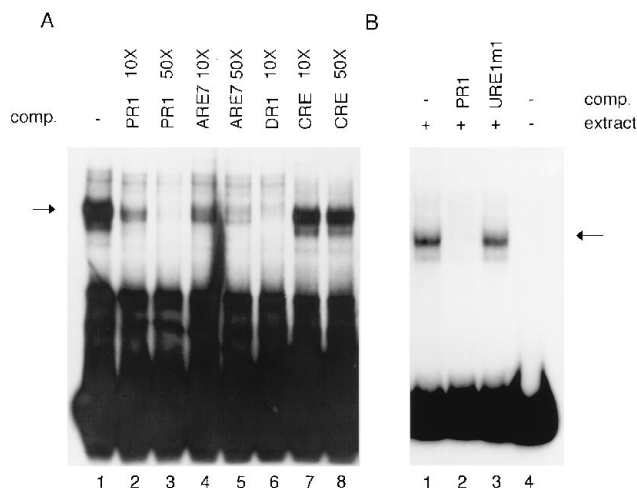


FIG. 4. Protein binding to PR1. (A) Competition analysis of HIB-1B C3 brown adipocyte nuclear extract binding to PR1, a double-stranded oligonucleotide containing URE1 and CRE2 (bp -2461 to -2500 of the UCP enhancer), is shown. The CRE2 site (TAGTCGTCA) differs from the consensus CREB-ATF binding site (TGACGTCA) (28). The arrow represents binding to the URE1 site. Competitors (comp.) are as described in the legend to Fig. 3. (B) Nuclear extract binding to the 120-bp fragment probe in competition with the 20-fold molar excess of a double-stranded PR1 oligonucleotide or URE1m1 oligonucleotide containing a mutation of the 5' half-site of URE1 (Fig. 3).

URE1 was required for protein binding to the 120-bp enhancer fragment, we used an oligonucleotide that contained a mutation of the 5' half-site of URE1 from TCACCC to TCACAA (URE1m1) (Fig. 3A). When included in an EMSA binding reaction mixture, the wild-type URE1 (PR1), but not the mutated URE1 sequence (URE1m1), inhibited binding of HIB-1B adipocyte nuclear extracts to the 120-bp probe (Fig. 4B, lanes 1 to 3). A mutation in the 3' half-site of URE1 from TGACCA to AGACCA (URE1m2) (Fig. 3A) also abolishes binding (data not shown). When oligonucleotides containing the m1 and m2 mutations of URE1 are used as probes in EMSA, nucleoprotein complex formation is not observed (data not shown). These studies establish URE1 as the DR-1 element that is bound by proteins from adipocyte nuclear extracts and, combined with the experiments presented in Fig. 3B, suggest that it can be recognized by a PPAR γ -RXR α heterodimer.

Next we used specific antisera to test directly whether PPAR γ was present in the PR1 binding complex. The binding to PR1 was examined with nuclear extracts from 3T3F442A and HIB-1B preadipocytes and adipocytes. Preadipocyte nuclear extracts exhibit less PR1 binding activity than adipocyte nuclear extracts (Fig. 5A, cf. lanes 1 and 7 with 4 and 10). The amount of nuclear extract included in these binding reaction mixtures was normalized to the amount required to produce equal amounts of mobility shift with ARE2 as a probe (data not shown); ARE2, a ubiquitously active *cis*-acting element from the aP2 enhancer, has been previously shown to be bound by a ubiquitous factor (16). Preincubation of the adipocyte nuclear extracts with PPAR γ antibody abolished the ability of the extracts to bind the PR1 probe; preimmune sera had no effect on binding (Fig. 5A, lanes 4 to 6 and 10 to 12). Treatment of the HIB-1B adipocyte nuclear extracts with RXR α antibody caused a supershift in the mobility of the URE1 complexes, confirming the presence of RXR α in these complexes (data not shown). The level of preadipocyte nuclear extract binding to PR1 was only slightly decreased by incubation with the PPAR γ antibody, and a significant amount of the

complex was resistant to the antibody (Fig. 5A, lanes 1 to 3 and 7 to 9).

To verify that PPAR γ is a major component of binding to URE1 *in vivo*, nuclear extracts were prepared from the BAT of cold-exposed mice. These extracts formed nucleoprotein com-

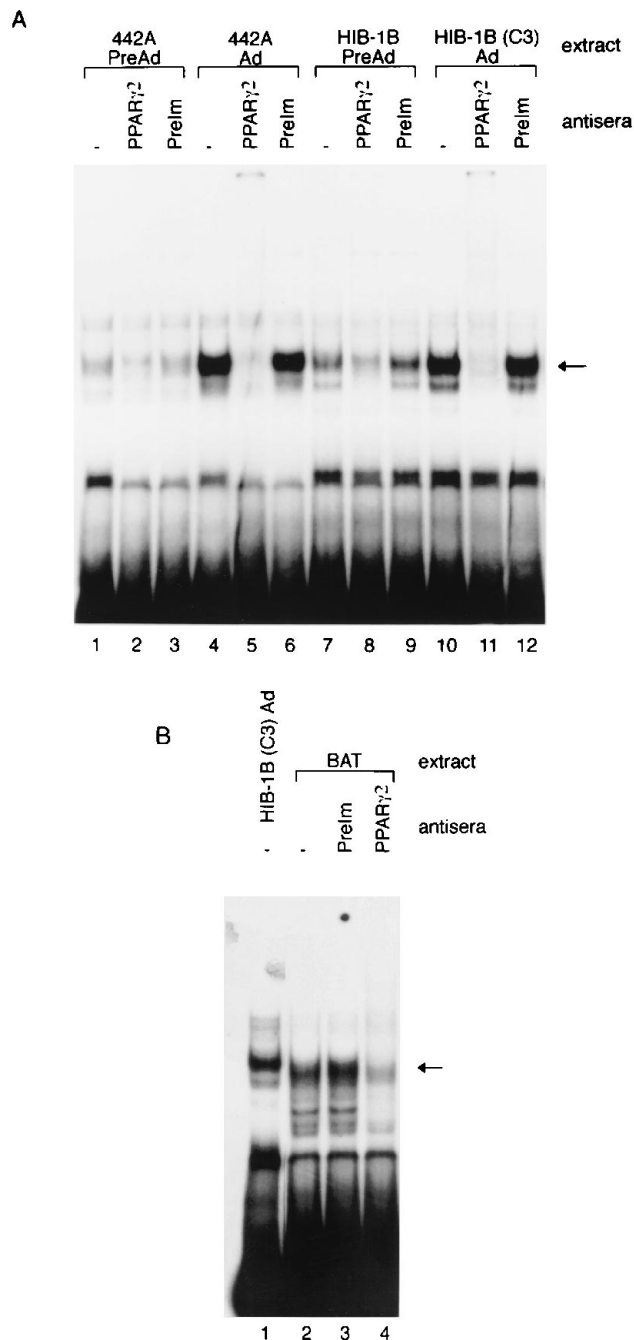


FIG. 5. Disruption of URE1 binding by PPAR γ antisera. (A) Nuclear extracts from 3T3F442A (442A) and HIB-1B preadipocytes (PreAd) or adipocytes (Ad) were incubated with 1 μ l of antiserum to PPAR γ or preimmune serum (PreIm) for 15 min at room temperature before use in EMSA with PR1 as the probe. Specific complexes are indicated by the arrow. (B) Nuclear extracts from the BAT of mice exposed to cold (4°C) for 18 h were incubated with 1 μ l of antiserum to PPAR γ or with preimmune serum for 15 min at room temperature before use in EMSA with PR1 as the probe. Specific complexes are indicated by the arrow. Also shown is binding to PR1 by HIB-1B adipocyte nuclear extract.

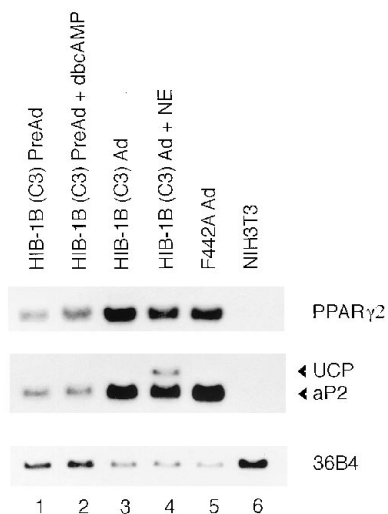


FIG. 6. Expression of PPAR γ 2 mRNA in HIB-1B differentiation. Total RNA (10 μ g) was isolated from cultured cells, blotted on a Zeta-probe membrane, and hybridized with 32 P-labeled cDNA probes. The probes include PPAR γ 2, UCP, and aP2. The 36B4 cDNA probe for human acidic ribosomal phosphoprotein PO (29) was used to control for RNA quality and blot transfer efficiency. Cultured cells include NIH 3T3 fibroblasts, 3T3F442A adipocytes (Ad), and HIB-1B brown preadipocytes (PreAd) and adipocytes. Cells were treated with NE or dbcAMP as indicated for 4 h immediately prior to RNA isolation.

plexes with the PR1 probe that had the binding specificity expected for PPAR γ -RXR α heterodimers (data not shown). When compared with HIB-1B adipocyte extract, the complexes from tissue have a slightly faster mobility (Fig. 5B, lanes 1 and 2); we suspect this is due to proteolysis during extract preparation. The BAT-derived complexes represent sequence-specific binding, since they are inhibited by PR1, ARE7, and DR-1 sequences but not by CRE or mutated URE1 sequences (data not shown). PPAR γ was required for binding of the tissue extracts to PR1, since preincubation of the BAT extracts with the PPAR γ antibody greatly reduced the level of binding activity, while the preimmune sera had no effect (Fig. 5B, lanes 2 to 4). These data show that PPAR γ binds URE1 in the nuclear extracts from both a brown adipose cell line and BAT.

The results presented above (Fig. 5A) suggested that PPAR γ expression is regulated during HIB-1B differentiation. We next compared PPAR γ mRNA levels in HIB-1B preadipocytes and adipocytes. Figure 6 shows an analysis of the RNA content for PPAR γ , UCP, the ribosomal protein 36B4, and the adipocyte lipid-binding protein (aP2) in NIH 3T3 fibroblasts, 3T3F442A adipocytes, and HIB-1B brown preadipocytes and adipocytes. As seen in Fig. 6, the differentiation state of the cells is indicated by expression of aP2 in HIB-1B and 3T3F442A adipocytes (lanes 1 to 5). Low levels of aP2 are observed in HIB-1B preadipocytes, but the levels dramatically increase when the cells differentiate (Fig. 6, cf. lanes 1 to 2 and 3 to 4). The level of PPAR γ expression is greatly increased during HIB-1B differentiation and is only modestly affected by NE treatment (Fig. 6, lanes 1 to 4). UCP expression is observed only in differentiated HIB-1B cells treated with NE (Fig. 6, lanes 3 and 4). UCP cannot be detected in the HIB-1B preadipocytes treated with dbcAMP, the second messenger for NE (Fig. 6, lanes 1 and 2), even at much longer exposure times (data not shown). The adipocyte-specific RNAs (aP2, PPAR γ , and UCP) were not detected in NIH 3T3 fibroblasts. The level of 36B4 was determined to control for loading equivalency

(Fig. 6). These data demonstrate that PPAR γ is regulated during the differentiation of HIB-1B cells and that the level of PPAR γ in HIB-1B adipocytes is similar to the level in 3T3F442A adipocytes. UCP expression is specific for differentiated HIB-1B cells and is not expressed in 3T3F442A adipocytes. These data suggest that PPAR γ 2 plays a critical role in regulating the differentiation-dependent expression of the brown adipocyte-specific gene product UCP.

We next examined the functional properties of URE1, the PPAR γ binding site from the UCP enhancer. The URE1-containing oligonucleotide PR1 was multimerized (three tandem copies) and inserted into the -73 CAT vector, and the activity of this construct (3PR1/ -73 CAT) was determined following transfection. To demonstrate transfection efficiency in preadipocytes, ARE3/SVCAT was also included in this experiment (Fig. 7A, lane 5). The expression of 3PR1/ -73 CAT, like that of 220/ -73 CAT, is restricted to NE-treated adipocytes (Fig. 7A). The differentiation-dependent activity of these constructs is not dependent on the -73 UCP promoter, since similar results are obtained when the enhancerless simian virus 40 early promoter is used as the minimal promoter (data not shown). Thus, PR1 contains both differentiation-dependent (URE1) and NE-responsive (CRE2) enhancing activity (see Discussion). If the URE1 site is required for the differentiation-dependent activity of the 220-bp enhancer, then a mutation of this site should dramatically reduce enhancer function. To determine the role of URE1 in enhancer activity, we mutated either the 5' or 3' half-site of URE1 (m1 and m2) (Fig. 3A) in the 220-bp enhancer fragment. As shown in Fig. 7B, either of these mutations reduced the level of enhancer activity about fivefold but did not completely abolish NE responsiveness. These data demonstrate that the URE1 site is critical for enhancer function.

Since PPAR γ and RXR α are ligand-activated transcription factors, we also asked if the natural PPAR γ ligand, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (10, 24), could activate URE1. We chose to do these experiments with HIB-1B preadipocytes because they express low basal levels of PPAR γ and because the URE1 reporter construct, 3PR1/ -73 CAT, has a low level of activity in these cells (Fig. 7A). Charcoal- and dextran-treated fetal calf serum was used for these experiments to remove potential PPAR activators present in the serum. Expression of 3PR1/ -73 CAT was stimulated 2.5-fold by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ treatment, while the promoter construct, -73 CAT, was unaffected (Fig. 8). These data demonstrate that ligand activation of PPAR γ in brown preadipocytes stimulates a differentiation-dependent enhancer element (URE1) and suggest that the presence or production of PPAR γ ligands may be a critical factor in controlling the terminal steps of differentiation (see Discussion).

Two other adipocyte-specific enhancers (aP2 and PEPCK [15, 33, 41, 44]) also are regulated by PPAR γ , and it has been shown that forced expression of PPAR γ and RXR α in preadipocytes and even in NIH 3T3 fibroblasts is sufficient to activate these normally adipocyte-specific enhancers (49, 50). To test directly whether PPAR γ could activate the UCP enhancer, we carried out transactivation experiments with HIB-1B preadipocytes and NIH 3T3 fibroblasts. Expression vectors for PPAR γ and RXR α were cotransfected with different reporter constructs, and the results are presented in Fig. 9. Reporter constructs containing either the 220- or 120-bp enhancer were transactivated by PPAR γ and RXR α expression (Fig. 9A) in HIB-1B preadipocytes. Transactivation was dependent on an intact URE1 site, since the 220m1/ -73 CAT construct containing the m1 mutation of URE1 (Fig. 3A) was not activated by PPAR γ -RXR α expression (Fig. 9A). Surprisingly, in NIH

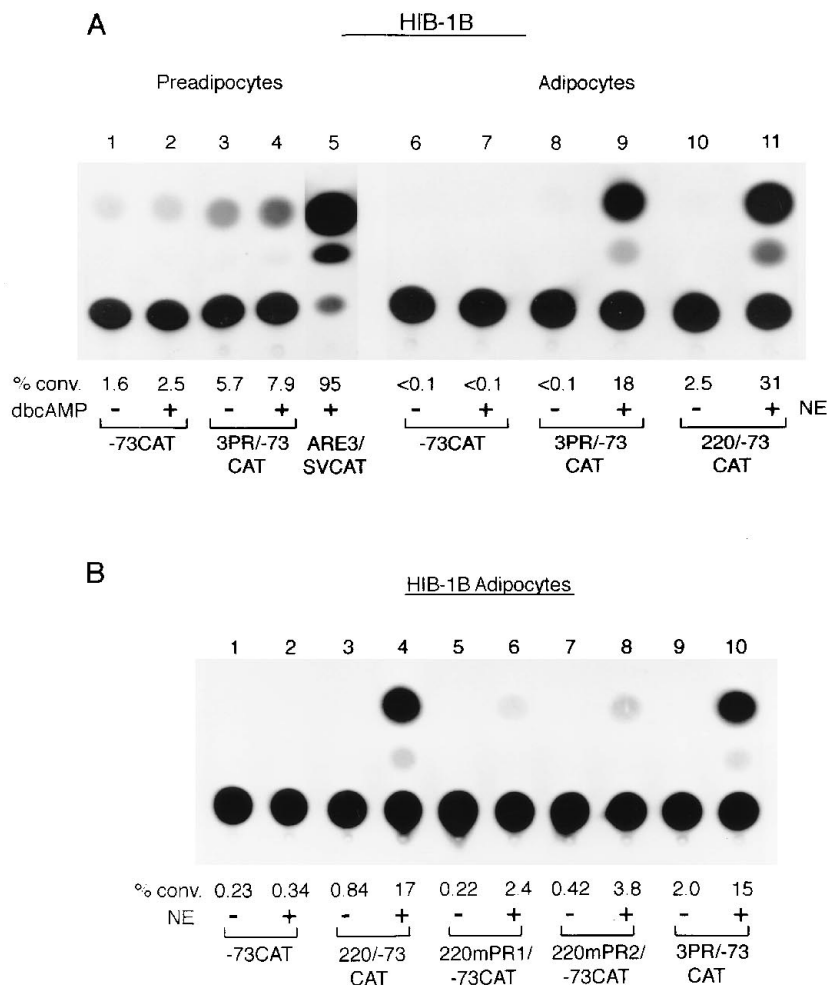


FIG. 7. Functional activities of URE1. (A) Three tandem copies of the URE1-containing oligonucleotide, PR1, were inserted into the *Xba*I site of -73 CAT. CAT activities resulting from transient transfection of HIB-1B preadipocytes (lanes 1 to 5) and adipocytes (lanes 6 to 11) as described in the legend to Fig. 2 are shown. Comparable results were obtained from six independent repetitions of this experiment with two different plasmid preparations. % conv., percent conversion of chloramphenicol to acetylated forms. (B) The results of transfecting wild-type and mutated enhancer-driven CAT constructs into HIB-1B adipocytes are presented. The mutations of the URE1 are 220mPR1 (TCACCC to TCACAA) and 220mPR2 (TGACCA to AGACCA); these correspond to URE1m1 and URE1m2 shown in Fig. 3A. Comparable results were obtained from three independent repetitions of this experiment with two different plasmid preparations.

3T3 cells the 220-bp UCP enhancer was not transactivated by PPAR γ and RXR α expression (Fig. 9B). This was not due to the inability of URE1 to be recognized by PPAR γ and RXR α in NIH 3T3 cells, since the URE1 construct, 3PR1/ -73 CAT, was readily transactivated in these experiments (Fig. 9B). As a positive control, we show that the 520-bp adipocyte-specific enhancer from the *aP2* gene could be transactivated in these cells (Fig. 9B). The transactivation of this enhancer in NIH 3T3 cells by PPAR γ and RXR α has been previously demonstrated (50). The promoter alone (-73 CAT) was not stimulated by cotransfection of the expression vectors in either cell type (Fig. 9A and B). These results show that PPAR γ -RXR α interaction at URE1 mediates the differentiation-dependent activity of the UCP enhancer. In addition, they strongly suggest that there are additional factors present in brown preadipocytes and adipocytes that are needed for enhancer function.

DISCUSSION

Adipose tissue mass is tightly regulated in response to the need for and availability of energy (food). Dysfunction result-

ing in obesity has serious health implications, including increased risk of cardiovascular disease, insulin resistance, and hypertension. Reduction of adipose tissue stores is a major goal in the treatment of obesity-related metabolic defects; however, the failure rate of clinical treatments that rely on controlling food intake and exercise approaches 100%. Understanding the mechanisms that control adipose tissue growth, development, and gene expression may provide novel insights leading to alternative treatments for obesity. Since obesity can result from increases in adipose cell number, the regulation of adipocyte differentiation is a crucial control point in determining the growth of adipose tissue mass.

A great deal has been learned about the molecular events and signals that regulate adipocyte differentiation and gene expression from cell culture studies. Most recently, two transcription factors, C/EBP α and PPAR γ , have been shown to transform nonadipogenic fibroblasts into cells that will differentiate into lipid-filled adipocytes. Much attention has been focused on PPAR γ , since it is the only known transcription factor that is expressed at high levels specifically in adipose tissue (50). Furthermore, because PPAR γ is a member of the

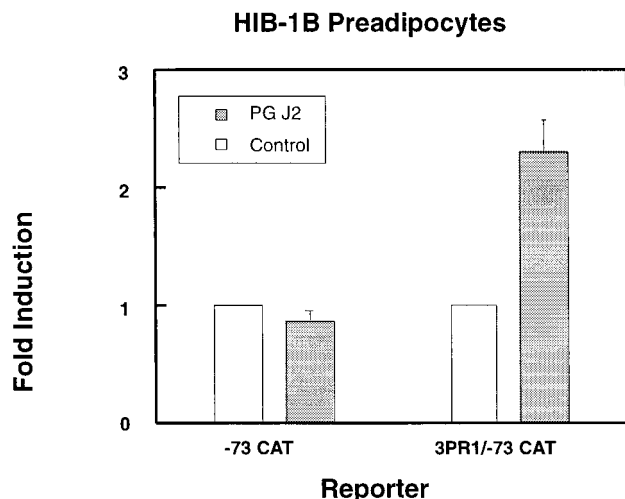


FIG. 8. Activation of URE1 by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 . CAT activity produced by HIB-1B preadipocytes transfected with reporter constructs -73 CAT and 3PR1/-73 CAT (8 μ g per plate) and treated or untreated for 24 h with 3 μ M 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (PG J_2 ; Cayman Chemical Co.), which was applied in three successive treatments at 2-h intervals beginning 24 h after transfection. Cells were cultured to confluence in 10% calf serum and then placed in 10% charcoal- and dextran-treated fetal calf serum (Irvine Scientific) 4 h prior to transfection. The data represent the averages of results from duplicate dishes, and the standard errors are indicated.

ligand-activated nuclear hormone receptor family, its activity can be modulated by external stimuli (51). In this regard, it has recently been demonstrated that PPAR activators, such as fatty acids and hypolipidemic drugs (fibrates), promote the differentiation of cultured preadipocytes (7). More intriguingly, the thiazolidinediones, a novel class of antidiabetic compounds that increase the level of peripheral insulin responsiveness, have been shown to be potent and specific activators of PPAR γ

(30) and to promote adipocyte differentiation in cell culture (23, 30). Most recently, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 has been identified as a potent adipogenic agent and shown to be a natural and specific ligand for PPAR γ (10, 24).

By the approach of combined analysis with transgenic mice and cultured hibernoma cells that express UCP, an important enhancer has been identified at kb -2.5 to -2.3 of the mouse and rat UCP genes (28). This 220-bp enhancer is located in a region that is required for brown fat specificity (4, 28). In our studies to identify the elements needed for brown adipocyte-specific gene expression, we found that the UCP enhancer is regulated in a differentiation-dependent manner in HIB-1B cells. This response was mapped to URE1, a nuclear hormone DR-1 element that is recognized by a heterodimer of PPAR γ -RXR α . Using PPAR γ antisera, we demonstrated that PPAR γ is a component in nucleoprotein complexes formed between URE1 and brown adipocyte nuclear extracts from cultured cells or cold-exposed mice. These results suggest a molecular explanation for previous studies showing that ciglitazone, a thiazolidinedione antidiabetic agent, increased energy expenditure in lean rats by increasing the thermogenic activity level of BAT (42). In genetically obese (*ob/ob*) mice, ciglitazone was shown to restore cold-induced UCP expression (34). The identification of a PPAR γ response element in the UCP enhancer raises the possibility that some of the antidiabetic effects of this class of drugs (thiazolidinediones) may be due to their effects on brown adipocyte differentiation and thermogenesis. We are currently studying the effects of these compounds on the differentiation of HIB-1B cells.

The localizations of three *cis*-acting elements (URE1, BRE1, and CRE2) within a 40-bp stretch of the 220-bp enhancer suggest an intimate interaction between the respective transcription factors. Indeed, enhancer activity in brown adipocytes requires all three sites (this report has shown that URE1 is required, and reference 28 showed the significance of CRE2 and BRE1). The URE1 provides the response to the

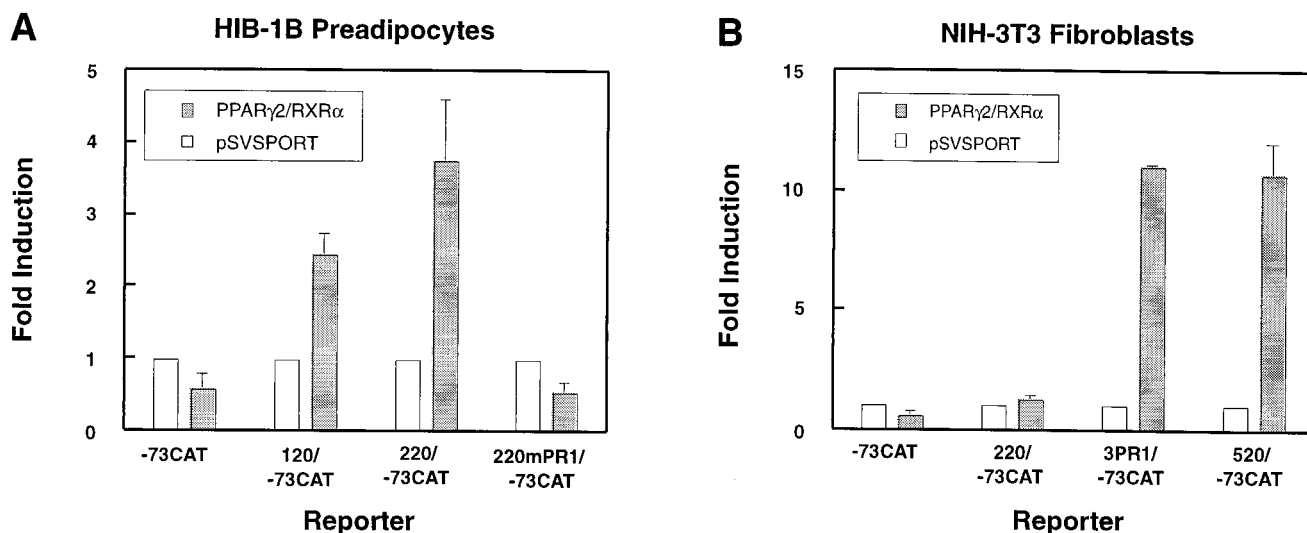


FIG. 9. Activation of the UCP enhancer by PPAR γ -RXR α expression. (A) CAT results from cotransfecting expression vectors for PPAR γ 2 (0.5 μ g) and RXR α (0.5 μ g) or from an empty expression vector (1.0 μ g; pSVSPORT) with 8 μ g of reporter constructs (Fig. 1) into HIB-1B preadipocytes. Cells were treated for 24 h with 1 mM dbcAMP. Comparable results were obtained from four independent repetitions of this experiment with two different plasmid preparations. The data shown represent the averages of three experiments, and the standard errors are indicated. (B) CAT results from cotransfecting expression vectors for PPAR γ 2 (0.5 μ g) and RXR α (0.5 μ g) or from an empty expression vector (1.0 μ g; pSVSPORT) with 1 μ g of reporter constructs (Fig. 1) into confluent NIH 3T3 fibroblasts. Cells were treated for 24 h with 1 mM dbcAMP. 520/-73 CAT contains the aP2 enhancer (kb -5.4 to -4.9 [15]) that has previously been shown to be transactivated in NIH 3T3 cells inserted into the minimal UCP promoter vector (-73 CAT). Comparable results were obtained from four independent repetitions of this experiment with two different plasmid preparations. The data shown represent the averages of two experiments, and the standard errors are indicated.

differentiation state of the cell, while CRE2 responds to NE treatment. Studies of white preadipocyte cell lines have shown that an increased expression level of PPAR γ accompanies differentiation (50). Ectopic expression to high levels of PPAR γ in nonpreadipocyte cell lines, such as NIH 3T3, requires treatment with PPAR γ ligands or activators in order to achieve adipocyte differentiation (51). By contrast, in preadipocyte cell lines, such as 3T3F442A, exogenous activators other than serum and insulin are not required (17, 18). This suggests that the latter cell line may produce activators. It will be important in future studies to identify the mechanism for PPAR γ activation in adipocyte cell lines.

The substantial increase in PPAR γ levels that accompanies HIB-1B cell differentiation may make the cells more responsive to activators, such as fatty acids, that are present in serum. In addition, it is also possible that the differentiated cells produce natural PPAR γ ligands or activators. In this regard, we have observed that the level of URE1 activity can be increased in preadipocytes by both mechanisms. Overexpression of PPAR γ and RXR α by cotransfection stimulates enhancer and URE1 activities in preadipocytes. In HIB-1B preadipocytes that express low levels of PPAR γ (Fig. 6), the natural PPAR γ ligand (15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂) is capable of activating URE1. The premature activation of URE1 in preadipocytes in response to 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ suggests that the availability of PPAR γ ligands is critical in the differentiation-dependent activation of URE1. It will be of interest to determine if PPAR γ also regulates UCP expression in response to physiologic signals. For example, fatty acids have been identified as activators of the PPAR family, and fatty acid levels rise dramatically because of the catecholamine-stimulated lipolysis accompanying cold-exposure and diet-induced BAT thermogenesis. Thus, PPAR γ could play a dual role in the regulation of UCP expression by also mediating fatty acid induction of UCP expression.

Although it has not been firmly established that the UCP enhancer is capable of directing gene expression specifically to BAT in transgenic mice, it does appear to function specifically in cell culture model systems (5, 28). Clearly, in our transactivation experiments we see that the enhancer can be transactivated in brown preadipocytes by PPAR γ -RXR α expression; however, this is not sufficient to achieve enhancer function in NIH 3T3 fibroblasts. This result implies either that there is a specific factor in brown preadipocytes (and adipocytes) that is required for activity or that there is a silencer activity in NIH 3T3 cells that prevents enhancer function. The notion that this factor is already present in the preadipocytes suggests that it may be a factor that is activated during the transition from a pluripotent proadipocyte to a preadipocyte destined to become a UCP-expressing brown fat cell. Indeed, there is evidence to suggest that preadipocytes are committed to either white or brown cell lineages (37). The identification of this factor is a goal of our current studies. The HIB-1B cell line can be grown in suspension and has already been used to purify adipocyte-specific transcription factors (48).

Although brown and white adipocytes have opposing functions, they complement each other in dealing with increased energy intake. Thus, it is not surprising that the signals mediating their growth and differentiation would be shared. PPAR γ may represent a key factor in sensing these signals and stimulating the differentiation of preadipocytes. For example, when an animal is challenged with increased energy intake, both white adipose tissue and BAT masses increase to store or burn, respectively, the excess energy intake. The properties and expression pattern of PPAR γ suggest that it may play a pivotal role in the response to increased energy intake. It has become

increasingly clear that obesity does not always result from gluttony but can also be due to increased energy efficiency that in some circumstances results from decreased BAT activity levels. A clear role for BAT in regulating energy balance has been well established for rodents; however, its role in adult humans remains controversial. BAT has been identified in adult humans, and it will be interesting if this tissue can be exploited to control obesity.

ACKNOWLEDGMENTS

We thank Peter Tontonoz, Chiayeng Wang, and Nick Davidson for helpful discussions and critical readings of the manuscript. We thank Lisa Choy for generously providing the HIB-1B C3 cell line, Erding Hu and Peter Tontonoz for the generous gift of PPAR γ antibody, and Peter Tontonoz for PPAR γ and RXR α expression vectors. We thank Bruce Spiegelman for generously sharing all of the above-described reagents and for helpful discussions. We thank Ron Evans for the RXR α clone.

This work was supported by a development award from the University of Chicago (to R.A.G.) and by a pilot and feasibility study award (to R.A.G.) from the Clinical Nutrition Research Unit (DK26678) of the University of Chicago. I.B.S. was supported by a USDA training grant (92-38420-7335).

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