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Upstream stimulatory factors regulate the C/EBPα gene during differentiation of 3T3-L1 preadipocytes

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Abstract

During adipocyte differentiation, CCAAT/enhancer-binding protein α (C/EBPα) functions as a pleiotropic transcriptional activator of numerous adipocyte genes. The promoter of the C/EBPα gene has an E-box upstream of C/EBP binding site. Deletion or mutation of the E-box decreases promoter activity, suggesting that the E-box participates in the regulation of C/EBPα expression. Protein binding to the E-box during the adipocyte differentiation is increased as indicated by EMSA and UV cross-linking. Purification of the E-box binding proteins from differentiated 3T3-L1 adipocytes, showed that USF and AP-4 are associated with the E-box. Supershift analysis showed that USF1 and USF2 bind to this element as heterodimers, whereas the addition of anti-AP-4 antibody enhanced the binding complex, suggesting that AP-4 negatively regulates the promoter activity. The expression of AP-4 is reciprocally regulated with USF-1 during adipocyte differentiation. These findings suggest that USF-1 and 2 play roles in C/EBPα expression, whereas the AP-4 represses it.

Keywords: USF; C/EBPα; E-box; Promoter; AP-4; DNA binding activity; Adipocyte differentiation

Differentiation of growth-arrested 3T3-L1 preadipocytes into adipocytes is induced by hormonal stimuli that trigger expression of numerous adipose-specific genes [1,2]. Since C/EBPα plays an essential role in this process [3,4], we focused on the identification of *cis*-elements and their cognate transcription factors that regulate the activity of the C/EBPα promoter [5,6]. DNase I footprinting assay of the proximal promoter of the C/EBPα gene with preadipocyte and adipocyte nuclear extracts revealed several apparent regulatory regions including C/EBP regulatory element [5], dual CUP (C/EBP undifferentiated protein) binding sites [7–10] and Sp1 binding site [11]. Extensive investigation demonstrated that C/EBPβ and C/EBPδ mediate activation driven by the C/EBPα gene promoter through binding to the C/EBP regulatory element [6,12]. Once expression of C/EBPα has occurred by this process, autoactivation of the gene by its own product maintains the level of expression of C/EBPα and thus, the terminally differentiated adipocyte phenotype [13]. Concurrently, the expression and binding of CUP and Sp1, repressors of the gene, are down-regulated further enhancing expression of the C/EBPα gene [6].

While the expression of C/EBPβ and C/EBPδ occurs rapidly (within 2 h) after induction of differentiation of growth-arrested preadipocytes, the expression of C/EBPα is delayed [12]. The expression of C/EBPα is delayed until mitotic clonal expansion (MCE) is initiated. Thus, upon the induction of differentiation preadipocytes remain growth-arrested for ~14 h and then synchronously re-enter the cell cycle and undergo several rounds of mitosis, i.e.,

Abbreviations: C/EBP, CCAAT enhancer binding protein; USF, upstream stimulatory factor; AP-4, activating protein-4; EMSA, electrophoretic mobility shift assay; Wt, wild type.

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MCE, a process required for subsequent differentiation [12,14]. We recently showed that during MCE dual phosphorylation of C/EBPβ occurs concomitant with the entry of S-phase and at which DNA binding activity is acquired [15]. These events account for the long lag period between expression of C/EBPβ, acquisition of DNA binding activity and transcriptional activation of the C/EBPα gene. During these studies the possibility was considered that other transacting factors might be needed to obtain the full activity of C/EBPα promoter.

In the present paper, we investigated the roles of the E-box and GC-box that lie upstream of the C/EBP regulatory element in the C/EBPα promoter. It was determined that USF1 and USF2 bind to the promoter and participate in the acquisition of full promoter activity.

Materials and methods

Cell culture, induction of differentiation, transfection. Induction of differentiation of post-confluent 3T3-L1 preadipocytes (on Day 0) was as described [16]. To examine C/EBPα promoter activity, transient transfections of 3T3-L1 preadipocytes were performed on Day 0 using Lipofectamine (Invitrogen), followed by the induction of differentiation. Six hours after transfection, media were removed and 4 h later differentiation was induced. Luciferase activity to assess promoter activity and the expression of endogenous C/EBPα were determined 1–3 days after induction.

Electrophoretic mobility shift assays (EMSA) and UV cross-linking. Nuclear extracts were prepared with NUN buffer as described [17]. Labeled probe (5′-CGGAC CACGT GTGTG CGGGG GCGAC AGC-3′) was incubated with 5 μg of nuclear extract in a reaction mixture containing 10 mM Hepes, pH 7.6, 1 mM EDTA, 7% glycerol, 1 μg of poly(dIdC), 100 mM NaCl and 1 mM DTT. For UV cross-linking the reaction mixture was exposed to UV light at 254 nm for the indicated time and DNA–protein complexes resolved on 12% SDS–PAGE gels.

Purification of E-box binding proteins. Nuclear extract was prepared from 100 dishes of 10-cm monolayers of 3T3-L1 adipocytes on Day 3. Diluted nuclear extract (containing ~30 mg of protein) was incubated with DEAE–Sepharose ion-exchange matrix (GE Healthcare) overnight at 4 °C and eluted with increasing NaCl concentration. To prepare an E-box affinity matrix, the sense strand of E-box oligonucleotide (Fig. 1B) was biotinylated and annealed with the antisense strand and 1 nmole of double stranded oligonucleotide was mixed with 1 ml of streptavidin–Agarose beads (Invitrogen). The fraction containing DNA-binding activity was eluted with 300 mM NaCl from DEAE ion-exchange chromatography and mixed with the affinity matrix in the presence of 30 μg of poly(dIdC). After overnight incubation, the matrix was packed into a 1-ml column and washed with 5-column volumes of buffer without salt (10 mM Hepes, pH 7.6, 1 mM DTT, 0.1% NP-40, 1 mM EDTA, 7% glycerol, protease inhibitor cocktail). Elution was carried out with stepwise increases in NaCl concentration. The E-box binding activity in the eluted fractions was monitored by EMSA and the active fractions pooled and concentrated by TCA precipitation. After solubilizing in sample buffer and SDS–PAGE on 4–10% gradient gels, proteins were visualized by Coomassie brilliant blue staining after which the bands were excised from the gel and subjected to mass analysis as described [9].

Western blot analysis. Cell extracts were prepared from 3T3-L1 preadipocytes or adipocytes at indicated time points in the differentiation program. Cell monolayers were washed once with cold phosphate-buffered saline (pH 7.4) and then scraped into lysis buffer containing 1% SDS and 60 mM Tris–Cl, pH 6.8. Lysates were heated at 100 °C for 10 min, clarified by centrifugation, and then subjected to immunoblotting. Specific proteins were visualized with anti-AP-4, USF1, USF2 antibodies (Santa Cruz) or anti-C/EBPα, C/EBPβ antibodies [12].

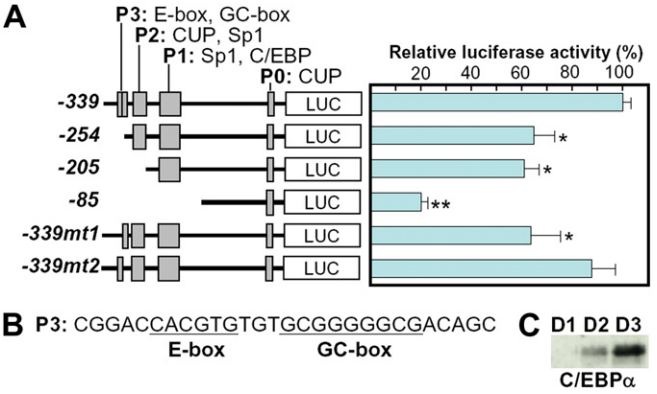

Fig. 1. Activity driven by the C/EBPα promoter is dependent on the E-box as well as the C/EBP regulatory element. (A) Schematic depiction of C/EBPα promoter constructs and their respective luciferase activities when expressed in 3T3-L1 cells. P0, P1, P2 and P3 indicate the location of known regulatory elements, and "mt" represents mutation of the E-box or GC-box. 3T3-L1 cells were transfected with the luciferase constructs by Lipofectamine, and then differentiated in the presence of IBMX, Dex and insulin. Three days after induction of differentiation cells were harvested and luciferase activity was determined. $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. wild type value. (B) Nucleotide sequence of the P3 region. E-box and GC-box are underlined. (C) Western blot analysis of C/EBPα in transfected cells. Cells were transfected as above, harvested at Day 1, 2 or 3 and the expression of C/EBPα assessed by SDS–PAGE and immunoblotting with anti-C/EBPα antibody.

Results and discussion

An E-box is required for maximal activity mediated by the C/EBPα promoter

Previous DNase I footprinting analysis of the C/EBPα promoter revealed a protected region between nucleotides –275 and –259 [5]. This region contains a canonical E-box (CACGTG) and a GC-box (GCGGGGGCG). To determine whether this region is important for C/EBPα promoter activity, deletion constructs of the promoter region fused to the luciferase gene were examined in 3T3-L1 cells (Fig. 1A). Since C/EBPα is expressed ~36 h after the induction of differentiation [18], cells were transfected at post-confluence, then treated with differentiation inducers and harvested 1–3 days after induction. As this is the period during which endogenous C/EBPα is expressed, luciferase assays were conducted during this time window (Fig. 1C). As shown in Fig. 1A deletion of P3, the region containing the E-box/GC-box, caused substantial reduction of luciferase activity. Eliminating the C/EBP regulatory element (in P1), the promoter activity was diminished to basal level, indicating that C/EBPβ and C/EBPδ are the main regulators of C/EBPα expression. However, the C/EBP regulatory element possessed only ~60% of the promoter activity without the E-box, as demonstrated by the mutation or deletion of E-box. The mutation of the GC-box (Fig. 1B) that had been suggested to bind Zif268 [5], did not affect the promoter activity. Together these results indicate that the E-box is required for full activity