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cAMP Activation of CAAT Enhancer-binding Protein-β Gene Expression and Promoter I of Acetyl-CoA Carboxylase*

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The acetyl-CoA carboxylase (ACC) gene contains two distinct promoters, denoted PI and PII. PI is responsible for the generation of class I ACC mRNAs which are induced in a tissue-specific manner under lipogenic conditions. PII generates class II ACC mRNAs which are expressed constitutively. During 30A5 preadipocyte differentiation, both promoters are activated; the preadipocytes must be pretreated with cAMP for this activation to occur. In this report, we present evidence that CAAT enhancer-binding protein-β (C/EBP-β) is induced and involved in the PI activation by cAMP. Expression of the reporter gene under the control of the PI promoter is activated within 3 h after treatment of 30A5 cells with a cyclic AMP analogue, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate, and 3-isobutyl-1-methylxanthine, in association with the accumulation of C/EBP-β mRNA and protein. These accumulations were inhibited in the presence of H8, a protein kinase inhibitor; H8 also inhibited activation of PI by cAMP. However, the induction of reporter gene expression and the increase of C/EBP-β mRNA by cAMP were not affected by treatment with tumor necrosis factor α, which completely inhibited the accumulation of C/EBP-α mRNA. Overexpression of C/EBP-β by transfection with the C/EBP-β gene led to increased binding of C/EBP-β to DNA and partial PI activation. cAMP did not affect the amount of C/EBP-β binding to the DNA but did promote phosphorylation of C/EBP-β and PI activation. As in the case of C/EBP-α, C/EBP-β bound to the CCAAT box of the PI promoter. These results indicate that cAMP not only induces, but also activates, bound C/EBP-β through phosphorylation for PI activation. Our studies also indicate that cAMP induces C/EBP-α. C/EBP-β induction, however, precedes that of C/EBP-α.

30A5 preadipocytes are derived from C3H 10T1/2 mouse fibroblasts that can be differentiated into adipocytes (1, 2). The induction conditions for differentiation are pretreatment of the cells at confluence with dexamethasone and insulin for 3 days, followed by incubation in basal medium containing insulin alone. Under these conditions, about 80% of the cells become laden with fat droplets by day 7 or 8 (1). The differentiation can be accelerated by at least 2–4 days when cells are pretreated with cAMP and 3-isobutyl-1-methylxanthine (IBMX)[1] at confluence for as little as 1 h instead of the dexamethasone and insulin pretreatment (3). Pretreatment of the cells can be omitted if the cells are kept in the same medium for 5 days, so that some nutrients become limited; these conditions also allow the cells to differentiate upon incubation in medium containing insulin. During the nutrient limitation of 30A5 cells, the intracellular level of cAMP increases to a level comparable to cAMP and IBMX concentrations used in the pretreatment (3). These studies indicated that cAMP is essential to induce differentiation of preadipocytes (3). Activation of acetyl-CoA carboxylase (ACC) gene expression, which is closely associated with the differentiation, also requires cAMP pretreatment of the cells (3, 4). ACC gene transcripts consist of multiple forms which are generated as a result of differential splicing of two primary transcripts from two distinct promoters, PI and PII (5, 6). Previously, we classified those mRNA species transcribed from PI as class I ACC mRNAs, whereas those transcribed from PII were designated class II mRNAs. Stimulated lipogenic conditions lead to induction of the class I ACC mRNAs in the liver (5). Likewise, induction of PI promoter expression occurs during differentiation of 30A5 preadipocytes into adipocytes (4). Based on these observations, together with others (5), we suggested that PI is an inducible promoter under stimulated lipogenic conditions, whereas PII is constitutively expressed (5). In a recent report we showed that accumulation of CCAAT/enhancer-binding protein α (C/EBP-α) mRNA occurred in close association with the PI gene products during the differentiation of 30A5 preadipocytes (4). Furthermore, we showed that C/EBP-α binds to a specific sequence, GCAAT, in the PI promoter and that binding allowed the expression of PI which was otherwise repressed (4). In this paper, we demonstrate that cAMP treatment of 30A5 cells activates both C/EBP-α and C/EBP-β gene expression, which lead to PI activation. However, in the case of C/EBP-β, increased binding of C/EBP-β to the CAAT box alone is not sufficient to activate PI unless the bound C/EBP-β is phosphorylated and activated by cAMP.

EXPERIMENTAL PROCEDURES

Materials—Commercial products were obtained as follows. Eagle's basal medium, donor calf serum, neomycin sulfate (G418), Nick translation kit and DNase I were from Life Technologies, Inc.; insulin was from Collaborative Research; 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (8-CPT cAMP), IBMX, poly(dI-dC), *N*-[2-(methylamino)ethyl]-5-isoquinolone sulfonamide (H8), monoclonal anti-phosphoserine (6), and chloroquine were from Sigma; restriction enzymes and T4 DNA kinase were from New England Biolabs; T4 DNA polymerase from International Biotechnologies; Klenow fragment of DNA polymerase I, dideoxynucleoside triphosphates, and calf intestinal al-

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[1] The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; ACC, acetyl-CoA carboxylase; C/EBP, CAAT enhancer-binding protein; TNF, tumor necrosis factor; G418, neomycin sulfate; 8-CTP cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; H8, *N*-[2-(methylamino)ethyl]-5-isoquinalone sulfonamide; bp, base pair(s); CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein.

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kaline phosphatase were from Boehringer Mannheim; deoxynucleoside triphosphates and T4 DNA ligase were from Pharmacia Biotech Inc.; [^{14}C]chloramphenicol (57 mCi/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [α-^{35}S]dATP (1000 Ci/mmol) were from Amersham Corp.; [γ-^{32}P]ATP (6,000 Ci/mmol) was from DuPont NEN; RNAzol™ was from Biotecx. Oligonucleotides were synthesized by an Applied Biosystems model 3800 DNA synthesizer and purified by high performance liquid chromatography. The plasmids pMSV-C/EBP-α and pMSV-C/EBP-β, which contain the full length of the rat C/EBP-α or β coding sequences under the control of murine sarcoma virus LTR, were generous gifts from Dr. Z. Cao and Dr. S. L. McKnight. Antibodies against the peptides of C/EBP-α were a generous gift from Dr. P. Cornelius and Dr. M. D. Lane. Antibodies against the peptides of C/EBP-β were purchased from Santa Cruz Biotechnology.

Construction of a Chimeric Gene of the PI Promoter and CAT Gene Coding Region and Generation of Deletion Mutants—A 1144-bp fragment flanking the 5′-end of exon 1 of the ACC gene (including 136 bp of exon 1) was inserted in front of the chloramphenicol acetyltransferase (CAT) gene of pUC-CAT3 by use of the *Xba*I site (7). This construct is designated pPI-CAT0 (Fig. 1). 5′-Deletion mutants of the PI promoter from pPI-CAT0 were generated by using exonuclease III and S1 nuclease (3). Clones with different degrees of deletion were selected, and the exact sites of deletion were determined by dideoxy nucleotide sequence analysis (8). The two deletion mutants (pPI-CAT2 and pPI-CAT5) are shown in Fig. 1. For pPI-997/CAT, a 997-bp fragment flanking the 5′ end of exon 1 of the ACC gene (−1008 to −12) was inserted upstream of the CAT gene of pUC-CAT3. pPI-D/CAT was constructed by restriction enzyme digestion of a 997-bp fragment (−1008 to −12) of PI promoter and ligation of the digested fragments in front of the CAT gene at the *Xba*I site of pUC-CAT3. pPI-TATA was constructed by insertion of a synthesized DNA fragment representing the fragment from −34 to −12 of PI in front of the CAT gene at the *Xba*I site of pUC-CAT3. To create a 5-base deletion mutant, pPI-997/CAT(mut), the 997-bp fragment was subcloned in pBluescript KS$^+$ and mutation was carried out by the Kunkel method (9) using the primer 5′-CTGACCTTCATTAT↓GGAATCTTGCCTTTCG-3′. 209 indicates the position of the 5-base deletion ($^{-67}$GCAAT^{-63}). The mutated 997-bp fragment was then inserted in front of the CAT gene of pUC-CAT3. The pPII-CAT22 construct was described previously (10).

DNA-mediated Cell Transfection and CAT Assay—Mouse 30A5 preadipocytes were cultured in 100-mm Petri dishes to about 80% confluence in Eagle's basal medium supplemented with 10% donor calf serum (Life Technologies, Inc.). Stable transfectants were obtained as described previously (3). Twenty-four hours after adding DNA precipitates to the culture plates, cells were split and fed with media containing G418. After cells had been incubated for 10–14 days, about 300 G418-resistant colonies were pooled as stable clones. CAT assays were performed as described by Gorman *et al.* (11).

Preparation of Nuclear Extracts from 30A5 Cells—Nuclear extracts were prepared basically according to the method of Dignam *et al.* (12) from 30A5 preadipocytes that had been incubated with or without 0.1 mM 8-CPT cAMP and 0.5 mM IBMX at confluence for a specified period as indicated. All the subsequent steps were carried out at 4 °C. Cells were harvested in 1 ml of phosphate-buffered saline/plate by spinning for 10 min in a clinical centrifuge at 2000 rpm. The cell pellet was resuspended in 5 times the pellet volume of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) and kept on ice for 10 min. The cell resuspension was centrifuged in a clinical centrifuge at 2000 rpm for 10 min and resuspended in 2 times the pellet volume of buffer A. Cells were homogenized in a glass Dounce homogenizer with a B pestle until the cells were broken (about 10 strokes). A crude nuclear pellet was obtained by centrifugation at 6,000 rpm in a Sorvall centrifuge (SS-34) for 20 min at 4 °C. The nuclear pellet was resuspended in one pellet volume of buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF). Nuclei were broken by using a Dounce homogenizer with a B pestle (about 10 strokes). The nuclear suspension was stirred for 30 min and then centrifuged for 20 min at 13,000 rpm in an SS-34 centrifuge. The supernatant was dialyzed against buffer D (20 mM HEPES, pH 7.9, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF). The final nuclear extract was obtained by centrifugation at 13,000 rpm for 20 min. Nuclear extracts were divided into aliquots, quickly frozen in liquid nitrogen, and stored at −80 °C. Two to three milligrams of nuclear proteins were routinely obtained from 10^8 cells. All buffers contained protease inhibitors (1 μg/ml aprotinin and pepstatin and 2 μg/ml leupeptin).

DNase I Footprinting—DNase I footprinting analysis was performed according to Dynan and Tjian (13) with the following modifications. A 20-μg sample of nuclear protein from 30A5 cells was incubated with approximately 1.5×10^4 cpm of the ^{32}P-labeled DNA probe at room temperature for 15 min in 50 μl of a buffer containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI-dC). The reaction mixture was then subjected to DNase I (0.2–1 unit) treatment at room temperature for 1 min. After the reaction, the samples were extracted and analyzed by 8% denaturing polyacrylamide gel electrophoresis.

Mobility Shift DNA-binding Assay—The 30-bp oligonucleotide (5′-CTTCATTATGCAATGGAATCTTGCCTTTCG-3′) was end-labeled using [γ-^{32}P] ATP and T4 DNA polynucleotide kinase (14). Typical binding conditions were the same as the footprinting assay except that the total volume was 20 μl. For the competition experiment, a molar excess of unlabeled DNA fragment was added to the binding mixture. For the supershift assay with antiserum, 2 μl of antiserum or preimmune serum was added to the reaction mixture and incubated for 10 to 15 min prior to adding labeled probes. After incubation at room temperature for 15 min, the reaction mixtures were directly subjected to electrophoresis in a 4% nondenaturing polyacrylamide gel in Tris-glycine buffer (25 mM Tris base, 190 mM glycine, and 1 mM EDTA, pH 8.5). The gel was then fixed with a mixture of 10% methanol and 10% acetic acid, dried, and autoradiographed.

Preparation of Total Cellular RNA from 30A5 Cells—Total RNA from 30A5 preadipocytes or differentiated adipocytes was prepared by the AGPC method as described by Chomczynski *et al.* (15). Briefly, 2 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) was added to each plate, and cells were scraped and transferred into a tube. 0.2 ml of 2 M sodium acetate, 2 ml of phenol, and 0.4 ml of chloroform-isoamyl alcohol mixture (49:1) were added to each tube. After vortexing, the mixture was kept for 15 min on ice before centrifugation in an HB-4 rotor at 10,000 rpm for 20 min. The aqueous phase was transferred into a new tube and an equal volume of isopropanol was added. After centrifugation at 10,000 rpm for 20 min, the pellet was dissolved in 0.3 ml of solution D and 0.3 ml of isopropanol was added. After spinning in a microcentrifuge for 10 min, total RNA precipitate was washed with 75% ethanol, dried briefly and resuspended in diethyl pyrocarbonate-treated water.

Northern Blot Hybridization—15 μg of total RNA was fractionated by electrophoresis on formaldehyde/agarose gels as described (14). RNA was then transferred onto nylon membranes and hybridized with ^{32}P-labeled probes (16). Filters were prehybridized in 0.5 M Na_2HPO_4, pH 7.0, 0.7% SDS, 0.1% bovine serum albumin, 1 mM EDTA, and 50 μg/ml yeast torular RNA at 65 °C for 4 h. Heat-denatured and nick-translated DNA probes (10^6 cpm/ml) were added to the hybridization bags, and incubation was continued for 18 h at 65 °C. Following hybridization, filters were washed in 250 ml of 40 mM Na_2HPO_4, 1 mM EDTA, 5% SDS, and 0.5% bovine serum albumin at 70 °C for 1 h and then in 40 mM Na_2HPO_4, 1 mM EDTA, and 1% SDS at 70 °C for 30 min. Finally, the filters were rinsed in 250 ml of 0.5 M Na_2HPO_4, pH 7.0, at room temperature and air-dried before exposure to Kodak XAR film. 1060 bp of the *Nco*I fragment of C/EBP-α (17) was used for detection of C/EBP-α mRNA and 1500 bp of the *Eco*RI/*Bam*HI fragment of C/EBP-β for detection of C/EBP-β mRNA (18). The probes were labeled using a labeling kit from Life Technologies, Inc.

Western Blot Analysis—Nuclear fraction was prepared by the procedures described above for DNase I footprinting and mobility shift DNA-binding assay. The cytoplasmic fraction was prepared from the supernatant fraction after the nuclear fraction was separated by centrifugation and concentrated by spinning the supernatant in a microconcentrator (Amicon) for 2 h at 4 °C. The cytoplasmic or nuclear fractions (30 μg) were mixed with an equal volume of SDS-sample buffer, heated to 100 °C for 5 min, and fractionated on 10% SDS-PAGE. Proteins were then transferred to nitrocellulose filters using mini transblot equipment. The filters were blocked with 1% bovine serum albumin (1 h) and 10% bovine serum (1 h) and probed with C/EBP-β antibody (C/EBP-β (Δ198), Santa Cruz Biotechnology, Inc.). Immunoreactive C/EBP-β protein was visualized by anti-rabbit IgG-alkaline phosphatase conjugate and Sigma Fast™ system following the procedure recommended by the supplier.

Determination of C/EBP-β Phosphorylation—To 250 μl of nuclear extract in buffer C (1 μg/μl), Triton X-100 (1 μl), and a 50% slurry of protein A-Sepharose (50 μl) were added and incubated for 4 h. Following this incubation, the mixture was centrifuged at 14,000 rpm for 5 min. The supernatant (250 μl) was transferred to an Eppendorf tube, anti-C/EBP-β (10 μl) was added, and the mixture was incubated, with shaking, for 2 h before 50 μl of protein A-Sepharose was added. The mixture was incubated for another 4 h, and the precipitate was obtained by centrifugation at 14,000 rpm for 5 min. The precipitate was

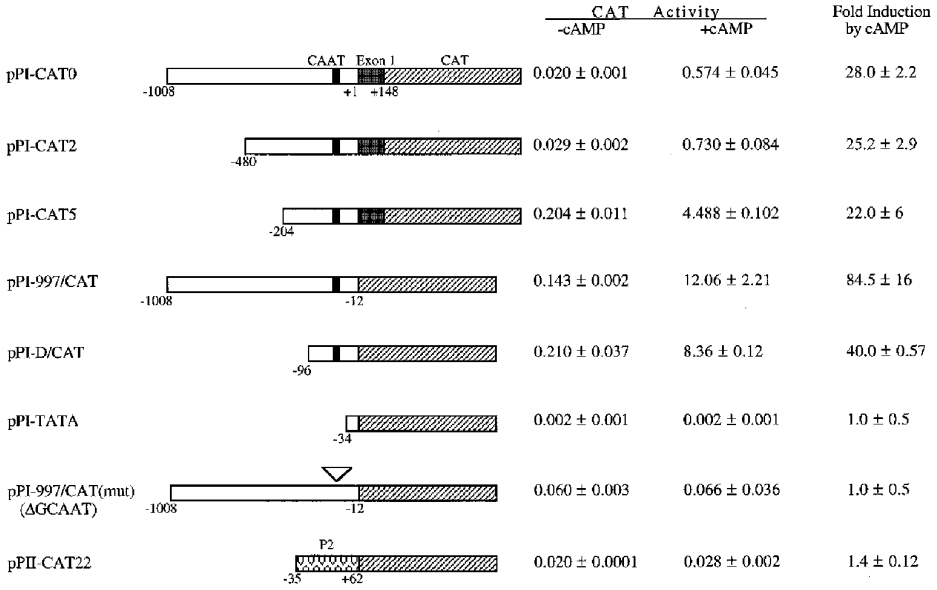

FIG. 1. **cAMP action on various subdeleted PI-CAT constructs.** The *numbers* in the diagram designate the subdeleted position of the promoter. pPI-997/CAT(mut) contains the PI promoter fragment (from −1008 to −12) from which the sequence GCAAT (the CCAAT box at −63/−67) was deleted. The construction of these plasmids is described under "Experimental Procedures." Stable clones containing these plasmids were cultured for 24 h with and without cAMP and IBMX before analysis of CAT activity. The basal promoter activities and those in the presence of cAMP were analyzed. The standard deviations were obtained from three independent experiments.

then washed three times with buffer I (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF, 10 mM NaF, 10 mM sodium pyrophosphate, 0.1% Triton X-100, 0.02% SDS, pH 7.5). The washed pellet was cleaned once more with buffer I without Triton X-100 and SDS. Bound C/EBP-β was eluted by washing twice with 25 μl of the elution buffer (0.5% SDS, 10 mM DTT, 10 mM Tris-HCl, pH 7.5). Thirty μl of the eluate was then mixed with 10 μl of the sample buffer, and proteins were separated on a 10% SDS-PAGE. Phosphorylation level was determined through Western blot analysis using monoclonal anti-phosphoserine antibody. To examine the specificity of the phosphoserine antibody, 80 μg (80 μl) of nuclear extract was incubated with and without 20 units (20 μl) of alkaline phosphatase at room temperature for 30 min. The treated nuclear extracts were incubated with 20 μl of C/EBP-β antibody to precipitate C/EBP-β. C/EBP-β in the immunoprecipitate was then subjected to SDS-PAGE and then to Western blot analysis using antibodies against C/EBP-β or phosphoserine.

RESULTS

cAMP Activation of PI Expression—As discussed in the Introduction, cAMP pretreatment of 30A5 cells is required for insulin to induce ACC and cell differentiation (3). Cells treated with 8-CPT cAMP and IBMX at confluence become shiny, but do not differentiate morphologically and do not accumulate lipid droplets until 8-CPT cAMP and IBMX are removed. Following the addition of insulin after the removal of 8-CPT cAMP and IBMX, cells start to accumulate lipid drops and become morphologically differentiated adipocytes by day 4. There is no identifiable consensus sequence for the cAMP-responsive elements (19, 20) in the 1 kilobase pair of the PI promoter. In order to locate the site of cAMP action on PI expression, we constructed a chimeric gene containing PI and the bacterial CAT gene. Diagrammatic representations of some of the relevant plasmids are seen in Fig. 1. To identify those cells with cAMP-responsive elements in the plasmids, the cells were treated with or without cAMP and the expression of the CAT activities were determined. Fig. 1 also shows the basal promoter activities and those activated by cAMP.

The induction of CAT activity by cAMP occurred only with those cells containing the plasmids with the CCAAT box sequence. For example, cAMP routinely induced CAT activities 20–30-fold over those in the absence of cAMP in the case of pPI-CAT0, pPI-CAT2, pPI-CAT5; and 40–80-fold in the case of pPI-997/CAT and pPI-D/CAT. The CAT expressions of cells with pPI-TATA (PI sequence from −34 to −12), which contain no CCAAT box, were hardly affected by the addition of 8-CPT cAMP and IBMX. The CAT expression of pPI-997/CAT(mut), in which a 5-bp sequence of the CCAAT box from pPI-997/CAT was deleted, also showed no effect of cAMP on the induction of CAT activity, although the basal promoter activity increased about 3-fold as observed previously (4). Finally, cAMP had no effect on cells containing the plasmid pPII-CAT22. This plasmid contains a small piece of the PII sequence, which shows promoter activity by itself but contains no CCAAT box. These results suggested that the CCAAT box sequence of the PI promoter is involved in the stimulation of PI promoter expression by cAMP.

Protein Factors Binding to the CCAAT Box Region of PI—To determine whether there are any nuclear binding proteins for the CCAAT box region, DNase I footprinting analysis was performed using the DNA fragment from −303 to −12 of PI and nuclear extracts from 30A5 cells incubated with or without 8-CPT cAMP, and IBMX at confluence for 48 h (Fig. 2). The nuclear extract from 30A5 cells treated with 8-CPT cAMP and IBMX was found to protect the probe around the CCAAT box sequence from DNase I digestion. The protected sequence is around −71 to −47 (5′-TTATGCAATGGAATCTTGCCTTTCG-3′) including a CCAAT box, which is also protected from DNase I digestion by C/EBP-α (4). On the other hand, when the DNA fragment of −303 to −12, in which 5 bp of the CCAAT box sequence (GCAAT) were deleted, was used for DNase I footprinting analysis with nuclear extract from 30A5 cells incubated with or without 8-CPT cAMP and IBMX, this mutated probe did not show any protected region regardless of the origin of the nuclear extract. These results agree with our conclusion based on studies using the deletion mutants that the GCAAT sequence of the PI promoter is a cAMP-responsive element.

In order to identify the binding factor(s), 30 base pair oligonucleotides, spanning this protected region, were synthesized and mobility shift DNA-binding assays were performed. The 30-base pair fragment generated 3 bands (Fig. 3, *lane 1*). All these bands were competed out in the presence of excess amounts of non-labeled probes, but not by either the mutated sequence, in which the AA of the GCAAT sequence in the CCAAT box was substituted for by GG, or an unrelated DNA sequence, a 30-base pair fragment from PII (data not shown; see also Ref. 4). Therefore, these three bands (B1, B2, and B3) appear to be specific to this 30-bp DNA fragment containing the GCAAT sequence.

C/EBP-α and C/EBP-β Binding to PI—In order to identify the factors that bind to this DNA fragment of PI, supershift assays were performed by using anti-C/EBP-α and anti-C/

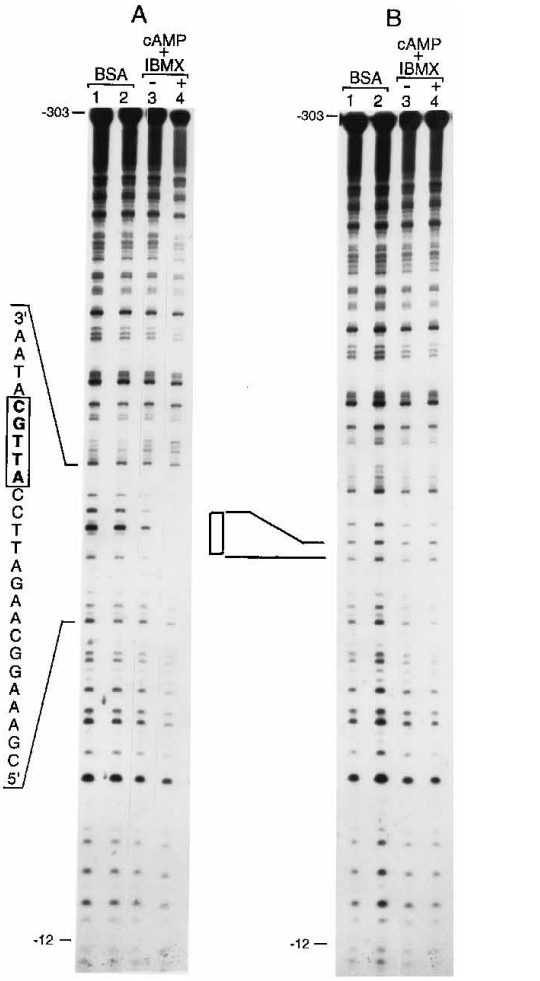

FIG. 2. **DNase I footprinting of PI with nuclear extracts from 30A5 cells.** The following DNAs were used: *A*, PI fragment (−303 and −12 bases) prepared from pPI-997/CAT; *B*, PI fragment (−303 and −12) from which the 5-bp GCAAT of the CCAAT box were deleted. This DNA fragment was prepared from pPI-997/CAT(mut). The plasmids were digested with *Bam*HI and labeled using [γ-^{32}P]ATP and T4 DNA kinase. One end-labeled fragment was prepared by digestion with *Nsp*I. 20 μg of nuclear extract from 30A5 cells that had been incubated with (*lane 4*) or without (*lane 3*) 100 μM cAMP and 500 μM IBMX for 48 h or 20 μg of bovine serum albumin (*lanes 1* and *2*), were incubated with 1.5 × 10^4 cpm of the ^{32}P-labeled DNA probe. The reaction mixtures were then subjected to DNase I (0.2–1 unit) treatment at room temperature for 1 min. After terminating the reaction, samples were extracted and analyzed in 8% denaturing polyacrylamide gels by electrophoresis and autoradiography. The protected region is shown at the *left* side of ladders with the sequences. The 5-bp deleted region is indicated by a *small box* between the ladders of the *A* and *B* digestion patterns. *Dotted lines* note corresponding regions between the two probes.

EBP-β (Fig. 3). The band B1 is shifted by the presence of anti-C/EBP-α, establishing that B1 is due to C/EBP-α binding to the DNA (Fig. 3, *lanes 2* and *6*). On the other hand, bands B2 and B3 are supershifted by anti-C/EBP-β (Fig. 3, *lanes 3* and *7*), indicating that bands B2 and B3 are generated by C/EBP-β in nuclear extracts of 30A5 cells. None of these bands is supershifted with anti-AP2 (Fig. 3, *lanes 4* and *8*). In addition, the binding activities of C/EBP-α and β were increased in nuclear extracts of 30A5 cells treated with 8-CPT cAMP and IBMX. Although the reason for the generation of two C/EBP-β-containing complexes, B2 and B3, is not clear at this time and will be the subject of further investigations, it appears that the B2 and B3 complexes do not contain C/EBP-α, because the complexes are not recognized by anti-C/EBP-α. Neither of these bands was shifted by antibodies against NFκB or CREB, suggesting that these complexes do not contain NFκB subunits or

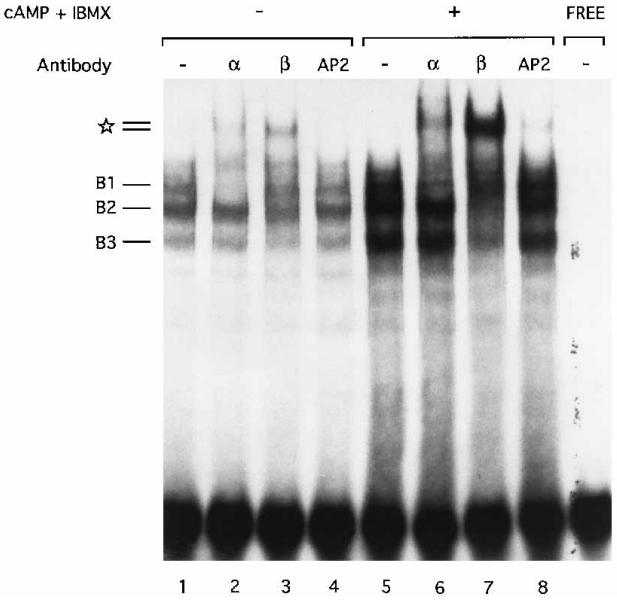

FIG. 3. **Supershift assay.** 10 μg of nuclear extract from confluent 30A5 cells that had been incubated without (*lanes 1–4*) or with (*lanes 5–8*) 100 μM 8-CPT cAMP and 500 μM IBMX for 48 h were then incubated with anti-C/EBP-α (*lanes 2* and *6*), anti-C/EBP-β (*lanes 3*, and *7*), anti-AP2 (*lanes 4* and *8*), or without antibody (*lanes 1* and *5*) prior to addition of the labeled 30-bp DNA fragment (−76 to −47) of PI. Then the mixtures were incubated further with the labeled 30-bp DNA fragment (1.5 × 10^4 cpm). *B1*, *B2*, and *B3* indicate shifted bands of the 30-bp DNA fragment with nuclear extracts. *Star* indicates the supershifted band with anti-C/EBP-α or anti-C/EBP-β.

CREB (data not shown).

Induction of C/EBP-α and C/EBP-β in 30A5 Cells Treated with cAMP and IBMX—The binding activities of both C/EBP-α and β proteins increased in nuclear extracts of 30A5 cells treated with 8-CPT cAMP and IBMX (Fig. 3). To examine whether or not this increase in the binding activity is due to increases in gene expression and the amount of C/EBPs, the respective mRNA levels were analyzed (Fig. 4). The levels of mRNAs for C/EBP-α and β increased considerably in 30A5 cells by treatment with 8-CPT cAMP and IBMX for 48 h (Fig. 4, *lanes 2* and *5 versus lanes 3* and *6*). The kinetics of accumulation of mRNAs for C/EBP-α and β are of considerable interest, particularly with respect to answering the question as to whether or not C/EBP-β can activate PI. While a small increase of C/EBP-α occurred within 12 h of treatment with 8-CPT cAMP and IBMX (Fig. 5*A*), accumulation of the C/EBP-β mRNA occurred to a greater extent and had already reached a maximum by 3 h (Fig. 5*A*). This maximum increase of the C/EBP-β mRNA was followed by a gradual decrease. This increase in the amount of C/EBP-β mRNA following cAMP treatment is indeed associated with an increase in the total amount of C/EBP-β in the nucleus as revealed by the Western analysis (Fig. 5*B*, *lane 4*). Three hours of cAMP treatment increased the amount of C/EBP-β in both the cytosol and the nucleus. *Panels A* and *B* in Fig. 5*B* represent the results of two separate experiments. If cAMP acted solely on the phosphorylation of the existing C/EBP-β and thus promoted translocation of C/EBP-β from the cytosol to the nucleus, an increase in the total amount of C/EBP-β would not be expected (Fig. 5*B*, *lanes 1* and *2 versus 3* and *4*). These observations indicate that cAMP induced C/EBP-β during a period when cAMP exerted no apparent effect on C/EBP-α. To further correlate cAMP activation kinetics of PI and the kinetics of increases in the two forms of C/EBP, cAMP activation of the PI promoter was carried out by assaying the CAT activity of the stable clone (pPI-997/CAT) at various time points during the incubation with 8-CPT cAMP

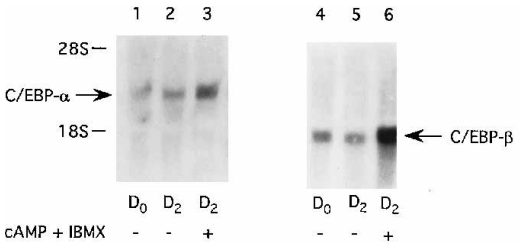

FIG. 4. **Northern blot analysis of C/EBP-α and β mRNAs.** 15 μg of total RNA from 30A5 cells (*lanes 1* and *4*) or 30A5 cells incubated without (*lanes 2* and *5*) or with (*lanes 3* and *6*) 100 μM 8-CPT cAMP and 500 μM IBMX for 48 h were fractionated in formaldehyde, 1% agarose gels and transferred onto Hybond nylon membranes. The membranes were hybridized separately with a nick-translated rat C/EBP-α cDNA probe (*lanes 1–3*) or a rat C/EBP-β cDNA probe (*lanes 4–6*).

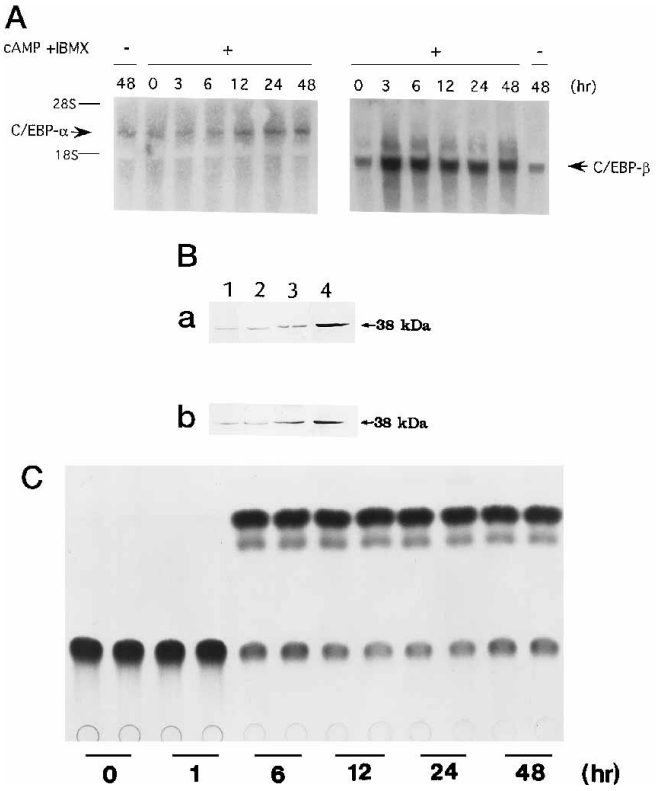

FIG. 5. **Time dependence of cAMP action on the activation of PI and the expression of C/EBP-α and C/EBP-β.** *A*, Northern blot analysis. Total RNAs were prepared from confluent 30A5 cells at various time points during incubation of the cells with cAMP and IBMX as described under "Experimental Procedures." The accumulation of mRNAs for C/EBP-α and C/EBP-β was analyzed at various time points by Northern blot analysis. *B*, Western analysis of C/EBP-β was carried out using the extracts (30 μg) prepared from the cytosolic (*1* and *3*) and nuclear fractions (*2* and *4*) of the control cells (*lanes 1* and *2*) and cells that had been treated with a cAMP analogue (*lanes 3* and *4*) for 3 h. The two panels (*a* and *b*) show the results of two independent experiments. *C*, CAT activity from the stable clone transfected with pPI-997/CAT (150 μg of proteins) was assayed at various time points during incubation of the cells with cAMP and IBMX.

and IBMX (Fig. 5*C*). The cAMP activation of PI expression occurs maximally by 6 h of treatment when the increase in the C/EBP-α mRNA is not yet detectable (Fig. 5*A*). These observations suggest that C/EBP-β may play a role in cAMP activation of the PI promoter. This conclusion is further supported by using an inhibitor of cAMP-dependent protein kinase as discussed below.

Effect of Suppression of C/EBP Gene Expression on PI Activation—H8 is a strong inhibitor of the catalytic subunit of cAMP-dependent protein kinase and blocks cAMP action in the

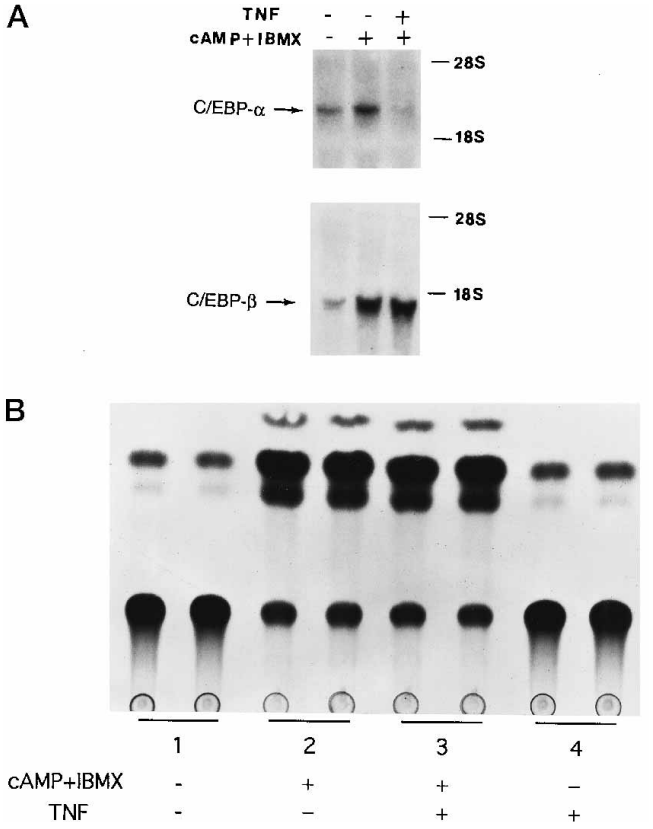

FIG. 6. **Effect of TNF-α on the expression of C/EBP-α and C/EBP-β, and cAMP activation of the PI promoter.** *A*, confluent 30A5 cells were incubated in medium without (*lane 1*) or with 0.1 mM 8-CPT cAMP and 0.5 mM IBMX, with (*lane 3*) or without (*lane 2*) 200 units of TNF-α/10 ml. Total RNAs were prepared as described under "Experimental Procedures," and the accumulation of mRNAs for C/EBP-α and C/EBP-β was analyzed by Northern blot analysis. *B*, cellular extracts were prepared from stable clones with pPI-997/CAT, and CAT activity was assayed as described under "Experimental Procedures."

cell (21). When stable clones with pPI-997/CAT were preincubated with 100 μM H8 for 30 min prior to the addition of 8-CPT cAMP and IBMX (3 h), H8 treatment completely blocked C/EBP- β mRNA accumulation and the cAMP activation of the PI promoter. Large increases in both mRNA and CAT activity were observed in cells that were not treated with H8 (data not shown). These observations support the hypothesis that cAMP not only induces C/EBP-β mRNA accumulation, but that C/EBP-β is the cAMP-responsive factor in the activation of PI promoter. As shown in Fig. 5*A*, a noticeable C/EBP-α mRNA accumulation by cAMP does not occur until almost 24 h later.

The effect of TNF-α further supports our thesis that C/EBP-β is responsible for cAMP activation of the PI promoter. When confluent 30A5 cells were treated with TNF-α together with 8-CPT cAMP and IBMX for 2 days, TNF almost completely repressed the induction of mRNAs for C/EBP-α, while C/EBP-β induction was not affected at all, as shown by Northern blot analysis (Fig. 6*A*). However, as shown in Fig. 6*B*, the same concentration of TNF-α had no effect on the induction of CAT activity, in spite of complete suppression of C/EBP-α induction. Therefore, cAMP can activate the PI promoter in 30A5 cells through the transcriptional activation of the C/EBP-β gene without cAMP activation of the C/EBP-α gene.

C/EBP-β Activation of PI—In order to establish further that C/EBP-β itself can activate PI expression, the effects of C/EBP-β expression on PI activation were assessed by co-transfection of expression vectors of C/EBP-β and pPI-997/CAT into 30A5 cells (Fig. 7). The extent of activation of CAT gene ex-

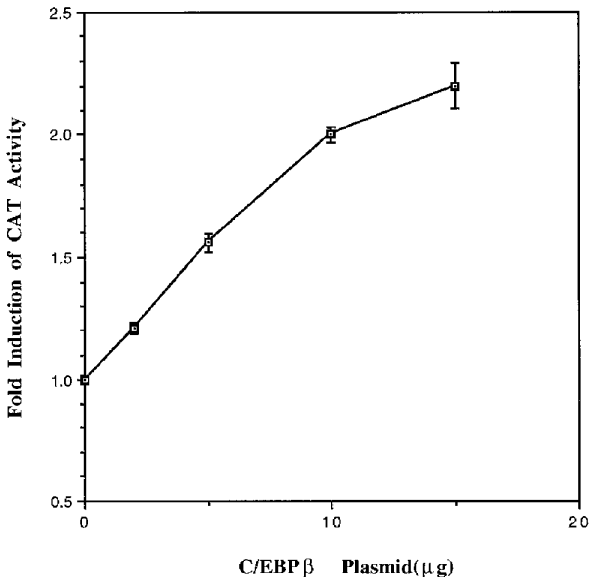

FIG. 7. **Transactivation of PI-CAT by C/EBP-β.** Five μg of pPI-997/CAT was co-transfected with increasing amounts of MSV-C/EBP-β as indicated. The total amount of DNA was adjusted to 15 μg in each case using pMSV. CAT activities were measured after 48 h as described under "Experimental Procedures." Each value represents the mean ± S.E. of three experiments.

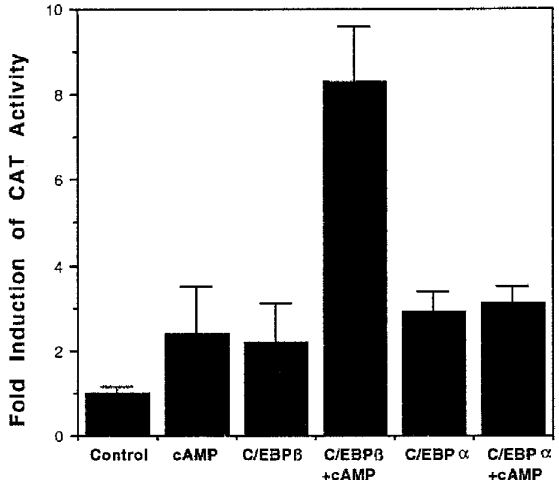

FIG. 8. **Effect of transient transfection with C/EBP and cAMP on PI expression.** 30A5 preadipocytes were co-transfected with 15 μg of pMSV/EBP-α or pMSV/EBP-β, and the cells were maintained for 48 h until 2 h before the CAT assay when cAMP was added to some of the samples, as follows: *column 1*, cells were transfected with 15 μg of PMSV vector; *column 2*, control cells were treated as described for *column 1*, except for a 2-h treatment with cAMP; *column 3*, pMSV/EBP-β-transfected cells; *column 4*, pMSV/EBP-β-transfected cells were treated with cAMP; *column 5*, cells were transfected with pMSV/EBP-α; *column 6*, pMSV-EBP-β-transfected cells from *column 5* were then treated with cAMP. The values represent the mean ± S.E. of 6 experiments.

pression is dependent on the amount of C/EBP-β plasmid used. The same qualitative effects of C/EBP-β expression were also observed in 30A5 cells stably transfected with pPI-997/CAT (data not shown).

One of our most interesting observations is that the activation of PI expression by overexpressed C/EBP-β was not fully achieved unless the overexpressed C/EBP-β was exposed to cAMP (Fig. 8). As shown in Fig. 8, cAMP treatment of 30A5 cells for 2 h increased CAT activity about 2-fold. The same levels of CAT activities were observed when pPI-997/CAT plasmids were co-transfected with either of the plasmids containing

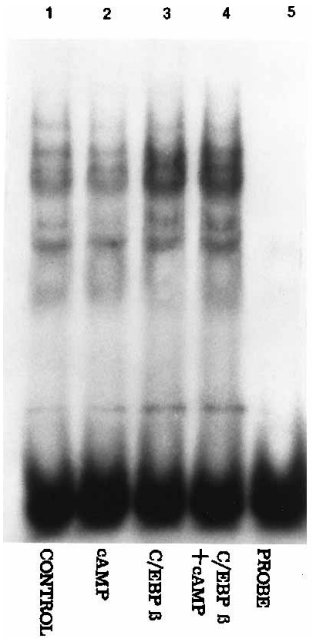

FIG. 9. **Effect of transient transfection of C/EBP-β and cAMP treatment on C/EBP-β binding activity.** The DNA-mobility shift assay was performed as described under "Experimental Procedures" using nuclear extract from cells that had been transfected with pMSV/EBP-β for 48 h before the treatments indicated. *Lane 1*, control; *lane 2*, treated with 8-CPT cAMP (100 μM, 30 min); *lane 3*, C/EBP-β transient-transfected (48 h); *lane 4*, C/EBP-β transient-transfected cells treated with 8-CPT cAMP (100 μM) for 30 min before nuclear extract was prepared; *lane 5*, free probe.

C/EBP-β or C/EBP-α genes. On the other hand, cAMP treatment of 30A5 cells that were co-transfected with both plasmids increased CAT activity between 8- and 10-fold. However, there was no effect of cAMP on CAT expression in cells with the C/EBP-α gene. This suggests that, with respect to the activation of CAT gene expression, cAMP acts on C/EBP-β, and not in a manner independent of C/EBP-β.

In order to examine whether or not cAMP treatment of C/EBP-β expressing cells affects the binding efficiency of C/EBP-β, nuclear extracts were prepared from cells that had been treated with or without cAMP and DNA-band shift analysis was performed (Fig. 9). There were no significant differences between the binding activities of nuclear extracts from cell preparations that had been treated with cAMP and cells that were not treated (Fig. 9).

Furthermore, the amounts of nuclear C/EBP-β (Fig. 10, *panel A*) are not affected by short term cAMP treatment, *i.e.* up to 2 h, in the control cells (Fig. 10, *lane 1 versus 2*), or in cells transfected with the C/EBP-β gene (Fig. 10, *lane 3 versus 4*). However, the short term cAMP treatment did induce phosphorylation of C/EBP-β (Fig. 10*A*, *panel B*).

As shown in Fig. 10*B*, the antibodies against phosphoserine are specific to phosphorylated C/EBP-β (Fig. 11, *lane 1 versus 2*), and thus discriminate between phosphorylated C/EBP-β and nonphosphorylated C/EBP-β. The total amount of C/EBP-β did not change as a result of phosphatase treatment (*lanes 3* and *4*).

Finally, in order to clearly establish that the phosphorylation of C/EBP-β does not affect its binding ability, nuclear extract from the cAMP-treated cells was treated with or without intestinal alkaline phosphatase-Sepharose, and the effect of phosphatase on the status of phosphorylation and DNA binding activity was examined. As shown in Fig. 11*A*, the binding activity of the phosphatase treated nuclear extract was not affected, while C/EBP-β was almost completely dephosphoryl-

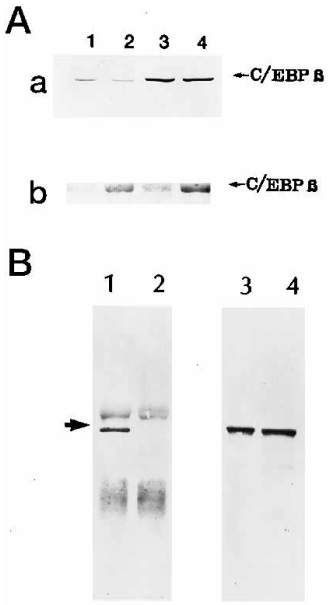

FIG. 10. **Effect of cAMP on C/EBP-β phosphorylation.** *A*, Western blot analysis for the amounts of C/EBP-β (*panel A*) and the extent of phosphorylation of C/EBP-β (*panel B*) were carried out as described under "Experimental Procedures." cAMP treatments were carried out for 30 min (*lanes 2* and *4*), and C/EBP-β expressions were for 48 h (*lanes 3* and *4*) as described under "Experimental Procedures." *Lane 1*, control; *lane 2*, control + cAMP; *lane 3*, C/EBP-β-transfected; *lane 4*, C/EBP-β-transfected and cAMP-treated. *B*, in order to examine the specificity of antibodies against phosphoserine, the effect of dephosphorylation of the phosphorylated C/EBP-β was examined. Nuclear extracts (80 μg) from cells expressing C/EBP-β that had been treated with cAMP were incubated with and without intestinal alkaline phosphatase (20 units) for 30 min. The reactivity of the samples to the antibodies was examined as described under "Experimental Procedures": *lanes 1* and *3*, control nuclear extracts; *lanes 2* and *4*, phosphatase-treated samples. *Lanes 1* and *2* were probed with anti-phosphoserine and *lanes 3* and *4* with anti-C/EBP-β. Each lane contained 40 μg of nuclear proteins.

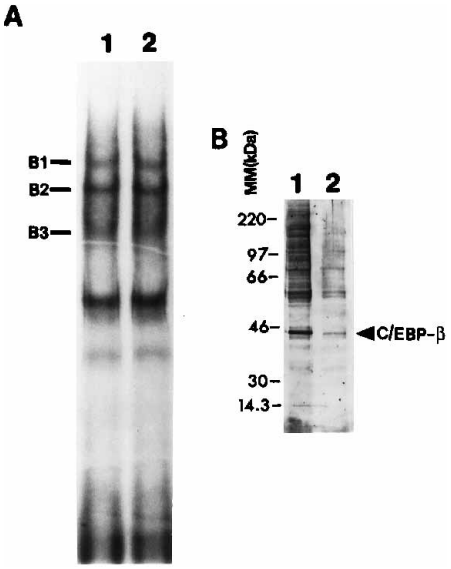

FIG. 11. **Phosphorylated status of C/EBP-β and its DNA binding ability.** Nuclear extracts were prepared from cells expressing C/EBP-β and were treated with cAMP as described in Fig. 10. Nuclear protein preparations (10 μg/sample) were then incubated with intestinal alkaline phosphatase-agarose conjugate (20 units of phosphatase) (*lane 2*) or with an equivalent amount of agarose (*lane 1*) for 30 min. Following these treatments, agarose was removed by centrifugation for 2 min at 14,000 × *g*. Supernatants were used for DNA-binding assay (*A*) and for Western analysis using anti-phosphoserine (*B*) as described under "Experimental Procedures."

ated, as shown in Fig. 11*B*.

These observations support the thesis that phosphorylation of C/EBP-β by cAMP-dependent protein kinase causes transcriptional activation as in the case of Ca^{2+}-dependent phosphorylation of C/EBP-β (23), rather than a covalent modification leading to facilitated translocation from the cytosol to the nucleus and thus DNA binding (22).

DISCUSSION

Previously, we reported that in 30A5 cell cultures both differentiation and activation of PI of the ACC gene require a short period of cAMP treatment before the cells are exposed to insulin (3, 4). While analyzing the role(s) played by cAMP in making the cells competent to respond to insulin, we observed that PI of the ACC gene was in a state of repression by virtue of the presence of a negative *cis*-element, and that C/EBP-α binding to the CCAAT box led to the activation of PI expression (4). We also presented evidence that the repressed state of PI involved interaction between the negative *cis*-element and the CCAAT box. Deletion of the CCAAT box (GCAAT sequence in PI) from PI, or masking the CCAAT box with C/EBP-α, resulted in the activation of PI. These results suggested that the interaction between the negative *cis*-element and the CCAAT box could control PI expression in either a negative or positive direction depending on the presence or absence of C/EBP-α. C/EBP is a family of proteins that belongs to a class of the basic region-leucine zipper proteins (bZIP class). C/EBP-β is an isoform of C/EBP-α. These proteins are capable of forming a complex within or without the family, but the relationships among these proteins in controlling specific genes has been difficult to assess.

In this report, we have demonstrated that the expression of the C/EBP-β gene is activated by cAMP and the increased level of C/EBP-β and modification of the increased level of C/EBP-β may be responsible for the activation of PI when 30A5 cells are treated with cAMP. This conclusion is based on the observation that overexpression of C/EBP-β alone, without cAMP treatment, caused only minimal activation of PI. In the latter case, cAMP treatment presumably modified the preexisting C/EBP-β. Although cAMP treatment led to the accumulation of both mRNAs of C/EBP-α and C/EBP-β, activation of PI could occur when cAMP only stimulated C/EBP-β accumulation. Furthermore, when C/EBP-β accumulation was inhibited by the use of an inhibitor of protein kinase, H8, there was no PI activation, and thus the C/EBP-β accumulation can be causally related to PI activation. Although H8 can inhibit other protein kinases as well as cAMP-dependent protein kinase, other kinases would not contribute to the effects of cAMP observed in the experimental setting being used here, since they are not stimulated by cAMP (21). TNF-α, which inhibited the accumulation of C/EBP-α, had no effect on C/EBP-β accumulation, and this accumulation activated PI.

Finally, we have shown that C/EBP-β gene transfection alone can activate CAT gene expression in the presence of cAMP. Although we have shown that cAMP treatment of the cells induces C/EBP-β expression and C/EBP-β phosphorylation, one could still argue that the site of cAMP action involves something other than C/EBP, and that such action of cAMP together with cAMP action on C/EBP-β leads to the activation of the promoter. If that were the case, the full activation of CAT gene expression would still require C/EBP-β. At this point, we cannot completely exclude such a possibility.

It had been reported that C/EBP-β was phosphorylated by Ca^{2+}-calmodulin-dependent protein kinase II. The phosphorylated C/EBP-β transactivated the gene containing the Ca^{2+}-calmodulin-dependent protein kinase II-responsive element in a pituitary cell line (G/C) (23). The phosphorylation of the

serine at position 276 of C/EBP-β did not alter its binding affinity for the Ca^{2+}-calmodulin-dependent protein kinase II-responsive element or its ability to form a homodimer. On the other hand, it has been shown that when C/EBP-β was phosphorylated, translocation of the phosphorylated C/EBP-β into the nucleus was stimulated (22). The mechanism of cAMP activation of c-*fos* gene transcription seems to have a similar explanation, *i.e.* C/EBP-β is translocated to the nucleus, where it activates transcription by binding to the serum-responsive element of the c-*fos* promoter. NF-IL6, a homolog of C/EBP-β, is phosphorylated by mitogen-activated protein kinase at threonine 235, which is essential for its transcriptional activity, and it has been suggested that differential phosphorylation at different sites may play a role under various physiological conditions (24). In this report, we established that cAMP induced C/EBP-β expression in the process of PI activation in 30A5 preadipocytes. Lack of C/EBP-β mRNA induction by cAMP in the presence of H8 (data not shown) and in the 30A5 cells expressing mutant regulatory subunit of cAMP-dependent protein kinase (data not shown) further support the thesis that cAMP induces C/EBP-β gene expression. Our present experimental results, however, contribute further to the controversial subject of how cAMP affects C/EBP-β action. Our results in this regard support the view that in the 30A5 system cAMP-mediated phosphorylation of C/EBP-β activates transcription, and not binding of C/EBP-β to DNA or translocation from the cytosol to the nucleus.

The C/EBP binding site appears to mediate cAMP control of certain promoters (25). For example, it has been reported that C/EBP-β activation of the phosphoenolpyruvate carboxylase gene involves an interaction between CRE and C/EBP-β (26). Transcription of the phosphoenolpyruvate carboxylase gene is rapidly stimulated by cAMP as in the case of the c-*fos* gene (27, 28). It appears that C/EBPs are capable of facilitating communication between enhancers and promoters based on their ability to interact with other factors, *e.g.* CRE (26). Since PI does not contain CRE, it is unlikely that this is how cAMP activates PI. Also, we did not detect any CRE-binding protein in the C/EBP-β containing complex. Whether or not the occurrence of the two C/EBP-β complexes is due to two forms of C/EBP-β (22) is not clear at this time.

In our present studies, using chimeric gene constructs, we demonstrated that an increased level of C/EBP-β is a factor in the activation of PI of ACC in response to cAMP. It would be simplistic to suggest that the observed effect of C/EBP-β on PI is the only cause of the activation of the endogenous gene. The nuclear extract from cAMP-treated cells showed increased amounts of both C/EBP-α and C/EBP-β. It is particularly interesting to note that there are two shifted bands of the DNA probe containing C/EBP-β (Fig. 3). How these two complexes are formed is not known, but these observations suggest the occurrence of heterodimeric protein complexes between C/EBP-β and other proteins (18). Even such putative heterodimeric protein complexes appear to be independent of cAMP treatment in this case. The expression of genes involved in differentiation and development of higher eukaryotes is presumably regulated by coordinated interaction of various *cis*-acting DNA regulatory sequences that interact with specific *trans*-acting factors (29). How the binding of a regulatory protein at a distant *cis*-element affects the binding and activity of RNA polymerase is still a subject of intensive investigation and speculation (30–32).

Finally, we also presented evidence that cAMP activates C/EBP-α gene expression as well. Such an action of cAMP had been suggested previously under unpublished results (25).

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