THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 34, pp. 24660–24669, August 24, 2007

C/EBPβ Reprograms White 3T3-L1 Preadipocytes to a Brown Adipocyte Pattern of Gene Expression*

Received for publication, April 12, 2007 Published, JBC Papers in Press, June 21, 2007, DOI 10.1074/jbc.M703101200

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cAMP-dependent protein kinase induction of PPARγ coactivator-1α (PGC-1α) and uncoupling protein 1 (UCP1) expression is an essential step in the commitment of preadipocytes to the brown adipose tissue (BAT) lineage. We studied the molecular mechanisms responsible for differential expression of PGC-1α in HIB1B (BAT) and 3T3-L1 white adipose tissue (WAT) precursor cell lines. In HIB1B cells PGC-1α and UCP1 expression is cAMP-inducible, but in 3T3-L1 cells, expression is reduced and is cAMP-insensitive. A proximal 264-bp PGC-1α reporter construct was cAMP-inducible only in HIB1B cells and was suppressed by site-directed mutagenesis of the proximal cAMP response element (CRE). In electrophoretic mobility shift assays, the transcription factors CREB and C/EBPβ, but not C/EBPα and C/EBPδ, bound to the CRE on the PGC-1α promoter region in HIB1B and 3T3-L1 cells. Chromatin immunoprecipitation studies demonstrated that C/EBPβ and CREB bound to the CRE region in HIB1B and 3T3-L1 cell lysates. C/EBPβ expression was induced by cAMP only in HIB1B cells, and overexpression of C/EBPβ rescued cAMP-inducible PGC-1α and UCP1 expression in 3T3-L1 cells. These data demonstrate that differentiation of preadipocytes toward the BAT rather than the WAT phenotype is controlled in part by the action of C/EBPβ on the CRE in PGC-1α proximal promoter.

Two specialized adipose tissues have evolved to enable mammals to adapt to varying metabolic demands. White adipose tissue (WAT)[2] is able to store energy in the form of triacylglycerol, whereas brown adipose tissue (BAT) dissipates energy in the form of heat to maintain body temperature, particularly in juvenile animals (1). The BAT phenotype is characterized by a high density of mitochondria expressing the BAT-specific protein, uncoupling protein 1 (UCP1) (2). WAT is also an endocrine organ that secretes adipocytokines, which have been implicated in the control of a wide range of physiological functions that under pathophysiological conditions may lead to the development of metabolic syndrome, diabetes, and cardiovascular disease (3). Therefore, understanding the molecular mechanisms regulating the conversion of progenitor cells to the WAT or BAT lineage will improve our understanding of processes that lead to WAT accumulation, obesity, and related pathological conditions.

The immortalized mouse brown HIB1B and white 3T3-L1 preadipocyte cell lines have been used to study the molecular events underlying the determination and differentiation of adipose tissue. 3T3-L1 cells can be induced to differentiate into lipid droplet accumulating cells by a mixture of hormones. White adipocyte differentiation is the result of a chain of transcriptional events involving CCAAT/enhancer-binding proteins C/EBPα, C/EBPβ, and C/EBPδ (4–6) and the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) (7, 8), which combine to induce adipogenic genes. The transcriptional events involved in brown adipocyte differentiation are not as well characterized, but genetic ablation studies have demonstrated that PPARγ, C/EBPβ, and C/EBPδ, but not C/EBPα, are also essential for this process (9, 10).

The nuclear receptor coactivator, PPARγ coactivator-1α (PGC-1α) has been shown to be involved in the expression of the BAT phenotype (11). PGC-1α is expressed in a number of tissues including brown adipose tissue, muscle, liver and brain (11). It may play a role in the stimulation of oxidative capacity via its interaction with the NRF1 and -2 family, which regulates mitochondriogenesis and the expression of mitochondrial oxidative enzymes (12). PGC-1α is induced by exposure to cold or adrenergic stimulation in BAT but not WAT, and ectopic expression of PGC-1α induces expression of UCP1 in WAT cells (11). PGC-1α is also expressed in non-adipose cells under circumstances in which increased metabolic energy expenditure is favored, for example in exercise-conditioned muscle tissue (13) or in the liver in response to glucagon stimulation during fasting (14).

The expression of PGC-1α is cAMP-dependent in liver and BAT, and a major role of PGC-1α is to augment cAMP-mediated transactivation of effector genes such as UCP1 in BAT, or PEPCK in the liver (11, 13). cAMP induction of PGC-1α appears to depend primarily on a single conserved cAMP response element (CRE) found within 200 bp of the transcription start site (14, 15). Although this proximal CRE can drive

* This work was supported by the Biotechnology and Biological Sciences Research Council and the Greek State Scholarship's Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; PPARγ, peroxisome proliferator-activated receptor γ; PGC-1α, PPARγ coactivator-1α; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; MSV, mouse sarcoma virus; PKA, cAMP-dependent protein kinase; PEPCK, phosphoenolpyruvate carboxykinase; RT, real time.

PGC-1α expression in both BAT and liver, additional unidentified mechanisms operate to silence cAMP-inducible PGC-1α expression in WAT, even though a range of other WAT expressed genes are cAMP-regulated (16).

A genome-wide analysis of CREs in the human genome has revealed that CREB-binding protein (CBP) and phosphorylated CREB (phospho-CREB) are widely bound to CREs without transactivation (17). Despite the association of phospho-CREB with a number of coactivators (CBP/p300), gene activation is weak and appears to require additional phospho-CREB regulatory partners for stable recruitment of the preinitiation complex. Studies on the PEPCK and interleukin-10 promoters have demonstrated an interaction between CRE and C/EBP binding sites in conferring tissue-specific and differentiation-dependent responses to cAMP (18–20).

To investigate the differential activation of PGC-1α and UCP1 gene expression by cAMP in WAT and BAT, we studied the control of the PGC-1α proximal promoter in HIB1B and 3T3-L1 adipogenic cell lines. Here we report that the CRE-containing proximal region of the PGC-1α promoter is sufficient to confer adipose cell-specific cAMP inducibility. We further report for the first time C/EBPβ expression and binding to the CRE as the mechanism that is able confer a BAT gene expression pattern on 3T3-L1 white preadipocytes.

EXPERIMENTAL PROCEDURES

Plasmids—Firefly luciferase reporter gene constructs containing 1873 bp (1873PGC1α-pGL3), 890 bp (890PGC1α-pGL3), and 264 bp (264PGC1α-pGL3) from the region upstream of the rat PGC-1α transcription start site were generated using the pGL3-Basic vector (Promega). These fragments were generated by PCR using primers 1873PGC1α sense (GTACGCGTACCATTCTGCTGTCTTGAAG), 890PGC1α sense (GTACGCGTCTGGGAGCCTATGAGAGCCA), and 264PGC1α sense (GTACGCGTAGTGTTTCCTTTCTTTCTTCTAT) and antisense (GTCTCGAGCAACTCCAATCCACTCTGAC) that had MluI or XhoI restriction sites at the 5′-end and were digested with the restriction enzymes MluI and XhoI to generate appropriate protruding ends. The pGL3-Basic vector (Promega) was also digested with the same enzymes, and the inserts were then ligated into these vectors.

Site-directed mutagenesis on the luciferase promoter (264PGC1α-pGL3) was performed using the QuikChange site-directed mutagenesis kit (Stratagene).To mutate the CRE the original sequence TGACGTCA was mutated to GACTACTG. Successful mutagenesis was monitored by sequence analysis (John Innes Sequencing Centre, Norwich, UK).

The pMSVC/EBPα (rat), pMSVC/EBPβ, and pMSVC/EBPδ (mouse) expression plasmids, which contain the respective cDNAs under the control of the mouse sarcoma virus (MSV) long terminal repeat, were kindly provided by S. McKnight (University of Texas Southwestern Medical Center, Dallas). The mock plasmid pcDNA3 was from Invitrogen. The CRE positive vector (6CRE-pGL3) was a kind gift from Robert Newton (Dept. of Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, London).

Cell Culture, Transfection, and Luciferase Assay—3T3-L1 cells (ECACC) and HIB1B cells (kindly provided by B. Spiegelman) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen) in 5% CO_2. For differentiation, cells were cultured to confluence (day 0) and then exposed to the differentiation mixture (0.5 mM 3-isobutyl-1-methylxanthine, 250 nM dexamethasone, 20 nM insulin, 1 nM T3). After 48 h, cells were maintained in medium containing 20 nM insulin and 1 nM T3 until day 7 for harvest.

The reporter plasmids were co-transfected using 3 μl of FuGENE 6 (Roche Applied Science)/μg of DNA or 2 μl of Lipofectamine 2000 (Invitrogen)/μg of DNA into 3T3-L1 and HIB1B cells at 80% confluence in combination with pMSVC/EBPα, pMSVC/EBPβ, pMSVC/EBPδ, or pcDNA3 as a control. The pRL-SV40 (from Promega) that carries *Renilla* luciferase was also co-transfected as an internal control for monitoring the transfection efficiency. At confluence (approx 24 h later), cells were treated with forskolin under serum-free conditions, and after 12 h cells were harvested and luciferase activities analyzed using the Dual-Luciferase assay kit (Promega) as recommended by the manufacturer. Values were expressed relative to the control *Renilla* to allow for differences in transfectional efficiency; there was no difference between cell type and treatments on the average *Renilla* values.

Electrophoretic Mobility Shift Assay and Supershift Assay—Preparation of nuclear extracts for electrophoretic mobility shift assays was performed in the presence of protease and protein phosphatase inhibitors using the nuclear extract kit from Active Motif. 10 μg of nuclear proteins extracted from confluent HIB1B or 3T3-L1 cells were incubated with ~10^6 cpm of radiolabeled oligonucleotide in a 20-μl reaction for 30 min at room temperature. The oligonucleotide spanned the PGC-1α-CRE regulatory element: 5′-TGCCTTGGAG*TGACGTCA*GGAGTTTGTGCA-3′ (CRE motif is in italics). Specific binding was established by co-incubating with 100-fold excess of either unlabeled oligo or oligo containing a mutated (underlined) CRE motif 5′-TGCCTTGGAG*AGGACTACT*GGAGTTTGTGCA-3′. Nuclear proteins were incubated with antibodies where indicated for 20 min prior to incubation with the radiolabeled oligonucleotides. Antibodies against CREB and C/EBPβ (LAP) were purchased from New England Biolabs, C/EBPα from Active Motif, and C/EBPδ from Santa Cruz Biotechnology. Competing unlabeled oligonucleotides were added 15 min prior to the addition of labeled probe.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed according to the manufacturer's protocol (Upstate). Briefly, HIB-1B and 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen) in 5% CO_2 until confluence (approx 48 h after plating). Following confluence, the cells were then stimulated with vehicle solution (Me_2SO) or forskolin (10 μM) for 1 h. For the cross-linking of transcriptional factors with genomic DNAs, cells were incubated with formaldehyde (1%) for 1 h at 37 °C. The cells were then washed with ice-cold phosphate-buffered saline twice, harvested with scrapers, and lysed with SDS lysis buffer. The whole-cell lysates were sonicated with a Soniprep 150 for 30 s at the maximum setting. This was repeated eight times with 1-min intervals between each 30-s pulse, yielding chromatin fragments between 200 and 500 bp in size. Lysates were centrifuged at 13,000 rpm (Eppendorf micro-

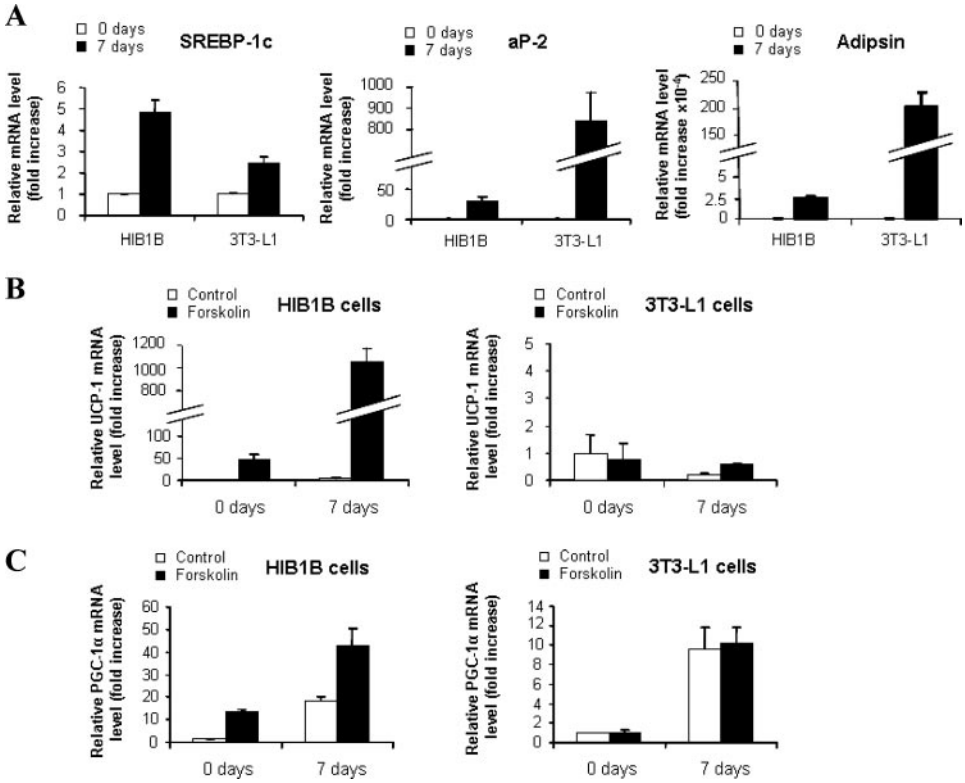

FIGURE 1. **Differential cAMP-inducibility of PGC-1α and UCP1 in HIB1B and 3T3-L1 cells.** *A*, adipogenic gene mRNA at 0 (confluence) and 7 days after induction to differentiate. *B*, UCP1 mRNA at 0 and 7 days after induction to differentiate and treated with or without forskolin for 3 h. *C*, PGC-1α mRNA at 0 and 7 days after induction to differentiate and treated with or without forskolin for 3 h. Values were analyzed by quantitative real-time PCR and normalized against β-actin expression. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

centrifuge) for 10 min, and the resulting supernatants were diluted 10-fold with ChIP dilution buffer in the presence of protease inhibitors. Normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) and salmon sperm DNA-50% protein A-agarose slurry (80 μl) was added to the lysates, which were incubated for 30 min at 4 °C to reduce nonspecific background. Agarose beads were precipitated by brief centrifugation, and the supernatant was collected. Antibodies against CREB (Upstate Biotechnology), C/EBPβ (C-19; Santa Cruz Biotechnology), or normal rabbit IgG (control samples) were added to the 2-ml supernatant fraction, and the mixture was incubated overnight at 4 °C with rotation. Immune complexes were then mixed with 60 μl of salmon sperm DNA-protein A-agarose slurry; the mixture was incubated for 1 h at 4 °C with rotation to collect the antibody-histone-DNA complex and washed sequentially with low salt buffer, high salt buffer, and lithium chloride wash buffer. After precipitates were washed with Tris-EDTA and extracted with elution buffer, reversal of cross-linking was done by heating at 65 °C overnight in the presence of NaCl. Purification of DNA was done with a QIAquick PCR purification kit (Qiagen), and the DNA fragments obtained were analyzed by PCR using the following primer pairs for the mouse PGC-1α promoter: forward (5′-GGGCTGCCTTGGAGTGACGTC-3′) and reverse (5′-AGTCCCCAGTCACATGACAAAG-3′).

Western Blotting—Cells were washed twice with ice-cold phosphate-buffered saline, lysed in modified radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 37 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5% SDS, and 0.5% sodium deoxycholate), and harvested with scrapers. The whole cell lysates were centrifuged at top speed for 15 min after 30 min of incubation on ice, and supernatants were mixed with 2× Laemmli sample buffer. An aliquot of 40–50 μg/well was fractionated by SDS-PAGE on 10% acrylamide gels and blotted onto polyvinylidene difluoride membrane. After 1 h of blocking in a buffer containing 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) nonfat milk powder, the membrane was incubated in fresh buffer with the appropriate antibody for 1 h at room temperature. The antibodies used were: 1:1000 dilution of anti-C/EBPα, 1:500 dilution of anti-C/EBPβ and anti-C/EBPδ, and 1:250 dilution of anti-actin (Sigma). The antigen-antibody complex was detected by incubating the membrane for 1 h at room temperature in a buffer containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Cell Signaling) and visualized with SuperSignal West Pico blotting substrate (Pierce, Perbio Science, Cramlington, UK).

Real-time PCR—Total RNA was isolated from cells by TRI reagent (Sigma). Quantitative RT-PCR was performed using SYBR Green (Qiagen) according to the manufacturer's instructions in Rotor Gene 3000 (Corbert Research). The sequences of the primers used for real-time PCR were: PGC-1α sense (GCGCCGTGTGATTTACGTT) and antisense (AAAACTTCAAAGCGGTCTCTCAA), UCP1 sense (CCTGCCTCTCTCGGAAACAA) and antisense (TGTAGGCTGCCCAATGAACA), C/EBPα sense (CCGGGAGAACTCTAACTC) and antisense (GATGTAGGCGCTGATGT), C/EBPβ sense (GCAAGAGCCGCGACAAG) and antisense (GGCTCGGGCAGCTGCTT), C/EBPδ sense (ACGACGAGAGCGCCATC) and antisense (TCGCCGTCGCCCCAGTC), aP2 sense (AACACCGAGATTTCCTT) and antisense (ACACATTCCACCACCAG), SREBP-1c sense (CTGGATTTGGCCCGGGGAGATTC) and antisense (TGGAGCAGGTGGCGATGAGGTTC), adipsin sense (CAGAGTGTCAATCATGAACCGG) and antisense (GATGTTTTCGATCCACATCCG) and β-actin sense (TCCTCCTGAGCGCAAGTACTCT) and antisense (GCTCAGTAACAGTCCGCCTAGAA).

Computational Analysis of the PGC-1α Promoter Region—Mouse and rat genomic sequences were retrieved from the NCBI browser (www.ncbi.nlm.nih.gov). Pairwise comparative analyses were performed with the program BLAST 2

sequences. The TESS analysis (www.cbil.upenn.edu/tess) was used to predict transcription factor binding sites. All of the positions in the promoter refer to the translation start site of the rat PGC-1α gene.

RESULTS

Differential cAMP Induction of PGC-1α and UCP1 Expression in Confluent and Differentiated HIB1B and 3T3-L1 Cells—HIB1B and 3T3-L1 cells were induced to differentiate into adipocytes by treating confluent cells with a standardized hormonal mixture as described under "Experimental Procedures." Differentiation status was verified by oil-red "O" staining (data not shown) and adipogenic gene expression profiling by quantitative RT-PCR (SREBP-1c, aP-2, adipsin, UCP1, PGC-1α; Fig. 1). To stimulate cAMP accumulation, forskolin (10 μM) was added to confluent cells immediately prior to (0 days) or 7 days following exposure to the differentiation mixture. Preliminary studies were performed with HIB1B and 3T3-L1 cells to establish the optimal time (3 h) for incubation with forskolin. In HIB1B cells, forskolin treatment increased UCP1 expression by ~50-fold in undifferentiated cells and by 1000-fold after 7 days of differentiation (Fig. 1*B*). By contrast, a lower base-line level of UCP1 expression was observed in 3T3-L1 cells, and forskolin had no effect on expression, regardless of differentiation status (Fig. 1*B*). Qualitatively similar differences in PGC-1α expression were also observed; higher basal expression levels in HIB1B cells, coupled with a pronounced inductive response to forskolin in both undifferentiated and differentiated states but no responses to forskolin were observed in 3T3-L1 cells at 0 and 7 days of differentiation (Fig. 1*C*). Responses to cAMP stimulation using 3-isobutyl-1-methylxanthine were similar to those observed for forskolin (results not shown). These results demonstrate that cAMP induction of PGC-1α and UCP1 mRNA can be observed in undifferentiated confluent HIB1B but not 3T3-L1 cells.

Cell-specific Expression of PGC-1α Promoter-Reporter Constructs: The Proximal 264 bp Is Sufficient for Specificity—To analyze the transcriptional mechanisms responsible for these cell-specific differences in PGC-1α expression, different lengths (from 264 to 1873 bp upstream of the predicted transcription start site) of the rat PGC-1α promoter were cloned into the luciferase reporter vector pGL3. These constructs were used in transient transfection experiments in HIB1B and 3T3-L1 cells. Preliminary experiments established that reporter activity measurements were optimal at 24 h after adding the hormonal mixture (differentiation mix) that is used to differentiate HIB1B and 3T3-L1 cells; these measurements were compared with levels in control undifferentiated Me_2SO-treated cells (Fig. 2). In HIB1B cells, the addition of the differentiation mix increased luciferase expression from constructs containing all three lengths of the PGC-1α promoter by 4–5-fold (Fig. 2*A*). By contrast, in 3T3-L1 cells the differentiation mix did not elicit any increase in expression for any of the promoter constructs (Fig. 2*B*).

This result suggested that factors within the proximal promoter region, encompassed by the 264-bp construct, accounted for cell-specific differences in PGC-1α induction by cAMP. This short proximal fragment exhibits very high

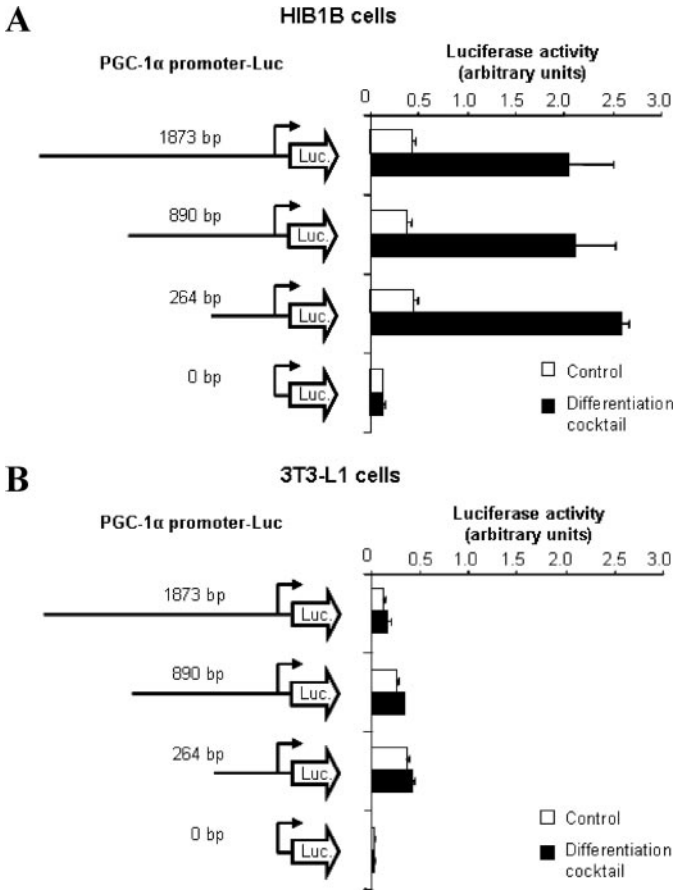

FIGURE 2. **Cell-specific expression of PGC-1α promoter-reporter constructs in which the proximal 264 bp is sufficient for specificity.** *A*, PGC-1α promoter-reporter assays in confluent HIB1B cells transiently transfected with 1873PGC1α-pGL3, 890PGC1α-pGL3, 264PGC1α-pGL3, or empty pGL3 luciferase reporter constructs treated with or without differentiation mixture of hormones for 24 h. *B*, PGC-1α promoter-reporter assays in 3T3-L1 cells transfected with 1873PGC1α-pGL3, 890PGC1α-pGL3, 264PGC1α-pGL3, or empty pGL3. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

sequence conservation across species (98% in rat/mouse/human) and contains a CRE, previously highlighted by studies of PGC-1α expression in hepatic cells (14). We therefore performed experiments in undifferentiated cells under serum-free conditions to analyze the effects of forskolin on the expression of the 264-bp promoter-reporter construct (Fig. 3, *A* and *B*). Under these conditions, forskolin induced reporter expression in both cell lines, but as predicted from the first experiment, the effect was markedly greater in HIB1B cells than in 3T3-L1 cells.

These results led us to consider the relative involvements of the core CRE and adjacent flanking regions in the cAMP-inducibility of the 264-bp construct. Site-directed mutagenesis of the CRE at positions −183 to −175 abolished completely the effect of forskolin on the proximal PGC-1α promoter-reporter activity in both cell types (Fig. 3, *A* and *B*). These data indicate that the CRE on the proximal PGC-1α promoter is able to give widely differing response characteristics to a cAMP-mediated stimulus in the two different adipocyte cell contexts. Importantly, this result was not due to the different sensitivities to forskolin, or the PKA pathway in the two cell types, because a luciferase reporter construct driven by six CREs in tandem

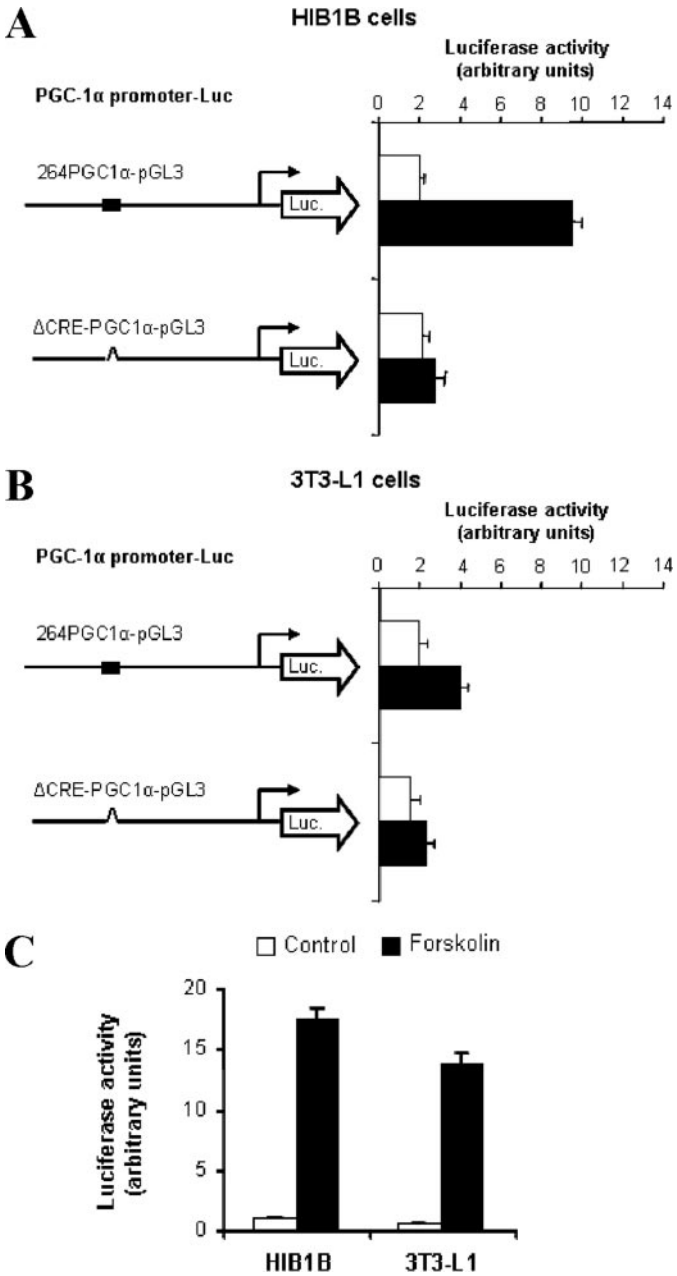

FIGURE 3. **A CRE on the 264-bp PGC-1α promoter is responsible for the adipose cell-specific response to a cAMP stimulus.** *A*, 264PGC1α-pGL3 or the same construct containing a mutated CRE assayed after transient transfection in confluent HIB1B cells and treated with or without forskolin for 12 h. *B*, PGC-1α promoter-reporter or the same construct containing a mutated CRE, assayed in transfected 3T3-L1 cells. *C*, luciferase construct (6CRE-pGL3) driven by a 6-CRE transfected in HIB1B and 3T3-L1 cells. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

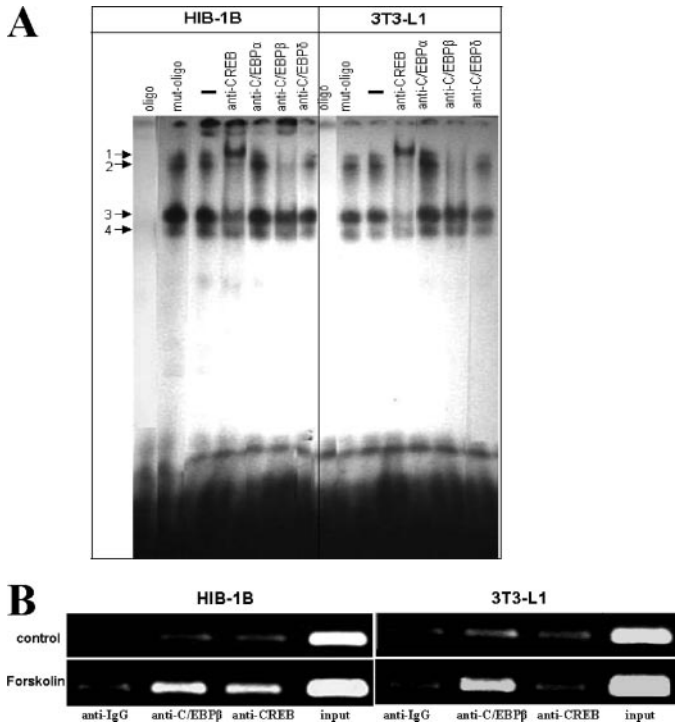

FIGURE 4. **Binding of CREB and C/EBPβ to PGC-1α-CRE.** *A*, electromobility shift assay showing specific binding for CREB and C/EBPβ, but not C/EBPα and C/EBPδ, in nuclear extracts from confluent HIB1B and 3T3-L1 cells incubated with a labeled PGC-1α-CRE oligonucleotide. *Oligo* and *mut-oligo* represent 100-fold excess of unlabeled oligonucleotide spanning the PGC-1α-CRE and the same oligonucleotide with the CRE mutated, respectively. *Numbered arrows* mark specific binding. *B*, chromatin immunoprecipitation demonstrates forskolin-dependent CREB and C/EBPβ binding to the CRE region of the PGC-1α proximal promoter in lysates from HIB1B cells. In 3T3-L1 cells only forskolin-dependent C/EBPβ binding is observed. Assays are of one of three independent experiments.

(21) was induced to the same extent in 3T3-L1 and HIB1B cells (Fig. 3*C*).

Previous studies have demonstrated that both CREB and ATF-2 can bind to the proximal CRE lying within the 264-bp region (14, 15). The C/EBP family of transcription factors has also been reported to modulate the activity of CREs in gene promoters (20). Therefore we performed gel shift experiments to identify the presence of CRE-binding proteins in nuclear extracts from HIB1B and 3T3-L1 cells. Specific binding of nuclear proteins to the PGC-1α-CRE oligonucleotide was demonstrated by either the disappearance of four bands when incubations included 100-fold excess of unlabeled oligonucleotide (Fig. 4*A*, *oligo*) or the lack of competition when the labeled PGC-1α-CRE oligonucleotide was incubated with 100-fold excess of a mutated CRE oligonucleotide (Fig. 4*A*, *mut-oligo*). Supershifts with the specific antibodies demonstrated that CREB (bands 1–4) and C/EBPβ (bands 1, 2, and 4) but not C/EBPα and C/EBPδ, were able to bind to the CRE in nuclear extracts from both HIB-1B and 3T3-L1 cells. The overlap in specific binding bands between CREB and C/EBPβ may indicate heterodimer formation between these leucine zipper transcription factors. We further demonstrated by ChIP assay that both C/EBPβ and CREB were part of a protein-DNA complex containing the CRE region of the PGC-1α proximal promoter (Fig. 4*B*). In HIB1B cells, forskolin increased the binding of both C/EBPβ and CREB, but this was only apparent for C/EBPβ in 3T3-L1 cells.

These data, along with those showing the cAMP induction of the CRE-positive control reporter in 3T3-L1 cells, suggested that the differential response in forskolin-induced PGC-1α expression between the two cell types was not related to the upstream sensitivity of the PKA pathway but rather to combinatorial transcription factor effects on the proximal CRE, which were able to confer adipocyte-specific differential expression of this promoter to cAMP.

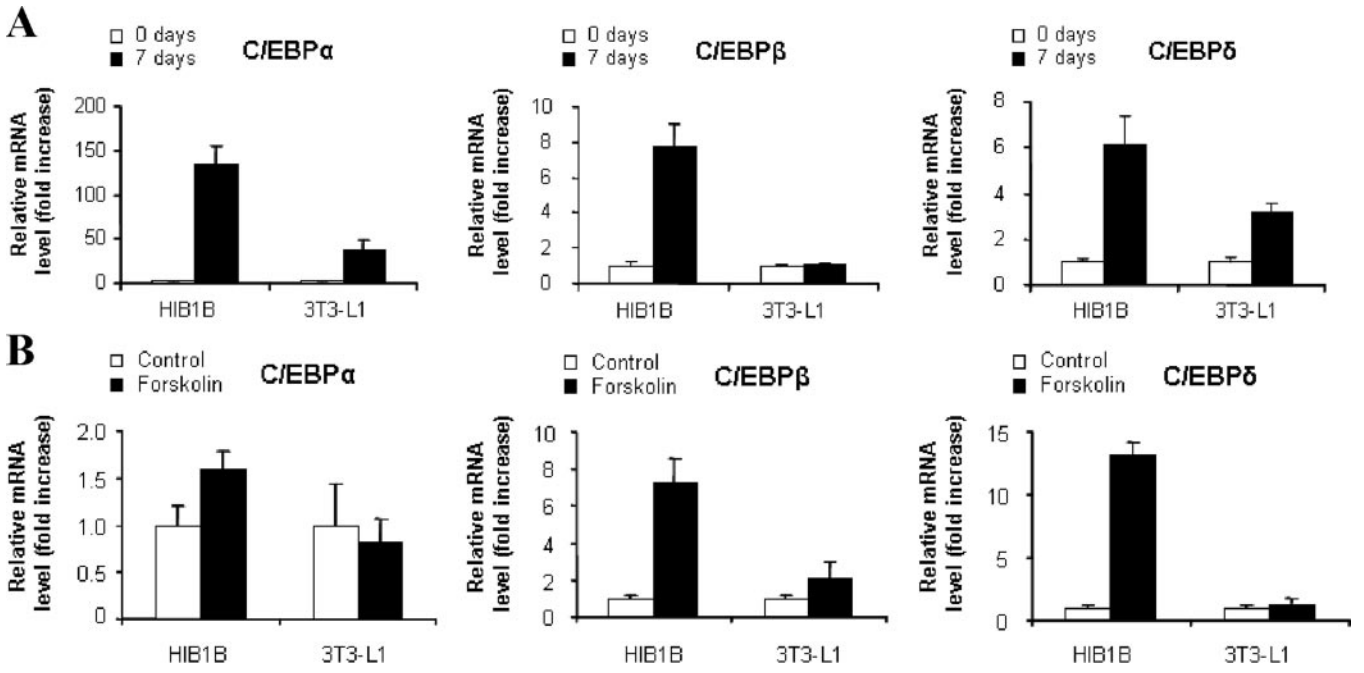

FIGURE 5. **Inducibility of C/EBP isomer mRNA during differentiation or by cAMP in HIB1B and 3T3-L1 cells.** *A*, C/EBPα/β/δ mRNA expression at 0 (confluence) and 7 days after induction to differentiate. *B*, confluent cells were treated with forskolin for 3 h. Values assayed by real-time PCR and normalized against β-actin expression. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

The Role of C/EBP Isoforms in Cell-specific cAMP Inducibility of PGC-1α—Evidence of the involvement of the C/EBP family of transcription factors in the modulation of the CRE in the promoter of other genes (20), coupled with the crucial role played by C/EBPs in commitment of progenitor cells to the adipocytes lineage (16), led us to consider whether differential expression of C/EBP isoforms might be involved in the cell context-specific cAMP inducibility of the 264PGC1α-pGL3 luciferase reporter construct.

To explore this possibility, we first used quantitative RT-PCR to compare expression patterns for three C/EBP isoforms (α, β, δ) in the two cell types during differentiation (Fig. 5*A*) and forskolin challenge (Fig. 5*B*). This showed that although there was no clear distinction between HIB1B and 3T3-L1 cells in the pattern of C/EBPα expression in response to either differentiation or forskolin, both C/EBPβ and C/EBPδ showed cell-specific differences. In the case of C/EBPβ, expression rose markedly during differentiation in HIB1B cells but did not change in 3T3-L1 cells. Additionally, both C/EBPβ and C/EBPδ were strongly induced by cAMP stimulation in confluent HIB1B compared with 3T3-L1 cells. These observations suggested that increased levels of C/EBPβ or C/EBPδ protein expression in HIB1B cells might be necessary for the induction of PGC-1α and UCP1 expression in response to cAMP stimulation during the BAT differentiation program.

To test the role of C/EBP transcription factors on PGC-1α gene regulation, we transfected both cell types with the 264-bp PGC-1α promoter-reporter construct and overexpressed the C/EBPα, β, and δ isomers. We first confirmed successful overexpression of the C/EBP isomers by Western blot (Fig. 6*A*). C/EBPβ overexpression more than doubled the activation of the 264-bp PGC-1α promoter-reporter construct. Importantly, this effect was observed in both HIB1B and 3T3-L1 cells (Fig. 6*B*). Overexpression of C/EBPα and C/EBPδ had a less marked effect on PGC-1α promoter activity than C/EBPβ (Fig. 6*B*). Overexpression of the three C/EBP isomers failed to influence the forskolin-induced expression of the 6CRE-pGL3 vector (Fig. 6*C*). These data suggested that C/EBPβ was responsible for the activation of the proximal PGC-1α promoter without affecting the sensitivity of the upstream PKA pathway.

To test the hypothesis that the CRE site mediates C/EBPβ modulation of PGC-1α cAMP-inducibility, we compared the reporter activity of the wild-type −264 bp promoter construct with that of a similar construct in which the CRE was mutated. As shown previously, C/EBPβ overexpression stimulated both basal and forskolin-stimulated activity of the wild-type PGC-1α reporter construct in both HIB1B and 3T3-L1 cells (Fig. 7). Mutation of the CRE effectively abolished any potentiating effects of C/EBPβ overexpression in either HIB-1B (Fig. 7*A*) or 3T3-L1 cells (Fig. 7*B*). These data demonstrate that C/EBPβ overexpression rescues cAMP-inducible PGC-1α expression in 3T3-L1 cells through effects within the proximal CRE containing region of the PGC-1α promoter.

To verify whether the observations of C/EBPβ overexpression on the PGC-1α promoter also corresponded to changes of the endogenous PGC-1α mRNA expression and cell commitment, we transiently overexpressed C/EBPβ protein in the two cell types and assessed the impact on cAMP-induced PGC-1α and UCP1 gene expression. As predicted from our earlier experiments, forskolin increased PGC-1α and UCP1 mRNA expression in HIB1B cells expressing a control "empty" p-cDNA3 vector but not in 3T3-L1 cells (Fig. 8). Furthermore, overexpression of C/EBPα, C/EBPβ, or C/EBPδ in HIB1B cells elicited no further increase in PGC-1α or UCP1 expression in response to forskolin (Fig. 8).

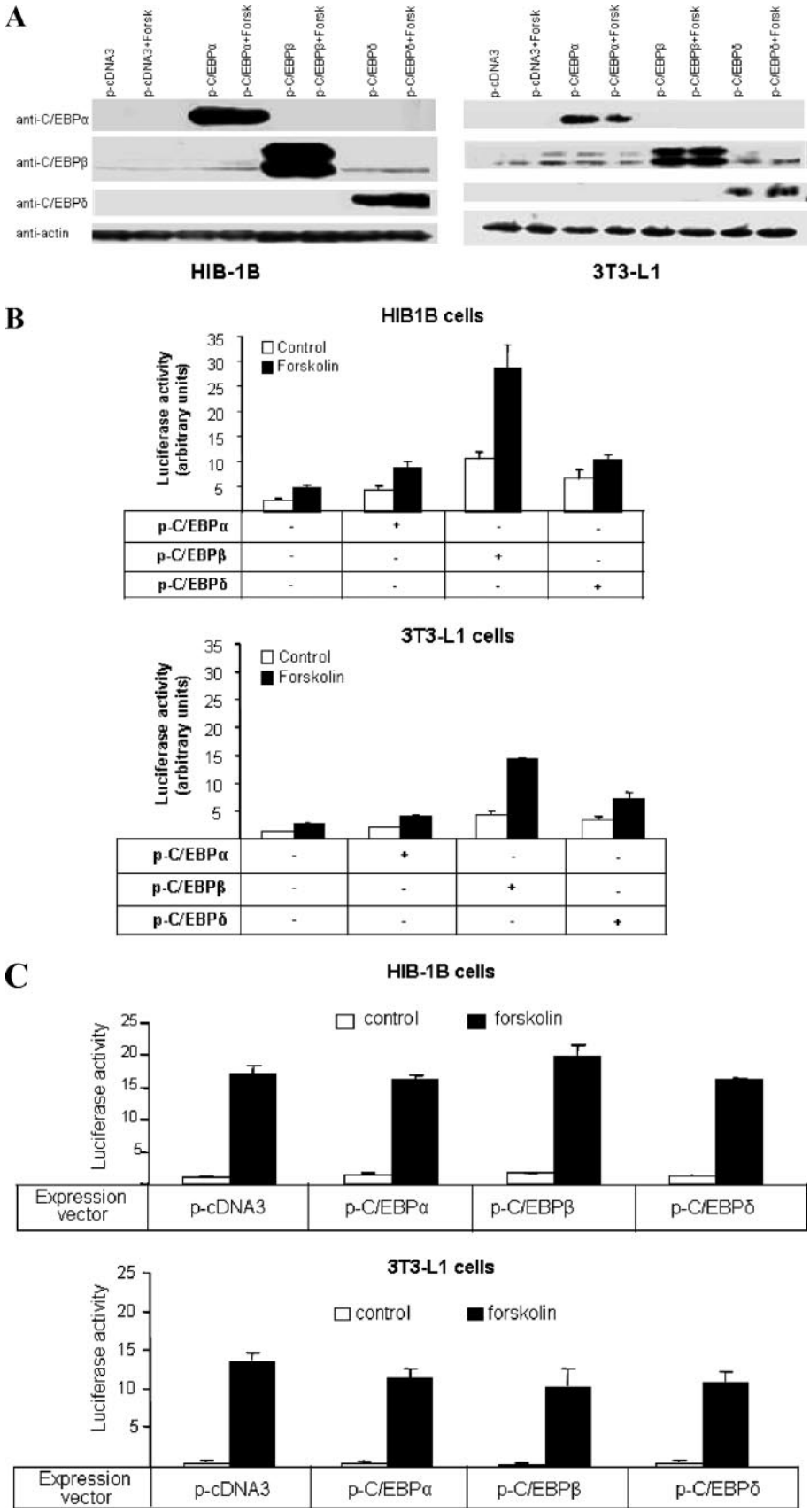

FIGURE 6. **Overexpression of C/EBPβ rescues PGC-1α proximal promoter transcriptional activity responses to cAMP in 3T3-L1 cells.** HIB1B or 3T3-L1 cells were transiently co-transfected with an empty control vector p-cDNA3 or 264PGC1α-p-GL3, p-MSV-C/EBPα, p-MSV-C/EBPβ, p-MSV-C/EBPδ, and 6CRE-pGL3 as indicated, and at confluence were treated with forskolin for 12 h. *A*, Western blot showing overexpression of C/EBP isomers. *B*, 264PGC1α-pGL3 promoter-reporter activity. *C*, 6CRE-pGL3 luciferase constructs driven by a 6-CRE in tandem. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

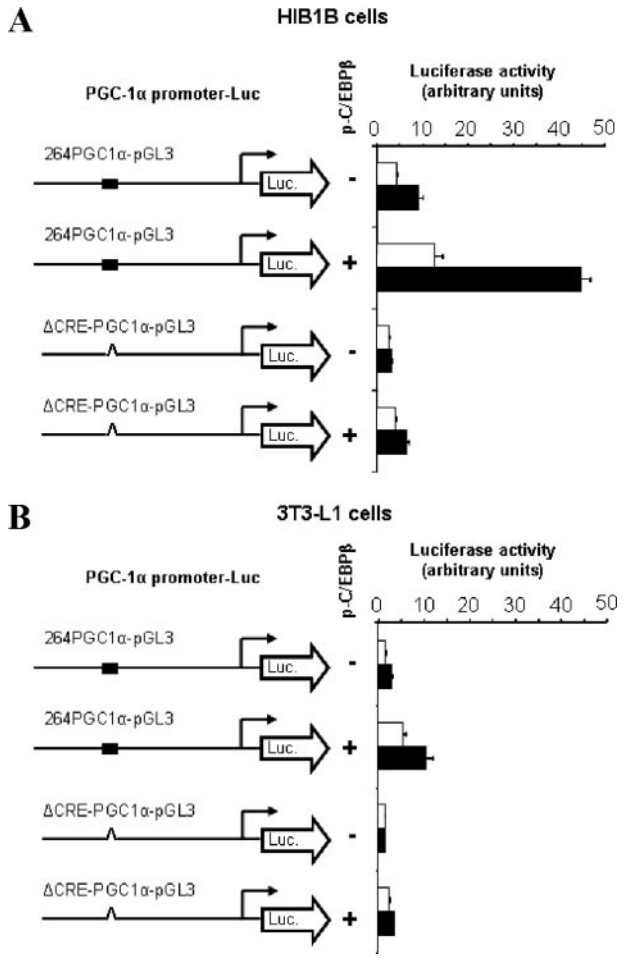

FIGURE 7. **Effect of C/EBPβ overexpression on cAMP-inducible PGC-1α promoter activity is decreased by mutation of the proximal promoter CRE in HIB-1B (*A*) and 3T3-L1 cells (*B*).** Cells were transiently co-transfected with the 264PGC1α-pGL3 luciferase reporter or the same construct containing a mutated CRE and pMSV-C/EBPβ overexpression constructs as indicated and at confluence were treated with forskolin for 12 h. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

Remarkably, overexpression of C/EBPβ caused 3T3-L1 cells to exhibit a BAT pattern of gene expression, *i.e.* highly forskolin-inducible PGC-1α and UCP1 mRNA expression. This large response was specific to C/EBPβ, because overexpression of C/EBPα and C/EBPδ had either no effect or much less effect on the PGC-1α or UCP1 mRNA in either cell type (Fig. 8).

In conclusion, we have provided evidence that C/EBPβ plays a pivotal role in the adipocyte-specific cAMP-induced expression of PGC-1α and UCP1 and is able to reprogram white preadipocyte 3T3-L1 cells to a BAT pattern of gene expression.

DISCUSSION

PGC-1α expression in brown fat is increased during cold exposure due to adrenergic activation of the PKA pathway (11). Our initial studies demonstrated that the forskolin-induced increase in PGC-1α and UCP1 mRNA in differentiated HIB1B cells was also observed in confluent HIB1B cells, suggesting that these genes become inducible early in the brown adipogenesis program. As expected, forskolin did not stimulate either PGC-1α or UCP1 mRNA in differentiated or confluent 3T3-L1 white preadipocyte cells. Here we demonstrate that the differential response in cAMP-induced PGC-1α expression between HIB1B and 3T3-L1 cells is caused by interaction of C/EBPβ with the CRE on the proximal promoter of PGC-1α.

In gel shift experiments we found that nuclear extracts from both HIB1B and 3T3-L1 cells bound CREB and C/EBPβ, but not C/EBPα or C/EBPδ, to the CRE in the PGC-1α proximal promoter. ChIP assays confirmed that in HIB1B cells, C/EBPβ and CREB bound to the CRE region of the PGC-1α promoter in a forskolin-dependent manner. In 3T3-L1 cell lysates, C/EBPβ but not CREB binding was forskolin-dependent. These experiments suggested that C/EBPβ may play an important role in mediating the differential effects of cAMP stimulation on PGC-1α expression in HIB1B and 3T3-L1 cells.

Evidence to support this hypothesis came from the demonstration that overexpression of C/EBPβ increased forskolin-induced transcriptional activity of the 264-bp PGC1α reporter construct in both HIB1B and 3T3-L1 cells. Furthermore, overexpression of C/EBPβ rescued the suppression of forskolin-induced PGC-1α expression in 3T3-L1 cells assessed by both reporter assays and direct mRNA measurements. This effect was specific to C/EBPβ, because similar experiments with C/EBPα or C/EBPδ overexpression failed to elicit the same increased sensitivity to cAMP stimulation. Mutation of the CRE on the PGC1α proximal promoter abrogated adipocyte nuclear protein binding and the potentiating effects of C/EBPβ overexpression on transcriptional activity of the 264-bp PGC1α reporter construct in both the HIB1B or 3T3-L1 cells. Importantly, overexpression of C/EBPβ, but not C/EBPα or C/EBPδ, in 3T3-L1 cells enabled cAMP to induce UCP1 expression by 260-fold. These data argue that the programming of preadipocytes to a BAT lineage is due to C/EBPβ interacting with CREB at the CRE on the PGC-1α proximal promoter.

C/EBP-related proteins have been found to potentiate cAMP-inducible expression of the PEPCK promoter (19) in a tissue-specific and differentiation-dependent manner. In contrast with the present study, this required CREB and C/EBPα or C/EBPβ to bind to separate sites on the promoter. Leucine zipper transcription factors also interact as homo- and heterodimers with CREs. Regulation of the interleukin-10 (20) and pre-interleukin-1β (22) promoters and human immunodeficiency virus type 1 long terminal repeat (23) by cAMP involves heterodimer formation between CREB and C/EBPβ, suggesting that the specific profile of transcription factors is capable of inducing tissue-specific patterns of gene expression (14, 19, 24). Cooperation between CREB and C/EBPβ has been suggested in rat C6 glioma cells transfected with tandem CRE sites in a pCRE-Luc reporter construct (25), but we were unable to observe this in our adipogenic cell lines.

An alternative mechanism, consistent with our results, is that C/EBPβ binds to CREB inducing a conformational change that recruits additional CBP/p300 into the preinitiation complex. C/EBPβ-CREB binding at a site independent of the CREB leucine zipper and CBP binding domains has been demonstrated (25). C/EBPβ interaction with other proteins as well as

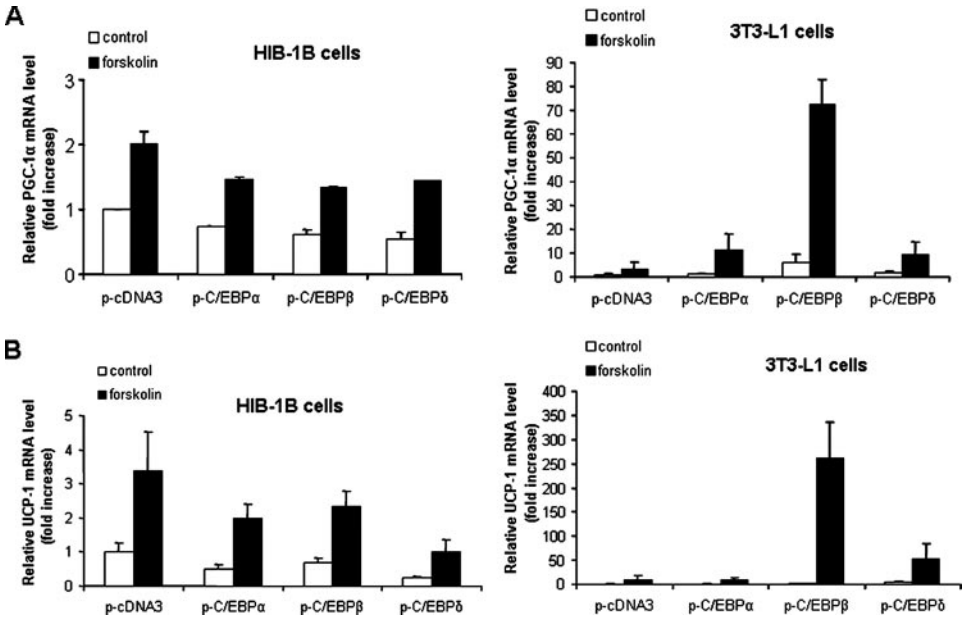

FIGURE 8. **Overexpression of C/EBPβ but not C/EBPα or -δ reprograms 3T3-L1 cells to a brown adipocyte pattern of gene expression.** Effect of C/EBPα, C/EBPβ, and C/EBPδ overexpression on PGC-1α mRNA (*A*) and UCP1 mRNA (*B*). HIB1B and 3T3-L1 cells were transiently transfected with an empty control vector p-cDNA3 or pMSV-C/EBPα, pMSV-C/EBPβ, and pMSV-C/EBPδ as indicated and at confluence were treated with forskolin for 3 h under serum-free conditions. Values were analyzed by quantitative real-time PCR and normalized against 18 S rRNA expression. *Error bars* represent S.E. of triplicate observations of one of three independent experiments.

subcellular localization, DNA binding, and transactivation potential are regulated by phosphorylation and require further investigation.

There is physiological evidence to support a role for C/EBPβ in BAT differentiation. Like PGC-1α, C/EBPβ expression in BAT is cold-inducible; during cold exposure, adrenergic stimulation and early development in rodents, C/EBPβ, but not C/EBPα or -δ, is increased in BAT but not WAT, in parallel with increases in UCP1 and PGC-1α mRNA (26, 27). We have confirmed that PGC-1α, C/EBPβ, and UCP1 mRNA increase in BAT but not WAT of cold-stressed mice (results not shown). Furthermore, in the present study we observed that C/EBPβ expression was increased in HIB1B but not 3T3-L1 adipocyte cell lines when challenged with forskolin to increase cAMP. C/EBPβ$^{-/-}$ mice have decreased brown adipose tissue and are cold-intolerant, but this is not because of a decrease in BAT UCP1 expression (28). However, this is likely to be because of a compensatory increase in C/EBPδ expression (28), because double knockout of C/EBPβ and -δ prevents BAT accumulation (10). In the present study, overexpression of C/EBPδ in 3T3-L1 cells increased forskolin-induced PGC-1α and UCP1 expression although to a much lesser extent than with C/EBPβ overexpression (Fig. 8). Genetic ablation studies have demonstrated that C/EBPβ and -δ, but not C/EBPα, play critical roles in brown fat differentiation (10) but it is not clear whether C/EBPs play a direct role in the expression of UCP1.

An outstanding puzzle is how C/EBPβ can be responsible for transduction to a BAT phenotype when it has been demonstrated that a transient increase in C/EBPβ initiates the adipogenic differentiation program in 3T3-L1 white adipose precursor cells. We speculate that this may be explained by the temporal dynamics of C/EBPβ expression with sustained C/EBPβ exposure resulting in the brown phenotype. Transgenic mice expressing C/EBPβ under the control of the C/EBPα promoter have 60% more brown adipose tissue and reduced white fat cell size than their wild-type littermates (29). During white adipocyte differentiation, C/EBPα is continously expressed after initiation by C/EBPβ, providing evidence that sustained C/EBPβ expression may be critical in committing cells to the brown phenotype (16). In summary, we have demonstrated that C/EBPβ plays a key role in regulating the expression of PGC-1α and UCP1 during early differentiation in adipocyte cell lines and in combination with adrenergic stimulation is able to reprogram white preadipocyte 3T3-L1 cells to a brown adipocyte lineage. These data provide new insights into the developmental mechanisms controlling cell differentiation and adipose tissue composition.

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