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 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)† at concentrations 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 CAAAT 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-phospho-kinase (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, dideoxynucleotides triphosphates, and calf intestinal alkaline phosphatase were from Promega. The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; ACC, acetyl-CoA carboxylase; C/EBP, CAAT enhancer-binding protein; C/EBP-α, C/EBP-β, CREB, CREB. The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; ACC, acetyl-CoA carboxylase; C/EBP, CAAT enhancer-binding protein; C/EBP-α, C/EBP-β, CREB, CREB.
kaline phosphatase were from Boehringer Mannheim; deoxynucleoside triphosphates and T4 DNA ligase were from Pharmacia Biotech Inc.; [32P]dCTP (3,000 Ci/mmol) and [α-32P]ATP (1000 Ci/mmol) were from Amersham Corp.; [γ-32P]ATP (6,000 Ci/mmol) was from Du Pont NEN; RNAaz™ was from Biotecx. Oligonucleotides were synthesized by 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. D. Lane. Antibodies against 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 Xbal site (7). This construct is designated pH-CAT (Fig. 1). 5'-Deletion mutants of the PI promoter from pFI-CAT0 were generated by using exonuclease III and S1 nucleases (8). Clones with different degrees of deletion were selected, and the exact sites of deletion were determined by dye deoxy nucleotide sequencing analysis (8). The two deletion mutants (pFI-CAT2 and pFI-CAT5) are shown in Fig. 1. For pFI-979/CAT, a 979-bp fragment flanking the 5' end of the ACC gene (−1088 to −108) was inserted upstream of the CAT gene of pUC-CAT3. pFI-D/CAT was constructed by restriction enzyme digestion of a 979-bp fragment (−1088 to −102) of PI promoter and ligation of the digested fragments in front of the CAT gene at the Xbal site of pUC-CAT3. pFI-TATA was constructed by insertion of a synthesized DNA fragment representing the fragment from −34 to −12 bp adjacent to the TATA box of the CAT gene of pUC-CAT3. To create a 5-base deletion mutant, pFI-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'-CTGACCTTCTATTGGAATCTTGCCTTTCG-3'. 209 indicates the position of the 5-base deletion (−5GCAAT−). The mutated 997-bp fragment was then inserted in front of the chloramphenicol acetyltransferase (CAT) gene of pUC-CAT3. The pFI-CAT22 construct was described previously (10).

DNA-mediated Cell Transfection and CAT Assay—Mouse 30A5 adipocytes were cultured in 100-mm Petri dishes to about 80% confluence in Eagle’s basal medium supplemented with 10% donorcalfserum7.0, 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 (106 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 m M Na2HPO4, 1 m M EDTA, 5% SDS, and 0.5% bovine serum albumin at 70 °C for 1 h and then in 40 m M NaHPO4, 1 m M EDTA, and 1% SDS at 70 °C for 30 min. Finally, the filters were rinsed in 250 ml of 0.5 m NaHPO4; pH 7.0, at room temperature and air-dried before exposure to Kodak XAR film. 1060 bp of the 5'-end region of pC/EBP-α (11) was used with pMSV-CAT for detection of C/EBP-α mRNA. The 1500 bp of total RNA was electrophoresed on formaldehyde/agarose gels as described (14). RNA was then transferred onto nylon membranes and hybridized with 32P-labeled probes (15). Filters were prehybridized in 0.5 M NaHPO4, 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 (106 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 m M NaHPO4, 1 m M EDTA, 5% SDS, and 0.5% bovine serum albumin at 70 °C for 1 h and then in 40 m M NaHPO4, 1 m M EDTA, and 1% SDS at 70 °C for 30 min. Finally, the filters were rinsed in 250 ml of 0.5 m NaHPO4; pH 7.0, at room temperature and air-dried before exposure to Kodak XAR film. 1060 bp of the 5'-end region of pC/EBP-α (11) was used with pMSV-CAT for detection of C/EBP-α mRNA. 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; ΔL198, 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 manufacturer.

Determination of C/EBP-β Phosphorylation—To 250 μl of nuclear extract in buffer C (1 μg/ml), 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
CAMP Activation of C/EBP-β Gene Expression and ACC Promoter I

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.

**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 kb upstream region 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 expression 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-1CAT0, pPI-1CAT2, pPI-1CAT5; and 40–80-fold in the case of pPI-997/CAT and pPI-1/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 pPI-1-CAT22. This plasmid contains a small piece of the PI 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 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-β (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 PI (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/
cAMP Activation of C/EBP-β Gene Expression and ACC Promoter I

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. These plasmids were digested with BamHI and labeled using [γ-32P]ATP and T4 DNA kinase. One end-labeled fragment was prepared by digestion with NspI. 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 × 106 cpm of the 32P-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 the lanes 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 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. 5A), accumulation of the C/EBP-β mRNA occurred to a greater extent and had already reached a maximum by 3 h (Fig. 5A). 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. 5B, 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. 5B 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. 5B, lanes 1 and 2 versus 3 and 4). These observations indicate that cAMP induced C/EBP-β during a period when C/EBP 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.
cAMP Activation of C/EBP-β Gene Expression and ACC Promoter I

**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-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.

**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 IBMX (lane 2) or with (lane 3) 0.1 mM 8-CPT cAMP and 0.5 mM IBMX, with (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.”

and IBMX (Fig. 5C). 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. 5A). 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 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, 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. 5A, 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. 6A). However, as shown in Fig. 6B, 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-
expression 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

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, C/AMP 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-bend 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 cell preparations that had been transfected with C/EBP-β gene (Fig. 10, lane 3 versus 4). However, the short term cAMP treatment did induce phosphorylation of C/EBP-β (Fig. 10A, panel B).

As shown in Fig. 10B, 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. 11A, 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.”

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 have 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 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 Ca²⁺-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/EBP-βs 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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REFERENCES
1. Pape, M. E., and Kim, K.-H. (1988) Mol. Endocrinol. 2, 395–403
2. Pape, M. E., and Kim, K.-H. (1989) Mol. Cell. Biol. 9, 974–9828
3. Park, K., and Kim, K.-H. (1991) J. Biol. Chem. 266, 12269–12256
4. Tae, H.-J., Luo, X., and Kim, K.-H. (1994) J. Biol. Chem. 269, 10475–10484
5. Lopez-Casillas, F., and Kim, K.-H. (1989) J. Biol. Chem. 264, 7176–7184
6. Leyne, E., Gijka, H. B., and Vunakis, H. V. (1989) J. Immunol. Methods 124, 239–249
7. Luo, X., Park, K., Lopez-Casillas, F., and Kim, K.-H. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 4042–4046
8. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467
9. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492
10. Luo, X., and Kim, K.-H. (1990) Nucleic Acids Res. 18, 3249–3254
11. Gorman, C. M., Mofat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
12. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1476–1489
13. Dyman, W. S., and Tjian, R. (1983) Cell 35, 79–87
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
16. Makino, T., Yamaoka, S. Y., Drobes, B. L., Manke, S. L., and Taparovski, E. J. (1989) Oncogene 4, 473–481
17. Birkenmeier, E. H., Gwynn, B., Howard, S. J., Jery, J., Gordon, J. I., Landsdulz, W. H., and McKnight, S. L. (1989) Genes & Dev. 3, 1146–1156
18. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) Genes & Dev. 5, 1338–1532
19. Roehle, W. J., Vanderbark, G. R., and Hanson, R. W. (1988) J. Biol. Chem. 263, 9063–9068
20. Zhu, F., Andriseanu, O. M., Pot, D., and Dixon, J. E. (1989) J. Biol. Chem. 264, 6550–6556
21. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23, 5036–5041
22. Metz, R., and Ziff, E. (1991) Genes & Dev. 5, 1754–1766
23. Wegner, M., Cao, Z., and Rosenfeld, M. G. (1992) Science 256, 370–373
24. Nakajima, T., Kinoshita, S., Sagawa, T., Sasaki, K., Naruo, M., Kimishita, T., and Akira, S. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2207–2211
25. McKnight, S. L., Lane, D. L., and Gueckoh-Waehls. (1989) Genes & Dev. 3, 2021–2024
26. Park, E. A., Gunery, A. L., Nizielskie, S. E., Hakimi, P., Cao, Z., Moorman, A., and Hanson, R. W. (1993) J. Biol. Chem. 268, 613–619
27. Lamers, W. H., Hanson, R. W., and Mesner, H. M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5137–5141
28. Sasaki, K., Cripe, T. P., Ishii, K., Konieczny, S. F., and Granner, D. K. (1984) J. Biol. Chem. 259, 10475–10484
29. Yutzey, K. E., Kline, R. L., and Konieczny, S. F. (1989) J. Biol. Chem. 264, 2207–2211
30. Ptashne, M. (1986) Nature 224, 697–701
31. Maniatis, T., Goodbourn, S., and Fisher, J. A. (1987) Science 236, 1237–1245
32. Adhya, S., and Garges, S. (1990) J. Biol. Chem. 265, 10797–10800