Role of CREB in Transcriptional Regulation of CCAAT/Enhancer-binding Protein β Gene during Adipogenesis*

Jiang-Wen Zhang‡, Dwight J. Klemm‡, Charles Vinson¶, and M. Daniel Lane‡

From the ‡Department of Biological Chemistry and Biochemistry, Cellular and Molecular Biology Program, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, the §Department of Medicine and Cardiovascular Pulmonary Research Laboratory, University of Colorado Health Sciences Center, Denver, Colorado 80262, and the ¶Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

The proximal promoter of the C/EBPβ gene possesses dual cis regulatory elements (TGA1 and TGA2), both of which contain core CREB binding sites. Comparison of the activities of C/EBPβ promoter-reporter genes with 5′-truncations or site-directed mutations in the TGA elements showed that both are required for maximal promoter function. Electrophoretic mobility shift and chromatin immunoprecipitation (ChIP) analyses with antibodies specific to CREB and ATF1 showed that these CREB family members associate with the proximal promoter both in vitro and ex vivo. Immunoblotting and ChIP analysis revealed that other CREB family members, CREM and ATF1, are up-regulated and associate with the proximal C/EBPβ promoter in mouse embryonic fibroblasts (MEFs) from CREB−/− mice. ChIP analysis of wild-type MEFs and 3T3-L1 preadipocytes revealed that interaction of phospho-CREB, the active form of CREB, with the C/EBPβ gene promoter occurs only after induction of differentiation of 3T3-L1 preadipocytes and MEFs. Consistent with the interaction of CREB and ATF1 at the TGA regulatory elements, expression of constitutively active CREB strongly activated C/EBPβ promoter-reporter genes, induced expression of endogenous C/EBPβ, and caused adipogenesis in the absence of the hormonal inducers normally required. Conversely, expression of a dominant-negative CREB blocked promoter-reporter activity, expression of C/EBPβ, and adipogenesis. When subjected to the standard adipocyte differentiation protocol, wild-type MEFs differentiate into adipocytes at high frequency, whereas CREB−/− MEFs exhibit greatly reduced expression of C/EBPβ and differentiation. The low level of expression of C/EBPβ and differentiation in CREB−/− MEFs appears to be due to up-regulation of other CREB protein family members, i.e. ATF1 and CREM.

The increased adipose tissue mass associated with obesity results from an increase in the number and size of adipocytes (1, 2). The increase in adipocyte number is due to recruitment of preadipose cells (including preadipocytes) that populate the vascular stroma of adipose tissue (3). This process is mimicked by the mitotic clonal expansion (MCE) of preadipocytes in cell culture that follows induction of differentiation (4–7). When growth-arrested 3T3-L1 preadipocytes or mouse embryo fibroblasts (MEFs) are treated with differentiation inducers, they synchronously re-enter the cell cycle, undergo −2 rounds of MCE, and then acquire adipocyte characteristics. Recent evidence has shown that this process is a prerequisite for terminal adipocyte differentiation (7, 8). C/EBPβ, a B-Zip transcription factor that is expressed prior to MCE, plays an essential role in this process (9) and in subsequent events of the differentiation program (10).

Following MCE, C/EBPβ initiates a cascade of transcriptional activation (11). C/EBPβ activates expression of the C/EBPα and PPARγ genes which function together as pleiotropic transcriptional activators of the large group of genes that produce the adipocyte phenotype (6, 12, 13). Both of the C/EBPα and PPARγ genes possess cis-C/EBP binding elements in their proximal promoters at which C/EBPβ binds and coordinately activates transcription (14–17). Once expressed, C/EBPα is thought to maintain expression of both the C/EBPα and PPARγ genes via transactivation mediated by their respective C/EBP regulatory elements (14, 16–18). As expression of C/EBPα increases (19), the expression of C/EBPβ declines. Presumably, this dual role facilitates maintenance of the terminally differentiated state.

Several lines of evidence have implicated CREB (cAMP response element-binding protein) as a transcriptional activator in the adipocyte differentiation program. Forced expression of CREB in 3T3-L1 preadipocytes promotes differentiation as evidenced by expression of adipocyte markers and the accumulation of cytoplasmic triglyceride (20). Factors, such as methylisobutylxanthine or forskolin that increase cellular cAMP or isobutylxanthine or forskolin that increase cellular cAMP itself (6, 21–24) in combination with other agents, i.e. glucocorticoid and IGF-1 or high levels of insulin, induce differentiation of 3T3-L1 preadipocytes (25). The cellular target of CAMP, protein kinase A, catalyzes phosphorylation of CREB on serine 133 and thereby, its capacity to activate transcription (26). Finally, there is circumstantial evidence that CAMP, acting through CREB, is responsible for the transcriptional activation of the C/EBPβ gene (27).

In the present investigation, we identify and characterize the roles of dual CRE-like cis regulatory elements (and their cognate trans-acting factors) in the C/EBPβ gene promoter early in the adipocyte differentiation program. We show that CREB is

* This work was supported by Research Grant DK38418 (to M. D. L.) and by Research Grants DK53989 and HL114985 and Veterans Affairs MERIT Review (to D. J. K.). 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.

† To whom correspondence should be addressed: Dept. of Biological Chemistry, Rm. 512 WBSB, Johns Hopkins University School of Medicine, Baltimore, MD 21205. Tel.: 410-955-3554; Fax: 410-955-0903; E-mail: dlane@jhmi.edu.

‡ The abbreviations used are: MCE, mitotic clonal expansion; MEF, mouse embryo fibroblast; PPARγ, peroxisome proliferator-activated receptor γ; IGF, insulin-like growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; wt, wild-type; E, embryonic day(s); ChIP, chromatin immunoprecipitation.

This paper is available on line at http://www.jbc.org

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 6, Issue of February 6, pp. 4471–4478, 2004

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 6, Issue of February 6, pp. 4471–4478, 2004

Received for publication, October 15, 2003
Published, JBC Papers in Press, October 30, 2003, DOI 10.1074/jbc.M311327200

4471
activated by phosphorylation and, along with ATF1, binds to the dual CRE-like elements in the proximal promoter of the C/EBPβ gene. Several lines of evidence indicate an essential role of the cis-elements and members of the CREB/ATF1 family in the adipocyte differentiation process.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Induction of Differentiation**—The 3T3-L1 preadipocytes were maintained and propagated in DMEM containing 10% (v/v) calf serum. Two-day post-confluent (designated day 0) cells were induced to differentiate (25) with DMEM containing 10% (v/v) fetal bovine serum (FBS) and 0.5 mM 1-methyl-3-isobutylxanthine (i.e. 3-isobutyl-1-methylxanthine). The DNA fragment containing the wild-type TGA site in the poly(dI-dC) mililiter (designated hereafter as MDI) until day 2. Cells were then fed DMEM supplemented with 10% FBS and insulin (1 μg/ml) for 2 days after which they were fed every other day with DMEM containing 10% FBS.

**Preparation of Mouse Embryo Fibroblasts (MEFs)—** CREF+/– females were crossed with CREF+/- males, and embryos were dissected 14.5 days after detection of vaginal plugs. After the head and internal organs were removed, embryos were minced and incubated in trypsin for 30 min at 37°C. Dispersed cells were harvested (and saved), and fresh trypsin was added to the dispersed clumps. This step was repeated for three times until the embryo was virtually completely digested. After a final wash, the pooled centrifuged cells were resuspended in DMEM containing 10% (v/v) fetal calf serum and propagated. MEF cells were induced into differentiation using the same protocol employed for 3T3-L1 preadipocytes.

**Gene Constructs and Mutations**—A DNA fragment containing the mouse C/EBPβ cDNA (sequence information from GenBank) was used as a probe to screen a FixII mouse (BALB/cJ) genomic DNA library (provided by D. Nathans, Johns Hopkins University School of Medicine). After three rounds of screening, positive clones were selected and amplified. The amplified phage DNA was subjected to restriction digestion and Southern blotting and probed with a 5′–proximal promoter fragment of mouse C/EBPβ cDNA (nucleotides –541 to +550). Positive bands of appropriate size were cloned and sequenced. The promoter sequences were then cloned into pGL3 basic luciferase reporter expression vector (Promega). Serial 5′-deleted reporter constructs of the CREF promoter were constructed using nested deletion techniques (Eco Mung Bean Deletion Kit, Stratagene).

TGA-site mutant constructs, e.g. MT1, MT2, and DMT, were generated by the PCR method as described previously (28). In TGAI, the core TGA site at –11 bp was mutated from ATGACGCG to AACTCGCG (mutated bases underlined), and in TGA2 the core TGA site at –65 bp was mutated from GTAGGCGG to GACTCGCG. To construct DMT with mutations on both TGA sites, a unique Apal restriction site between two TGA sites was utilized. After double digested with Apal and XhoI, the DNA fragment containing the wild-type TGA sites in construct MT1 was replaced with a mutated TGA2 fragment from construct MT2. The authenticity of the mutations was verified by sequencing.

**Transfection and Reporter Gene Analysis**—Proliferating (–70% confluent) 3T3-L1 preadipocytes were transiently transfected with 2 μg of promoter-reporter construct along with 8 μg of carrier DNA using the calcium phosphate precipitation method (29). After 2 days post-confluent (about 72 h or more after transfection) in culture, cells were treated with 1% formaldehyde for 30 min at 37°C. Dispersed cells were harvested (and saved), and fresh trypsin was added to the dispersed clumps. This step was repeated for three times until the embryo was virtually completely digested. After a final wash, the pooled centrifuged cells were resuspended in DMEM containing 10% (v/v) fetal calf serum and propagated. MEF cells were induced into differentiation using the same protocol employed for 3T3-L1 preadipocytes.

**Chromatin Immunoprecipitation Analysis**—ChIP analyses were performed essentially as described (31, 32) with the following modifications. The 3T3-L1 preadipocytes were transiently transfected with 2 μg of construct MT2. The authenticity of the mutations was verified by sequencing. Anti-CREF was generously provided by Dr. David Ginty (Dept. of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD); anti-ATF1 and anti-ATF1/2 were from Dr. Wolfgang Schmid (Division of Molecular Biology of the Cell I, German Cancer Research Center, Heidelberg, Germany). The same antibodies were used for ChIP assays.

**Oil Red O Staining**—Cell monolayers were washed three times with PBS and then fixed for 2 min with 3.7% formaldehyde. Oil red O (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 1 h at room temperature. Cells were washed with water, and the stained fat droplets in the cells were visualized by light microscopy and photographed.

**Edcsylene-inducible VP16-CREB and KCREB Expression System**—The Ecdysone-inducible expression system was employed to prepare stably transfected 3T3-L1 preadipocytes that could be induced to express VP16-CREB, CREF-DIEELM, Lacz, or KCREB. The open reading frame for KCREB was isolated from the plasmid, pBsv-KCREB, as a HindIII-EcoRI fragment. This fragment was subjected to PCR with a set of primers that introduced a HindIII site and the following translation initiation sequence (GCCAC) immediately upstream of the first methionine codon. The resulting PCR product was purified by electrophoresis on a 1% agarose gel and ligated into the HindIII and EcoRI sites of the plasmid, pNiD. The open reading frame for VP16 (amino acids 412–490) was excised from the plasmid, pVP16 (Arthur Goldstein, University of Colorado Health Sciences Center, Denver, CO) as a HindIII-BamHI fragment. This fragment was also subjected to PCR to introduce a Kozak sequence immediately upstream of the translation start site and was then directly ligated to a BglII-HindIII fragment containing the DNA binding domain (amino acids 217–327 of the translation domain linked to the (non)DNA binding domain of KCREB. The CREB-DIEELM open reading frame was excised from a plasmid provided by...
Analysis of the proximal promoter of the C/EBPβ gene—Mediation of reporter gene expression early in the adipocyte differentiation program—A 300-bp cDNA probe corresponding to the 5\'-untranslated region of the C/EBPβ mRNA was used to screen a mouse genomic library to obtain the 5\'-flanking region of the gene (results not shown). An insert of ~6 kb was isolated, mapped, and sequenced. Alignment with the sequence of the C/EBPβ gene from the NCBI mouse genome database verified its location in the 5\'-flanking region of the gene. A series of 5\'-truncated promoter fragments (beginning at −2912 and terminating at +33 relative to the start site of transcription) were isolated and inserted into the pGL3basic luciferase reporter vector (Fig. 1A).

To identify promoter sequences necessary for activation of reporter gene expression during adipogenesis, the 5\'-truncated promoter-luciferase constructs were transiently transfected into 3T3-L1 preadipocytes when cells reached ~70% confluence. Two days after achieving confluence the preadipocytes were subjected to our standard adipocyte differentiation protocol (25), which involved exposure to medium containing MDI (methylisobutylxanthine, dexamethasone, and insulin). Fig. 1B illustrates the protocol for analysis of reporter gene activity. Thereafter, cells were harvested every 2 h during the early phase (0–14 h) of the differentiation program, and cell extracts were prepared for luciferase activity measurements. Maximal luciferase activity (~10-fold greater than non-induced controls) occurred ~8 h following induction after which activity began to decline (results not shown). As shown in Fig. 1C, deletions between −2912 bp and −117 bp promoter had little effect on reporter activity. However, deletion of the region between −117 bp and −107 bp resulted in a reduction of luciferase activity of ~50%. Further deletion of the segment between −107 bp and −50 bp virtually abolished reporter activity (results not shown). As shown in Fig. 1C, deletions between −2912 bp and −117 bp promoter had little effect on reporter activity. However, deletion of the region between −117 bp and −107 bp resulted in a reduction of luciferase activity of ~50%. Further deletion of the segment between −107 bp and −50 bp virtually abolished reporter activity. These findings indicated that two regions in the proximal promoter, between −117 bp and −107 bp and between −107 bp and −50 bp, were crucial for reporter activity and therefore, were controlled by the differentiation inducers. Using the TESS (transcription element search system) to scan the promoter sequence of the C/EBPβ gene, two CRE-like elements were identified. Both elements share the same half-CRE core sequence TGAAC, located at −111/−107 bp (referred to as TGA1) and at −65/−61 bp (referred to as TGA2).

The importance of the two CRE-like elements for promoter depression was illustrated in Fig. 1D. As shown in Fig. 1D, the transcriptional activity of both sites (MT1 and MT2) was reduced to 50% in response to adipocyte differentiation inducers. However, co-transfection of a CREB antisense oligonucleotide with the luciferase reporter construct targeted to the TGA1 site (MT1) resulted in a greater reduction of luciferase activity (~65%) than the wild-type sequence (WT). These results suggest that CREB is involved in the regulation of C/EBPβ expression during adipogenesis. Similar results were obtained with the TGA2 site (MT2).

In summary, the findings presented here demonstrate the importance of the two CRE-like elements for promoter activation of the C/EBPβ gene during adipogenesis. These elements are crucial for the expression of the gene in response to adipocyte differentiation inducers, and their regulation by the transcription factor CREB is critical for the expression of C/EBPβ during this process.
activity was verified by site-directed mutagenesis. Consistent with the results of the 5′-deletion experiments, mutation of each of the core CRE consensus sequences of the TGA1 and TGA2 sites (TGA → ACT) resulted in a 50–60% reduction of promoter activity (Fig. 1D). Mutation of both sites virtually abolished promoter activity. The two CRE-like elements are located just upstream of a consensus TATA box (Fig. 1). In the experiments described below the −117-bp construct was used as a minimal promoter-reporter.

**CREB and ATF1 Form Complexes with Oligonucleotides Corresponding to the TGA Promoter Elements—**Electrophoretic mobility shift analyses (EMSA) were performed with 32P-labeled TGA1 (−117/−96) and TGA2 (−71/−50) oligonucleotide probes, and nuclear extracts from 3T3-L1 preadipocytes were prepared at 0, 2, and 8 h following MDI treatment. As shown in Fig. 2A both probes produced band-shifts at all three times (Fig. 2A, lanes 1–3 and 10–12). Specificity was verified by the abolition of binding with a 20-fold excess of unlabeled CRE-consensus oligonucleotide (Fig. 2A, lanes 4–6 and 13–15). Mutations (TGA → ACT) in the core CRE-consensus sequences in TGA1 and TGA2 also blocked complex formation (Fig. 2A, lanes 7–9 and 16–18).

![Fig. 2. Electrophoretic mobility shift and supershift analyses with wt and mutant oligonucleotides corresponding to the two C/EBPβ promoter TGA sites and nuclear extracts of 3T3-L1 cells following induction of differentiation. A, EMSA with wt and mutant TGA-site oligonucleotides. Where indicated a 20-fold excess of an unlabeled consensus CREB binding-site oligonucleotide probe was used. B, supershift-EMSA with wt TGA-site oligonucleotides with anti-CREB antibody. C, supershift-EMSA with wt TGA-site oligonucleotides with antibody that recognizes CREB, CREB and ATF1 (ATF1/2), or with ATF1-specific antibody. PI serum refers to preimmune serum.](image)

**Expression of C/EBPβ Is Correlated with Phosphorylation of CREB—**The expression of CREB, ATF1, phospho-CREB, and C/EBPβ was analyzed following treatment of 3T3-L1 preadipocytes to differentiation inducers. Cell lysates, prepared at various times after treatment with MDI, were subjected to immunoblotting with antibodies against each of the three proteins (Fig. 3). CREB and ATF1 were expressed constitutively over the entire time course (up to 48 h after induction) even prior to exposure to differentiation inducers and did not correlate with the onset of expression of C/EBPβ. C/EBPβ was rapidly expressed, i.e., at 30 min, and reached a maximum between 2 and 4 h after induction. Correlated with the expression of C/EBPβ was the appearance of phospho-CREB and phospho-ATF1. Phospho-CREB and phospho-ATF1 were detected as early as 5 min after induction, remained high until 2–4 h and then declined. Chromatin immunoprecipitation (ChIP) experiments (shown below) revealed that phospho-CREB binds to the proximal promoter of the C/EBPβ gene in 3T3-L1 preadipocytes within 1 h after induction.

![Fig. 3. Expression of C/EBPβ, CREB/phospho-CREB, and ATF1/phospho-ATF1 following induction of differentiation of 3T3-L1 preadipocytes. Two-day postconfluent 3T3-L1 preadipocytes were treated with differentiation inducers after which cell extracts were prepared at the times (hours) indicated and subjected to SDS-PAGE and immunoblotting with anti-C/EBPβ, CREB, phospho-CREB, and ATF1 antibodies.](image)
Effect of Constitutively Active CREB (VP16-CREB) and Dominant-negative CREB (A-CREB) on C/EBPβ/H9252 Promoter-reporter Gene Activity—The effect of constitutively active CREB on transcription driven by the C/EBPβ/H9252 promoter was further evaluated in co-transfection experiments with 3T3-L1 preadipocytes. Constitutively active CREB (VP16-CREB) was co-transfected with the minimal (−117 bp) C/EBPβ/H9252 promoter-reporter constructs along with: empty vector, VP16-CREB, or VP16-CREBdLZ (lacking the CREB DNA binding domain). B, preadipocytes were co-transfected with the minimal (−117 bp) wt C/EBPβ promoter-reporter construct along with empty vector or dominant negative A-CREB. Two days later the cells were treated or not with differentiation inducers.

Effect of Constitutively Active CREB (VP16-CREB) and Dominant-negative CREB (A-CREB) on C/EBPβ Promoter-reporter Gene Activity—The effect of constitutively active CREB on transcription driven by the C/EBPβ promoter was further evaluated in co-transfection experiments with 3T3-L1 preadipocytes. Constitutively active CREB (VP16-CREB) was co-transfected with the minimal (−117 bp) C/EBPβ promoter-reporter construct containing wt or mutated TGA1 and TGA2 regulatory elements. VP16-CREB, which possesses the CRE binding domain of CREB, strongly activated reporter gene expression (Fig. 4A). Deletion of the DNA binding domain in VP16-CREB dLZ prevented activation of the reporter gene (Fig. 4A). Moreover, mutation of the TGA sites abrogated transactivation by VP16-CREB. It can be concluded that activation of the C/EBPβ promoter-reporter gene requires interaction between CREB and the CREs.

Dominant-negative A-CREB possesses a leucine zipper that allows dimerization with CREB family members but lacks a functional DNA binding domain. Instead, A-CREB contains an acidic region that replaces the basic DNA binding region. The acidic extension forms a heterodimeric coiled-coil with the basic region of endogenous CREB stabilizing the interaction. Thus, heterodimers formed cannot bind to CRE elements (33). As shown in Fig. 4B, co-transfection of the A-CREB expression vector with the −117-bp C/EBPβ promoter-reporter construct inhibited reporter gene expression by preadipocytes treated with differentiation inducers. The fact, that A-CREB markedly inhibits the ability of the minimal promoter to drive reporter gene expression, provides additional evidence that CREB/ATF1 transcriptionally activates the C/EBPβ gene.

CREB(−/−) Mouse Embryonic Fibroblasts (MEFs) Exhibit Reduced Expression of C/EBPβ and Adipogenesis—When subjected to the same protocol that triggers differentiation of 3T3-L1 preadipocytes, MEFs can be induced to differentiate into adipocytes (9). In a previous study we showed that disruption of the C/EBPβ gene in MEFs prevented adipogenesis (9). Given that CREB activates transcription of the C/EBPβ gene in 3T3-L1 preadipocytes, we suspected that disruption of the CREB gene would block expression of C/EBPβ and thereby, differentiation. To test this hypothesis CREB(−/−) MEFs were employed. MEFs were isolated from both E14.5 CREB(−/−) and wt littermate mouse embryos. The genotypes of the MEFs were verified by PCR of genomic DNA (not shown), and disruption of CREB expression was assessed by Western blotting. Expression of C/EBPβ (Fig. 5A) and the accumulation of cytoplasmic triglyceride as assessed by Oil Red O staining (Fig. 5B) were markedly reduced in CREB(−/−) MEFs compared with wt MEFs.
that in wt MEFs. The fact that expression of C/EBPβ and the accumulation of triglyceride were not totally blocked was apparently due to compensatory up-regulation of ATF1 and CREM (Fig. 5A). The latter CREB family members might be expected to partially compensate for the loss of CREB protein, because they both can transactivate, albeit less strongly, the C/EBPβ gene promoter (shown below).

**Binding of CREB Family Members to Chromatin-associated C/EBPβ Promoter Sequences in 3T3-L1 Preadipocytes and MEFs and the Effect of CREB Family Members on Transcription Driven by the C/EBPβ Promoter**—The binding of CREB family members to chromatin-associated C/EBPβ promoter TGA elements was investigated by ChIP analysis. Two-day post-confluent 3T3-L1 (or MEF) cells were treated (or not) with adipocyte differentiation inducers for 1 h and then with formaldehyde to cross-link DNA-protein complexes. After sonication, chromatin fragments were immunoprecipitated with CREB, ATF1, ATF1/2 (which can recognize both CREB and ATF1), phospho-CREB, or CREM antibody, and the immunoprecipitated fragmented DNA was subjected to PCR to amplify C/EBPβ promoter DNA (between −140 bp and +33bp) containing the dual TGA regulatory elements.

As illustrated in Fig. 6A, antibodies to CREB, ATF1, or ATF1/2 (which recognizes both CREB and ATF1) immunoprecipitated TGA element-containing promoter fragments from both 3T3-L1 preadipocytes and MEFs, whereas preimmune IgG did not. These results indicate specific association of these proteins with the proximal C/EBPβ gene promoter. In all cases, the CREB family members associated with the promoter fragments both before and after exposure to differentiation inducers, although in some cases the association was stronger after exposure to differentiation inducers. Consistent with the results of EMSA showing that these homologues bind to TGA regulatory elements in vitro (Fig. 2), the results of ChIP analysis show that most of the CREB family members associate with the TGA-containing region of the C/EBPβ gene promoter ex vivo.

Similar findings were obtained with anti-phospho-CREB antibody. However, because phosphorylation of CREB is dependent upon kinase activation by the differentiation inducers, the association of phospho-CREB with the C/EBPβ gene promoter was detected only after exposure of the cells to differentiation inducers.
inducers (Fig. 6A). Although CREB family members can bind to the C/EBPβ promoter regardless of prior treatment with differentiation inducers (Fig. 6A), their capacity to drive reporter gene expression mediated by the C/EBPβ gene promoter is dependent upon “activation” by differentiation inducers (Fig. 1C). This and the fact that the differentiation inducers activate phosphorylation of CREB indicate that binding to the promoter is not dependent upon phosphorylation. These findings are consistent with those of others (34, 35) that phosphorylation of CREB facilitates its interaction with CBP/p300, a component of the RNA polymerase II transcriptional initiation complex.

Although CREM did not appear to associate with C/EBPβ promoter sequences in 3T3-L1 preadipocytes as detected by ChIP analysis (Fig. 6A), CREM was found to associate with C/EBPβ promoter sequences in MEFs upon treatment with differentiation inducers as detected by ChIP analysis (Fig. 6A). It should be noted, however, that both CREM and ATF1 were overexpressed by CREB(−/−) MEFs (Fig. 5A). The interaction of CREM with the C/EBPβ promoter sequences was increased in CREB(−/−) MEFs before by treatment with differentiation inducers as detected by ChIP analysis (Fig. 6A).

Members of the CREB family of transcription factors not only bind to the C/EBPβ promoter ex vivo but also activate reporter gene expression driven by the promoter in 3T3-L1 preadipocytes. As shown in Fig. 6B expression vectors for CREB, CREM, and ATF1, co-transfected into 3T3-L1 preadipocytes with a minimal C/EBPβ promoter-reporter, strongly activate reporter gene expression.

**Constitutively Active CREB (VP-16CREB or CREBDIEDML) Activates, and Dominant-negative CREB (K-CREB) Inhibits, Expression of C/EBPβ and Adipogenesis—**To verify that CREB activates the expression of C/EBPβ and adipogenesis, the edysone-inducible system was employed to control expression of CREB proteins in 3T3-L1 preadipocytes. 3T3-L1 preadipocyte lines were established that harbor expression vectors in which constitutively active CREB (VP16-CREB and CREB-DIEDML), dominant-negative CREB (K-CREB), or a control vector (LacZ) are under the control of the edysone promoter. The effects of expression of the transgenes, induced by treatment of growth-arrested preadipocytes with ponasterone for 36 h, were compared with preadipocytes induced to differentiate using the standard differentiation protocol. As illustrated in Fig. 7 (A and C), expression of constitutively active VP16-CREB or CREB-DIEDML (Fig. 7B) was sufficient to induce the expression of C/EBPβ and adipogenesis (as indicated by Oil Red O staining of cytoplasmic triacylglycerol) without induction of differentiation with the inducers (MDI) normally required. In contrast, expression of dominant active K-CREB inhibited the expression of C/EBPβ protein and adipogenesis induced by treatment of the preadipocytes with differentiation inducers (KCREB plus MDI in Fig. 7C). Expression of LacZ by control cells had no effect on expression of C/EBPβ or adipogenesis in cells treated or not with differentiation inducers. Taken together, these findings demonstrate that active CREB protein is sufficient and required for the expression of C/EBPβ protein and the induction of adipocyte differentiation.

**Effect of CREB, ATF-1, ATF-2, or c-Jun Antisense Oligonucleotides on Adipogenesis by 3T3-L1 Preadipocytes—**To gain further insight into the dependence of adipogenesis on members of the CREB family of transcription factors, 3T3-L1 preadipocytes were treated with antisense oligonucleotides corresponding to sequences in the CREB, ATF-1, ATF-2, CREM, or c-Jun mRNAs. As expected, the CREB antisense oligonucleotides virtually abolished adipogenesis as indicated by the accumulation of cytoplasmic triglyceride (Fig. 8). ATF-1, and to a lesser extent ATF-2 antisense oligonucleotides decreased but did not eliminate the expression of cytoplasmic triglyceride. CREM and c-Jun antisense oligonucleotides had no detectable effect on triglyceride accumulation (Fig. 8). These findings further document the requirement of CREB and ATF-1 for the differentiation of 3T3-L1 preadipocytes into adipocytes.

**DISCUSSION**

In this study, we investigated the mechanism by which CREB activates expression of C/EBPβ early in the adipocyte differentiation program (7, 9, 11, 36). Once activated (10), C/EBPβ triggers a cascade of transcriptional events, notably activation of the C/EBPα and PPARγ genes (14, 15, 17). C/EBPα and PPARγ then activate the transcription of the set of genes that give rise to the adipocyte phenotype (6, 12, 13).

We identified dual CRE-like elements (i.e. TGA1 and TGA2 (Fig. 1A)) within the proximal promoter of the C/EBPβ gene to which CREB family members, i.e. CREB and ATF1, bind (Fig. 2, A–C) and activate transcription (Figs. 1D and 4A). CREB and ATF1 are constitutively expressed by 3T3-L1 preadipocytes and MEFs throughout the differentiation program, even in growth-arrested cells, prior to induction of differentiation.
EMSA and supershift experiments show that, although CREB and ATF1 together account for virtually all binding to the TGA-1 and -2 elements, CREB accounts for most of the binding activity (Fig. 2, A–C).

Nuclear extracts from CREB(−/−) MEFs exhibit greatly reduced binding to oligonucleotide probes corresponding to the TGA regulatory elements in the C/EBPβ promoter (data not shown). The remaining residual binding activity is due to CREM and ATF1 (Fig. 6A), whose expression is markedly upregulated in CREB(−/−) MEFs (Fig. 5A). Likewise, the expression of C/EBPβ and adipogenesis are greatly reduced in CREB(−/−) MEFs (Fig. 5, A and B). The fact that the reduction of the level of C/EBPβ and adipogenesis correspond closely to the reduction of DNA binding activity to the TGA elements provides compelling evidence that CREB and its cis regulatory elements play important roles in the early phase of the adipocyte differentiation program. In an analogous manner CREM has been shown to compensate for CREB deficiency in human adrenocortical carcinoma cell line H295R.

Other protein families, notably AP1 (Fos/Jun) protein, share similar DNA consensus binding sites with CREB/ATF1 family. Nevertheless, reduction in expression of Jun using antisense Jun had no effect on adipogenesis (Fig. 8). It has also been shown that overexpression of DeltaFosB inhibits adipogenesis (37). Likewise, overexpression of Finkel-Biskis-Reilly osteosarcoma virus v-Fos inhibits adipocyte differentiation (38).

Immediately following induction of differentiation CREB and ATF1 undergo phosphorylation (Fig. 3). This is followed by the phosphorylation of C/EBPβ (18). Whereas phosphorylation of CREB and ATF1 enhances binding to regulatory elements in the C/EBPβ gene promoter (Fig. 6A), the primary effect of phosphorylation of CREB and ATF1 appears to be transactivation of the C/EBPβ gene. This is consistent with our findings that activation of reporter gene expression and phosphorylation of CREB and ATF1 occur only after treatment with differentiation inducers (Figs. 1C, 1D, and 3). Chromatin immunoprecipitation experiments with anti-phospho-CREB antibody showed that phospho-CREB, which occurs rapidly upon induction (Fig. 3), binds to the proximal promoter of the C/EBPβ gene (Fig. 5A). Furthermore, expression of constitutively active CREB, in which the CREB transcription activation domain is replaced by VP16, a strong viral transcriptional activator, activated expression of C/EBPβ and differentiation (Fig. 7A).

Phosphorylation of CREB is known (39–46) to be activated by two components, i.e. cAMP and insulin, of the inducer mixture used to trigger differentiation of 3T3-L1 preadipocytes and MEFs (25). Thus, it appears that protein kinase A and a downstream kinase of the IGF-1 receptor signal transduction pathway phosphorylate CREB early in the differentiation program. CREB is known to be phosphorylated by several protein kinases, including protein kinase A and AKT (26, 47, 48). Consistent with involvement of the IGF-1 receptor signal transduction pathway, preadipocytes are known to possess a high level of IGF receptors but few insulin receptors (49). Moreover, it has been shown that in preadipocytes insulin (at the high level used in the differentiation inducer mixture) acts through the IGF receptor (49, 50). Recently, it was shown (51) that RhoVRho kinase blocks adipogenesis and promotes myogenesis in mesenchymal precursor cells. This observation also suggests that blockade of the IGF-1 signaling pathway blocks activation of CREB.

Acknowledgment—We thank Dr. David Ginty (Dept. Neuroscience, Johns Hopkins University School of Medicine) for providing CREB(−/−) mouse embryos for preparation of MEFs.

REFERENCES

1. Shepherd, P. R., Gruoli, L., Tozon, E., Yang, H., Leach, P., and Kahn, B. B. (1993) J. Biol. Chem. 268, 22443–22446
2. Gruoli, L., Shepherd, P. R., and Kahn, B. B. (1996) Proc. Nutr. Soc. 55, 191–199
3. Van, R. L., Bayliss, C. E., and Roncar, D. A. (1976) J. Clin. Invest. 58, 699–704
4. Boulat, D. A., Bolos, M. A., Kelly, T. J., and Lane, M. D. (1985) J. Biol. Chem. 260, 5565–5567
5. Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994) Annu. Rev. Nutr. 14, 91–129
6. MacDougald, O. A., and Lane, M. D. (1995) Annu. Rev. Biochem. 64, 345–373
7. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 44–49
8. Patel, Y. M., and Lane, M. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1279–1284
9. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 850–855
10. Tang, Q. Q., and Lane, M. D. (1999) Genes Dev. 13, 2231–2241
11. Yeh, W.-C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Genes Dev. 9, 168–181
12. Everts, E. D., Walkley, C. J., Puigserver, P., and Spiegelman, B. M. (2000) Genes Dev. 14, 1293–1307
13. Hwang, C., Loftus, T., Mandrup, S., and Lane, M. (1997) Annu. Rev. Cell Dev. Biol. 13, 385–448
14. Brandes, R., Arad, R., Benvenisty, N., Weil, S., and Bar-Tana, J. (1990) Mol. Cell. Biol. 10, 4685–4686
15. Zhu, Y., Qi, C., Koenenberg, J. R., Chen, X. N., Noya, D., Rao, M. S., and Reddy, J. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7921–7925
16. Clarke, S. L., Robinson, C. E., and Gimble, J. M. (1997) Biochem. Biophys. Res. Commun. 240, 99–103
17. Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. (1999) J. Biol. Chem. 274, 16741–16746
18. Tang, Q. Q., Jiang, M. S., and Lane, M. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13571–13575
19. Reusch, J. E., Colton, J. L., and Klemm, D. J. (2000) Mol. Cell. Biol. 20, 9858–1029
20. Brandes, R., Arad, R., Benvenisty, N., Weil, S., and Bar-Tana, J. (1990) Biochim. Biophys. Acta 1054, 219–224
21. Gasimir, D. A., Miller, C. W., and Ntambi, J. M. (1996) Differentiation 60, 203–210
22. Schmidt, W., Poll-Jordan, G., and Loffler, G. (1990) J. Biol. Chem. 265, 15489–15495
23. Yang, V. W., Christy, R. J., Cook, J. S., Kelly, T. J., and Lane, M. D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3629–3633
24. Student, A. K., Hsu, R. Y., and Lane, M. D. (1980) J. Biol. Chem. 255, 4745–4750
25. Gonzalez, G. A., and Montminy, M. R. (1989) Cell 59, 675–680
26. Niewoh, M., Manns, M. P., and Trautwein, C. (1997) Mol. Cell. Biol. 17, 3600–3613
27. Saito, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Science 239, 487–491
28. Hwang, C.-S., Mandrup, S., MacDougald, O. M., Geiman, D. E., and Lane, M. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7175–7180
29. Lavery, D. J., and Schibler, U. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6525–6529
30. Raymondjean, M., Cereghini, S., and Yaniv, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 757–761
31. Raymondjean, M., Cereghini, S., and Yaniv, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1871–1875
32. Fried, M., and Crothers, D. M. (1981) J. Biol. Chem. 256, 1037–1042
33.已经被重新排序为：2023-07-24 09:32:28
Role of CREB in Transcriptional Regulation of CCAAT/Enhancer-binding Protein β Gene during Adipogenesis
Jiang-Wen Zhang, Dwight J. Klemm, Charles Vinson and M. Daniel Lane

J. Biol. Chem. 2004, 279:4471-4478. doi: 10.1074/jbc.M311327200 originally published online October 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M311327200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 51 references, 30 of which can be accessed free at http://www.jbc.org/content/279/6/4471.full.html#ref-list-1