Regulation of Cyclin D1 and Wnt10b Gene Expression by cAMP-responsive Element-binding Protein during Early Adipogenesis Involves Differential Promoter Methylation*

Received for publication, August 19, 2008, and in revised form, October 27, 2008 Published, JBC Papers in Press, October 27, 2008, DOI 10.1074/jbc.M806423200

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Cyclin D1 expression is elevated and Wnt10b is repressed by cAMP during the first few hours of adipogenesis. cAMP-responsive element-binding protein (CREB) is a primary target for cAMP signaling, and we have shown that activation of CREB promotes adipogenesis and adipocyte survival. Here we tested the impact of CREB on expression of cyclin D1 and wingless-related mouse mammary tumor virus integration site 10b (Wnt10b) in 3T3-L1 cells. Forced depletion of CREB blocked Bt2cAMP-stimulated cyclin D1 expression and basal Wnt10b gene expression. Two CREB-binding sites were identified in the Wnt10b promoter region. Ablation of either site partially blocked promoter activity, while mutation of both sites completely suppressed promoter activity. These results suggest that CREB activates transcription from both the cyclin D1 and Wnt10b gene promoters. What accounts for the differential regulation of cyclin D1 and Wnt10b genes by cAMP? Chromatin immunoprecipitation revealed CREB bound to the Wnt10b promoter in untreated preadipocytes but not following treatment with Bt2cAMP. CREB binding to the cyclin D1 promoter was detected in untreated cells and post-Bt2cAMP. Differences between CREB binding to the two genes correlated with increasing methylation of the Wnt10b promoter following Bt2cAMP treatment, whereas no methylation of the cyclin D1 promoter was observed. Treatment of cells with the methylase inhibitor 5-azacytidine restored CREB binding to the Wnt10b gene promoter and prevented the inhibition of Wnt10b RNA expression by Bt2cAMP. We conclude that cAMP stimulates phosphorylation and binding of CREB to the cyclin D1 gene promoter. Simultaneously, hypermethylation of the Wnt10b gene promoter suppresses binding of CREB, allowing adipogenesis to proceed.

Adipogenesis is the process by which mature, insulin-responsive adipocytes are generated from undifferentiated preadipocytes and mesenchymal progenitor cells (1). This process is crucial to the normal development of adipose tissue and its expansion in response to excess dietary energy intake. Alternatively, most lipodystrophic syndromes are characterized by a suppression of adipogenesis and an increase in adipocyte death.

Cells destined to the adipocyte lineage arise late in development from multipotential stem cells of mesodermal origin (1–4). The commitment of the multipotent stem cells to the adipocyte lineage is a poorly understood process. However, once committed to the adipocyte lineage, nonproliferating preadipocytes become responsive to external stimuli that induce their differentiation to mature adipocytes. These stimuli include insulin-like growth factor-1 or insulin (which appears to work through the insulin-like growth factor-1 receptor), glucocorticoids, and agents that elevate intracellular cAMP levels (1).

Exposure of these cells to adipogenic inducers initiates a temporally orchestrated cascade of gene expression events that characterize adipogenic differentiation. These agents initially induce a period of mitotic expansion during which expression of CCAAT/enhancer binding proteins (C/EBPs)β and δ is increased, whereas expression of factors like Pref-1, necdin, and Wnt10b are diminished. Following mitotic expansion, differentiation begins, during which peroxisome-proliferator-activated receptor γ (PPARγ) and C/EBPα are up-regulated. These transcription factors regulate the expression of many of the factors that characterize the mature adipocyte phenotype like GLUT4 (5), adiponectin, aP2 (6), and perilipin (7).

We previously reported that the activity of the transcription factor CREB was stimulated by cAMP mimetics and insulin in both preadipocytes (8), suggesting that CREB might play a role in adipogenic conversion. Subsequent experiments demonstrated that ectopic expression of constitutively active forms of CREB could induce adipogenesis of 3T3-L1 cells (9, 10) and prevent their apoptotic death in response to insulin and/or serum deprivation and tumor necrosis factor α (11). Alternatively, ectopic expression of dominant negative forms of CREB blocked adipogenic conversion and stimulated apoptosis of mature adipocytes. Recent studies using these techniques as well as CREB-specific antisense and siRNA confirm these results and indicate that CREB may promote adipogenesis by...
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stimulating the expression of C/EBP β (12) and PPARγ (13). However, ectopic expression of C/EBPs α and β or PPAR-γ, which drive adipogenic differentiation in the presence of CREB, are unable to do so in the absence of CREB (and ATF-1) (13). These results suggest that CREB may modulate other pathways or processes involved in adipogenesis apart from the regulation of C/EBP β and PPAR-γ.

For example, cyclin D1 is up-regulated during the period of mitotic expansion that precedes adipogenic differentiation of 3T3-L1 cells (14, 15). Here we show that depletion of cyclin D1 inhibits preadipocyte proliferation and ultimately adipogenic conversion. Cyclic AMP-induced cyclin D1 expression and MDI-stimulated preadipocyte proliferation were suppressed in CREB-deficient cells. This indicated that CREB was required for cyclin D1 expression in response to cAMP.

Results reported herein also demonstrate that Wnt10b expression is partly regulated by CREB during early adipogenesis. Of the ~19 members of the Wnt family of signaling proteins, six are either expressed in preadipocytes or during adipogenic conversion (16, 17). Wnts 1, 6 and 10b are expressed in preadipocytes and inhibit initiation of adipogenesis. Alternately, Wnts 4, 5a, and 5b are expressed transiently during adipocyte differentiation and promote the differentiation cascade. These factors control adipocyte differentiation via canonical and noncanonical Wnt signaling pathways.

Expression of Wnt10b is suppressed by cAMP mimetics but not insulin/insulin-like growth factor-1 or glucocorticoids in 3T3-L1 cells (18). Because cAMP mimetics regulate CREB transcriptional activity and suppress Wnt10b expression, we hypothesized that both events may be linked. We found that depletion of CREB inhibits Wnt10b gene promoter activity. The effect of CREB on Wnt10b gene expression appears to be mediated by two putative cAMP response elements (CREs) in the Wnt10b gene promoter. This suggested that CREB was required to maintain basal Wnt10b gene transcription rather than repress Wnt10b expression in response to cAMP mimetics.

These results raise two related questions. First, how does activation of CREB transcriptional activity lead to inhibition of Wnt10b gene transcription. Second, how does activation of CREB simultaneously stimulate cyclin D1 gene transcription while attenuating Wnt10b gene transcription? Our results indicate that CREB binds the cyclin D1 gene promoter before and after cAMP or MDI treatment, whereas CREB binds the Wnt10b gene promoter only before induction with adipogenic agents. The differential binding of CREB to these gene promoters appears to be modulated in part by DNA methylation.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and supplies were from Invitrogen, Gemini Bioproducts (Gaithersburg, MD), and Specialty Media, Inc. (Lavallette, NJ). 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA). Three Double-stranded siRNA oligonucleotides to CREB (5′GAAATTCAACAGGATGCTTGTGG305, 5′28AATA-CAGCTGGCTAAACAATGG549, and 5′67GAAACATGGTTGTCAAGCT528) and a control siRNA oligonucleotide to firefly luciferase (CGTAGCGGAGAATCTTGA) were from Dharmaco (Lafayette, CO). Single-stranded DNA primers for PCR amplification of β-actin (forward, CCAACCGTGAAAGAGATGACC, and reverse, TCTAGGGGACATAGACACG), the Wnt10b gene promoter (19) (for cloning: forward, CGCGAGCTCTTAATACGAC, and reverse, GTTTAGGTCCAGGATTCCAC; for chromatin immunoprecipitation: forward, TCAGCTGACAGAACCAC, and reverse, GTTTCAGGTGTCGAGATCCAC; and for promoter methylation: forward, CCAAGCCCAATTCTGGAGCGCAA, and reverse, GTGTTGTTCTGTCCAGGCTTGA), the mouse cyclin D1 gene promoter (20) (forward, CTAAGGTTGGGCAACACCAC, and reverse, TCCGGTGGCTGGCTGCTGAG), and reverse, ACAACAGGAAGGAGAAAGCTC single-stranded DNA oligonucleotides for site-directed mutagenesis of the Wnt10b gene promoter CREs (CRE 5: forward, GCTCTGGAGGCGCGCTGCTGCTGAG, and reverse, GGTTGTCTCGCTGAGCTGATCGACTGCGGCTTCCACAGGCT; and CRE 6: forward, GCCCCCAAGAGCCCGGGCGAGTCTGCTGCTGCAGG, and reverse, CAGATGCCACAAACGCTCGGACTTCAACCAGGCTCGGCCGGCGCTTTGGG) or the single-stranded, biotinylated oligonucleotides listed in Fig. 6 were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Rabbit polyclonal antibodies for CREB, cyclin D1, and Cdk-4 were purchased from Cell Signaling (Danvers, MA). A mouse monoclonal neutralizing antibody to Wnt10b was purchased from R&D Systems (Minneapolis, MN). Luciferase assay reagents and the plasmid pRL-TK, containing the enhancerless thymidine kinase gene promoter driving a Renilla luciferase reporter gene, were obtained from Promega (Madison, WI). 5-azacytidine was purchased from Sigma. SuperSignal West Pico chemiluminescent substrate was purchased from Pierce. Horseradish peroxidase-conjugated secondary antibodies were obtained from Vector Laboratories (Burlingame, CA). Oligofectamine, Lipofectamine, and Plus reagent were obtained from Invitrogen, and [3H]thymidine was from MP Biomedicals (Solon, OH). Plasmids for the expression of VP16-CREB, CREB DIEDML, and KCREB were described previously (9, 10).

Cell Culture and Adipocyte Differentiation in Vitro—3T3-L1 and 3T3-F442A preadipocytes were passaged in low glucose Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum, 1 mM l-glutamine. 3T3-L1 fibroblasts were differentiated into adipocytes after siRNA and/or plasmid transfection, upon reaching confluency by the addition of high glucose DMEM containing 10% fetal calf serum, 1 mM l-glutamine, and M isobutylmethylxanthine or Bt2cAMP, 1 mM dexamethasone, and 1 μg/ml insulin (MDI). After 2 days, the 3T3-L1 cells were transferred to high glucose DMEM plus 10% fetal calf serum, 1 mM l-glutamine, and 1 μg/ml insulin and refed every 2 days. Differentiation of preadipocytes to mature adipocytes was confirmed by Oil Red O staining of lipid vesicles.

Western Blotting—Whole cell lysates were prepared in 20 mM Tris-HCl containing 10% glycerol, 0.3% Nonidet P-40, 300 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (Complete Mini Protease Inhibitor Tablets; Roche Applied Science). After correcting for protein concen-
trations, the cell lysates were mixed with an equal volume of Laemmli SDS loading buffer, and equal amounts of lysate protein were resolved on 10% polyacrylamide-SDS gels and transferred to polyvinylidene difluoride membranes. The blots were blocked with phosphate-buffered saline containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that recognize the target proteins indicated in each figure overnight at 4 °C. The blots were washed and subsequently treated with appropriate secondary antibodies conjugated to horseradish peroxidase. After the blots were washed, specific immune complexes were visualized with SuperSignal West Pico Chemiluminescent Substrates.

**siRNA and Plasmid Transfection and Luciferase Assays**—For siRNA transfection, 3T3-L1 cells were plated at 30–50% confluency on 6-well plates in complete medium. Twenty-four hours later the cells were transferred to OptiMem for transfection. Double-stranded siRNA oligonucleotides were complexed with Oligofectamine reagent and applied to the cells according to the manufacturer’s recommendations at a final concentration of 200 nM. Where more than one siRNA oligonucleotide was used, equimolar amounts of the individual oligonucleotides were combined before use. After 3 h, an equal volume of DMEM containing 30% fetal calf serum was added to the wells. The cells were allowed to recover for 24 h before subsequent manipulations.

For plasmid transfections, plates of 3T3-L1 preadipocytes were grown to 70–80% confluency and transfected with the indicated plasmids with Lipofectamine Plus reagents according to the manufacturer’s recommendations. Luciferase assays were conducted on lysates from cells transfected with plasmid (pGL3-Basic; Promega) containing a 900-bp region of the Wnt10 gene promoter (see Fig. 5) linked to the firefly luciferase gene. The cells were cotransfected with the plasmid pRL-TK as an internal control. Cell lysates and luciferase reactions were performed with a dual luciferase reporter assay system on a Turner Designs 20/20™ luminometer (Turner Designs, Sunnyvale, CA).

**Microscopy**—Microscopy was performed on a Nikon TE2000-U inverted epifluorescent microscope. Phase contrast, brightfield images were captured to a personal computer with a Spot Insight color camera (Diagnostic Imaging, Sterling Heights, MI). The images were analyzed and processed with MetaMorph 6.1 Software (Molecular Devices, Sunnyvale, CA).

**Chromatin Immunoprecipitation for CREB Promoter Binding**—Chromatin immunoprecipitation assays for CREB binding to the cyclin D1 and Wnt10b gene promoters were performed on 3T3-L1 cells treated with MDI for 48 h using kits from Upstate (Charlottesville, VA) according to their directions. The procedure was modified as described previously (12).

**Semiquantitative RT-PCR**—cDNA was prepared from cells using Cells-to-cDNA II (Ambion, Austin, TX) reagents according to the manufacturer’s instructions. PCR amplification was performed with 3 µl of transcribed cDNA and 1 pmol of each primer for 30 cycles: hot start at 94 °C for 1 min, denaturation at 94 °C for 1 min, annealing at appropriate temperature for 45 s, and elongation at 72 °C for 2 min, followed by a final elongation for 8 min at 72 °C. Negative PCR controls included omission of reverse transcriptase or omission of cDNA. β-Actin primers were used to validate each batch of template before use. PCR products were resolved on 2% agarose gels containing ethidium bromide and photographed under ultraviolet illumination.

**[3H]Thymidine Uptake**—3T3-L1 preadipocytes were cultured as described above, incubated in serum-reduced medium for 24 h, and then transfected as indicated with control or cyclin D1-specific siRNA. During the final 24 h of incubation, half of each cell treatment group was exposed to MDI. During the final 3 h of the 24 h treatment, 2 μCi of [3H]thymidine/ml were added to the cultures. The cells were assayed for thymidine incorporation by standard methods. The results are expressed as 3H dpm/µg of protein.

**Antisense Oligonucleotides and Treatments**—Phosphorothioate oligonucleotides were synthesized by Integrated DNA Technologies, Inc. The sequence of the antisense oligonucleotide was: CREB, 5’-GGTCATCTAGTCACCGGT-3’, and a random sequence oligonucleotide (Scrambled) with sequence 5’-GTCTGACGTGACCTAG-3’ was used as a control. Each antisense or control oligonucleotide was introduced into 3T3-L1 preadipocytes using Lipofectin reagent (Invitrogen). Oligonucleotide-Lipofectin complexes were prepared in OptiMem medium (Invitrogen) and placed on cells at a final concentration of 15 μg/ml Lipofectin and 3 mM oligonucleotide. After 4 h, the cells were refed with conventional growth medium containing 20 μM of the individual oligonucleotides.

**CREB DNA Binding Measurements**—CREB DNA binding was measured using TransAM enzyme-linked immunosorbent assay kits. For direct DNA binding, biotinylated double-stranded DNA oligonucleotides containing each of the putative CREs in the Wnt10b gene promoter (Figs. 5 and 6) were individually bound to the wells of a TransAM assay strip. Control wells were prepared using oligonucleotides containing a consensus CRE (positive control) or a scrambled oligonucleotide (negative control). Competition binding assays were performed with consensus CRE oligonucleotides bound to the assay wells, but binding reactions contained a 10-fold molar excess of each Wnt10b CRE oligonucleotides. All other assay parameters followed the supplier’s instructions. Chromatin immunoprecipitation analysis for DNA binding to the Wnt10b or cyclin D1 gene promoters was conducted as described previously (13).

**pWnt10b-Luciferase Plasmid**—A 900-bp region of the Wnt10b gene promoter (from −705 to 216 relative to the primary transcription start site) was PCR-amplified from a mouse genomic library. The fragment was inserted upstream of the firefly luciferase gene in the plasmid pGL3-Basic. Two putative CREs in the promoter were mutated to a non-CREB-binding sequence (TCTGGTT) individually or in combination by the method of Huang and Sorkin (21).

**DNA Methylation**—The methylation status of the cyclin D1 and Wnt10b gene promoters was measured using DNA methylation assay kits from Panomics (Fremont, CA). Basically, 3T3-L1 cells were grown on 10-cm dishes and treated as described in the figure legends. The medium was removed from the cell monolayers, which were rinsed once with phosphate-buffered saline. The cells were recovered by scraping into microcentrifuge tubes and pelleted at 500 × g. Genomic DNA was isolated with Genomic DNA extraction reagents from Panomics. Approximately 5 μg of genomic DNA was cleaved as described in the figure.
with MseI restriction enzyme for 2 h at 37 °C. The DNA fragments were then incubated with the methylation binding protein, MeC2P, for 30 min at 15 °C. The binding reactions were applied to separation columns, and DNA retained on the column was isolated for PCR amplification with primers listed above. The presence of a PCR-amplified product indicates binding of MeC2P to methylated target DNA fragments.

RESULTS

Cyclin D1 Is Required for Preadipocyte Proliferation and Adipogenesis—Prior to entering the adipogenic differentiation cascade, 3T3-L1 cells undergo a period of mitotic expansion, which is obligatory for their adipogenic differentiation. This period of proliferation is not essential for adipogenesis of primary preadipocytes or mesenchymal progenitor cells. Treatment of 3T3-L1 preadipocytes with the differentiation-inducing mixture MDI stimulates expression of cyclin D1, a key regulator of G1 entry and the G1-S transition, within 6 h (Fig. 1). Cyclin D1 levels remain elevated for 48 h. No change in expression of another cell cycle-related factor, Cdk-4, was observed. Actin levels diminished as differentiation progressed as previously reported (22, 23). To demonstrate the importance of cyclin D1 to mitotic expansion and adipogenesis, 3T3-L1 preadipocytes were transfected with nonspecific (control) or cyclin D1-specific siRNA. Fig. 2A shows that cyclin D1 is easily detected in cells transfected with control siRNA and exposed to MDI but not in cells receiving cyclin D1-specific siRNA and MDI 36 h post-transfection. Mitotic expansion, which occurs during the 48 h following induction with adipogenic agents was measured by [3H]thymidine uptake with MDI-treated cells. The data averaged from three experiments shows that depletion of cyclin D1 inhibits preadipocyte proliferation. C, siRNA-transfected cells were treated with MDI for 48 h and then transferred to medium supplemented with 1 μg/ml insulin for 7 days. Medium plus insulin was replaced every 48 h. On day 9, the cells were fixed and stained with Oil Red O. Representative brightfield micrographs show that cyclin D1 depletion suppresses adipogenesis and lipid accumulation.
prevented adipogenic conversion as evidenced by an absence of lipid accumulation (Fig. 2C).

It is unlikely that CREB induces cyclin D1 expression in preadipocyte and mesenchymal stem cell models that do not require mitotic expansion for PPARγ expression and adipogenesis. Although this cyclin is expressed in these models, it is not induced by pro-adipogenic agents (15, 24). Thus, the induction of cyclin D1 expression and its obligatory impact on preadipocyte proliferation and adipogenic conversion may be restricted to the 3T3-L1 model.

**CREB/ATF-1 Promote Cyclin D1 Gene Expression in 3T3-L1 Preadipocytes Treated with Differentiation-inducing Agents**—Because CREB plays an important role in cyclin D1 gene expression in other cell types and tissues (26–29), we tested whether forced depletion of both CREB and the closely related factor, ATF-1, would block cyclin D1 expression. CREB/ATF-1 siRNA effectively reduced CREB levels in 3T3-L1 preadipocytes 48–72 h post-transfection (Fig. 3A). We found that forced loss of CREB/ATF-1 repressed basal and MDI-stimulated cyclin D1 expression. No changes in Cdk-4 gene expression were observed with MDI and/or siRNA treatments. Likewise, the loss of CREB did not prevent MDI induction of C/EBP β expression as we previously reported (13). Thus, activation of CREB/ATF1 by MDI, in part, stimulates cyclin D1 gene expression in preadipocytes.

**Cyclic AMP-induced Repression of Wnt10b Promotes Adipogenesis**—MacDougald and co-workers (18, 30) have shown that Wnt10b is expressed in preadipocytes wherein it suppresses adipogenic conversion. Treatment of 3T3-L1 cells with MDI or, more specifically, with cAMP mimetics suppresses Wnt10b expression, thereby permitting initiation of adipogenic differentiation. We confirmed these observations by assessing Wnt10b RNA levels from subconfluent and confluent preadipocytes prior to MDI treatment and in fully differentiated adipocytes. Wnt10b protein was detected in both preadipocyte samples, but not in adipocytes (data not shown). In addition, MDI treatment of confluent 3T3-L1 preadipocytes with Bt2cAMP reduced Wnt10b RNA levels within 6 h (Fig. 4A).

To begin to understand the regulation of Wnt10b RNA synthesis, we cloned a ~900-bp region of the mouse Wnt10b gene...
CREB Regulates Transcription of the Wnt10b Gene—Because Wnt10b gene expression is regulated by cAMP and CREB is activated by cAMP in 3T3-L1 cells, we examined the impact of CREB on the Wnt10b promoter. First, Wnt10b gene promoter activity was measured in 3T3-L1 cells stably expressing various CREB isoforms. Ectopic overexpression of wild type CREB-321 had no effect on Wnt10b promoter activity (Fig. 5A). Overexpression of dominant negative KCREB (31) inhibited luciferase expression, whereas expression of constitutively active VP16-CREB (10) or CREB-DIEDM (32) stimulated transcriptional activity.

In a second series of experiments, Wnt10b gene promoter activity was assayed in 3T3-L1 cells transfected with scrambled or CREB-specific antisense oligonucleotides (12). Scrambled antisense had no effect on luciferase levels, but luciferase was reduced in cells transfected with antisense to CREB (Fig. 5B). For a third set of studies, the cells were transfected with control or CREB/ATF1-specific siRNA to test their effect on Wnt10b gene promoter activity. Although the nonspecific siRNA had no effect, CREB/ATF1 siRNA strongly inhibited promoter activity (Fig. 5C). Forced depletion of CREB also repressed Wnt10b RNA levels in confluent preadipocytes (Fig. 5D). These data indicate that CREB regulates transcription of the Wnt10b gene in a positive manner.

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**FIGURE 5. CREB stimulates Wnt10b gene transcription.** A–C, 3T3-L1 preadipocytes were transfected with plasmids containing the Wnt10b gene promoter linked to firefly luciferase and the enhancerless thymidine kinase promoter linked to Renilla luciferase (transfection control). A, cells were cotransfected with plasmids for the expression of wild type (WT), dominant negative (KCREB), or constitutively active (VP16-CREB or CREB DIEDML) forms of CREB. B, plasmid-transfected cells were subsequently transfected with no (Cntrl) oligonucleotide or single-stranded scrambled or CREB-specific phosphorothioate antisense oligonucleotides as described under "Experimental Procedures." C, plasmid-transfected cells were cotransfected with luciferase or CREB-specific siRNA and then treated with MDI or 0.3 mM Bt2cAMP or left untreated (Cntrl). A–C, 48 h post-transfection/treatment, firefly luciferase was measured in cell lysates and corrected for Renilla luciferase levels. A, data shows that dominant negative KCREB suppresses while constitutively active CREB isoforms stimulate Wnt10b gene promoter activity. Additional results indicate that depletion of CREB with antisense (B) or siRNA (C) inhibits Wnt10b gene promoter activity. D, preadipocytes were transfected with luciferase (Cntrl) or CREB-specific siRNA. 48 h later the cells were treated with 0.3 mM Bt2cAMP for 0 or 6 h. Total RNA was recovered and subjected to RT-PCR for Wnt10b or β-actin. The data indicate that CREB depletion reduces Wnt10b RNA levels. The figure shows data averaged from three experiments.

**FIGURE 6. CREs Are Present in the Wnt10b Gene Promoter—CREs have been identified in the promoter region of the cyclin D1 (20) but not the Wnt10b gene promoter. We screened the Wnt10b gene promoter region used in our transfection studies for the presence of potential CREs with the TFSEARCH program (33). We identified seven putative sites with significant homology to either the consensus CRE or 12-O-tetradecanoylphorbol-13-acetate response element sequences (Fig. 6). 20-bp oligonucleotides were prepared to each site and tested for their ability to bind purified recombinant CREB protein. We found that Wnt10b oligonucleotides 5 and 6 bound CREB, albeit to a lesser extent than an oligonucleotide containing a consensus CRE (Fig. 7A). Other Wnt10b gene CREs exhibited limited interaction with CREB. A similar assay in which each Wnt10b oligonucleotide was used to compete CREB binding to the consensus CRE oligonucleotide revealed that CREs 5 and 6 effectively competed CREB binding, whereas other sites had little effect (Fig. 7B).

Site-directed mutagenesis was performed on the Wnt10b promoter fragment to ablate the CREs at -73 to -54 and 184 to 203 (corresponding to CREs/oligonucleotides 5 and 6, respectively) individually or in combination. Transient transfections assays with the wild type and mutated promoters showed that individual ablation of the CREs reduced Wnt10b promoter activity with mutation of CRE 6 being somewhat more potent (Fig. 7C). However, this inhibition was only partial and not as
great as observed with Bt₃cAMP treatment. In fact, Bt₃cAMP reduced transcription from each of the individually mutated promoters to a level comparable with transcription from the wild type promoter in cells exposed to Bt₃cAMP. This suggests that binding of CREB to the nonmutated site partially stimulates promoter activity until cAMP signaling attenuates this activity. Combined mutation of both sites inhibited promoter activity to the same extent as treatment of the cells with Bt₃cAMP. These data indicate that binding of CREB to the Wnt10b promoter stimulates rather than represses transcription of this gene. The results raise two related questions. First, if cAMP activates CREB transcriptional activity and CREB binds the Wnt10b gene promoter, how does cAMP lead to repression of Wnt10b gene expression? Second, if CREB binds to both the cyclin D1 and Wnt10b gene promoters and is activated by cAMP, why is cyclin D1 expression up-regulated, whereas Wnt10b expression is suppressed during the early phase of adipogenesis?

Differential Binding of CREB to the Cyclin D1 and Wnt10b Gene Promoters—One possible explanation to the conundrum of CREB involvement in cyclin D1 and Wnt10b gene expression may be differences in the binding of CREB to the promoters for these two genes. Chromatin immunoprecipitation analysis was performed to assess the interaction of CREB with each promoter in untreated preadipocytes or after the cells were exposed to MDI for 6 and 24 h. CREB binding to the Wnt10b gene promoter was detected in untreated preadipocytes, but no interaction (signal in CREB lanes is equal or less than in IgG lanes) was detected in cells treated with MDI at either time (Fig. 8). Alternately, little or no interaction between CREB and cyclin D1 gene promoter was observed in untreated preadipocytes. However, CREB binding to this promoter was noted in both the 6- and 24-h MDI-treated cells. These data suggest that the differential impact of CREB on these two genes is largely due to differences in binding of CREB to their respective promoter regions.

Cyclic AMP Induces Methylation of the Wnt10b Gene Promoter, Which Results in Repression of CREB Binding—Promoter methylation is one of the primary mechanisms by which transcription factor binding is modulated. We examined...
the methylation of both the cyclin D1 and Wnt10b gene promoters in untreated 3T3-L1 cells and after treatment with Bт2cAMP for 6 and 24 h. Methylation of the Wnt10b gene promoter was virtually undetectable in untreated cells, but an increasing signal for methylation was seen in the 6 and 24-h samples (Fig. 9). Methylation of the cyclin D1 gene promoter region was not detected under any condition.

To determine whether Wnt10b gene promoter methylation regulates CREB binding to the promoter, preadipocytes were treated with or without the general methyltransferase inhibitor 5-azacytidine in the presence or absence of Bт2cAMP. Chromatin immunoprecipitation was employed to evaluate CREB DNA binding. As expected, CREB was associated with the Wnt10b promoter in untreated preadipocytes with or without the methyltransferase inhibitor (Fig. 10A). Bт2cAMP alone inhibited CREB binding in the absence of 5-azacytidine, but CREB binding was retained in cells treated with the 5-azacytidine and Bт2cAMP.

Next we tested whether methylation of the Wnt10b gene promoter resulted in changes in Wnt10b gene expression. 3T3-L1 preadipocytes were treated with Bт2cAMP for 0, 6, and 24 h, with some receiving 5-azacytidine. In the absence of methyltransferase inhibitor, Bт2cAMP repressed Wnt10b mRNA levels at 6 and 24 h, with greater inhibition at 24 h (Fig. 10B). However, no repression of Wnt10b was observed in cells treated with 5-azacytidine. The data demonstrate that Bт2cAMP-stimulated methylation of the Wnt10b gene promoter regulates both CREB binding and gene expression.

Finally, we examined the impact of azacytidine on Bт2cAMP-induced CREB binding to the cyclin D1 gene promoter. We found that Bт2cAMP stimulated binding to this promoter in the absence or presence of azacytidine and that the level of binding.
was not enhanced by azacytidine (Fig. 10C). Thus, CREB binding to the cyclin D1 gene promoter is not sensitive to promoter methylation.

**DISCUSSION**

Previous studies indicate that CREB participates in adipogenesis at both the early and late stages of the process (9–12). At early time points CREB regulates the expression of C/EBP β (12), necdin (34), and PGC-1α (35), all of which promote adipogenic conversion. At later times CREB regulates Akt, which participates in adipocyte differentiation (36, 37) and adipocyte survival (11), and stearoyl-CoA desaturase-1 (38). More recently we demonstrated that CREB binds the promoter of the PPARγ gene and participates in expression of this factor (13). Here we show that CREB/ATF1 at least partially controls cyclin D1 and Wnt10b expression during early adipogenesis (Fig. 11). Increases in cyclin D1 expression appear to be due to the binding of CREB/ATF1 to the cyclin D1 gene promoter in response to cAMP or MDI treatment. Suppression of Wnt10b expression by cAMP appears to involve methylation of the Wnt10b gene promoter region, leading to a loss of CREB binding. Our data explain how cAMP can simultaneously activate CREB and stimulate the transcription of one gene while inhibiting the transcription of a second gene by stimulating promoter methylation.

A wide variety of epigenetic processes contribute to commitment to the adipocyte lineage and adipogenic conversion. These include changes in chromatin structure elicited by histone modifications including methylation, acetylation, phosphorylation, ubiquitination, and sumoylation (reviewed in Ref. 39). In addition, covalent modification of specific DNA-binding trans-acting factors and transcriptional coregulators occur throughout the adipogenic cascade. However, direct methylation of promoter/regulatory regions of adipogenic genes has received less attention. Early studies demonstrated that C3H/10T1/2 fibroblasts/stem cells treated with azacitidines underwent stable commitment to the adipocyte lineage. In the case of C2H/10T1/2 cells, this appears to correlate with demethylation of the BMP4 gene promoter and increased BMP4 expression (42). Final adipogenic differentiation of 3T3-L1 cells and other committed preadipocytes is generally stimulated by methyltransferase inhibitors (39, 43). This seemingly contradicts our results indicating that methyltransferases suppress Wnt10b expression, allowing adipogenic conversion to proceed. However, global inhibition of methyltransferases clearly regulates a large number of genes, many of which may act downstream of Wnt10b or elicit effects dominant over Wnt10b to force progression of the adipogenic cascade. It will be interesting in future studies to follow the expression and impact of Wnt10b in differentiating preadipocytes treated with methyltransferase inhibitors.

More recent studies have reported sequential or temporal changes in DNA methylation throughout the genome during adipogenesis (44). Analysis of individual adipocyte-specific genes also reveals dynamic changes in the methylation status of their promoter regions. Tissue-specific and maturation-related changes in the methylation of the leptin gene promoter account in part for the restricted expression of this adipokine to mature adipocytes (45). In particular, demethylation of CpG sites within a C/EBP β sequence in mature adipocytes plays a major role in leptin expression (46). Likewise, the promoter for the GLUT4 gene is highly methylated in preadipocytes and less methylated in mature adipocytes, which correlates with its expression in the latter (47). It seems likely that promoter methylation is a common mechanism for regulating gene expression during adipogenesis and in mature adipocyte function. Our results support this contention by demonstrating that methylation of the Wnt10b gene promoter prevents binding of CREB, resulting in decreased Wnt10b expression.

A perplexing question remains: what accounts for the differential methylation of the cyclin D1 and Wnt10b (and other) gene promoters in response to cAMP and other adipogenic inducers? Little is known about the mechanisms that direct DNA methyltransferases to their targets (25). Differences in chromatin structure appear to be responsible for differential methylation in certain cases. Another potential mechanism involves the recruitment of repressor proteins. Because cAMP signaling can alter chromatin structure and the binding and activity of various trans-acting factors, neither mechanism can be excluded in this case of the Wnt10b gene promoter.

**Acknowledgment**—We thank Nikhil R. Oak for technical assistance.

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