

## Sequential gene promoter interactions of C/EBP $\beta$ , C/EBP $\alpha$ , and PPAR $\gamma$ during adipogenesis

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### Abstract

Treatment of 3T3-L1 preadipocytes with differentiation inducers triggers a cascade in which C/EBP $\beta$  is rapidly expressed, followed by C/EBP $\alpha$  and PPAR $\gamma$ . C/EBP $\alpha$  and PPAR $\gamma$  then activate the expression of adipocyte genes that produce the differentiated phenotype. Circumstantial evidence indicates that C/EBP $\beta$  activates transcription of the C/EBP $\alpha$  and PPAR $\gamma$  genes, both of which possess C/EBP regulatory elements in their proximal promoters. Although C/EBP $\beta$  is expressed immediately upon induction of differentiation, acquisition of DNA binding activity is delayed for  $\sim 14$  h. Chromatin immunoprecipitation (ChIP) analysis conducted 24 h after induction revealed that C/EBP $\beta$  binds to C/EBP regulatory elements in the proximal promoters of the C/EBP $\alpha$  and PPAR $\gamma$  genes. After an additional delay ChIP analysis showed that C/EBP $\alpha$  binds to its own promoter and to the promoters of the PPAR $\gamma$  and 422/aP2 genes. These findings support the view that once expressed, C/EBP $\alpha$  is responsible for maintaining the expression of PPAR $\gamma$  and C/EBP $\alpha$ , as well as adipocyte proteins (e.g., 422/aP2) in the terminally differentiated state. Together these findings provide compelling evidence that C/EBP $\beta$ , C/EBP $\alpha$ , and PPAR $\gamma$  participate in a cascade during adipogenesis.

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The 3T3-L1 preadipocyte cell system has proven to be a faithful model with which to investigate the adipocyte differentiation program [1–4]. Using this system it has been determined that the C/EBP family of transcription factors, notably C/EBP $\beta$ , C/EBP $\alpha$ , and CHOP-10, as well as PPAR $\gamma$ , have important roles in the differentiation program [1–5]. Circumstantial evidence suggests that these factors function sequentially in a signaling cascade that gives rise to the adipocyte phenotype [2,4]. However, definitive proof is lacking. Early in the differentiation program growth-arrested preadipocytes undergo  $\sim 2$  rounds of cell division [1,2,6–8], referred to as mitotic clonal expansion (MCE). This process is required for progression through the terminal steps of the differentiation program [1,2,8–10] and mimics the hy-

perplasia associated with the increase of adipose tissue mass that accompanies obesity.

C/EBP $\beta$  is expressed immediately ( $\leq 2$  h) following the treatment of 3T3-L1 preadipocytes with differentiation inducers [7,11]. However, at this point in the program newly expressed C/EBP $\beta$  lacks DNA binding activity and thus, cannot yet activate gene transcription. About 14 h later, however, C/EBP $\beta$  undergoes phosphorylation [7], loses its association with CHOP-10 [5], gains DNA binding activity, and binds to centromeres [7]. Concurrently, the preadipocytes synchronously re-enter the cell cycle and undergo  $\sim 2$  rounds of MCE. The coincidence of these events suggests that C/EBP $\beta$  plays a role in this process in MCE. That this is the case was verified recently by experiments in which C/EBP $\beta$  was found to be required for MCE [12,13].

Considerable evidence indicates that C/EBP $\beta$  acts as a transcriptional activator for both C/EBP $\alpha$  [14,15] and PPAR $\gamma$  genes [16,17]. This belief is supported by the fact

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that the proximal promoters of both of these genes possess C/EBP regulatory elements that are essential for transactivation of C/EBP $\alpha$  and PPAR $\gamma$  promoter-reporter transgenes by C/EBP $\beta$  in 3T3-L1 preadipocytes. Following their expression, C/EBP $\alpha$  and PPAR $\gamma$  appear to serve as pleiotropic transcriptional activators that coordinately induce expression of a large group of adipocyte genes including the 422/aP2, SCD1, Glut4, and *obese* genes, as well as others [15,18–20]. Together these actions lead to acquisition of adipocyte characteristics.

Proof that C/EBP $\beta$ , C/EBP $\alpha$ , and PPAR $\gamma$  interact with the appropriate gene promoters in intact cells is lacking. Here we report the sequential binding of C/EBP $\beta$ , C/EBP $\alpha$ , and PPAR $\gamma$  to their respective target gene regulatory elements by ex vivo cross-linking with chromatin immunoprecipitation analysis. Our findings provide compelling evidence for a transcriptional activation cascade initiated by the induction of differentiation of 3T3-L1 preadipocytes.

## Materials and methods

**Cell culture and induction of differentiation.** 3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (vol/vol) calf serum as described [21]. To induce differentiation, 2-day postconfluent preadipocytes (designated day 0) were fed DMEM containing 10% (vol/vol) fetal bovine serum (FBS), 1  $\mu$ g/ml insulin (I), 1  $\mu$ M dexamethasone (D), and 0.5 mM 3-isobutyl-1-methylxanthine (M) until day 2. Cells were then fed DMEM supplemented with 10% FBS and 1  $\mu$ g/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Adipocyte gene expression and acquisition of the adipocyte phenotype begins on day 3 and is maximal by day 8.

**Electrophoretic mobility shift analysis.** Nuclei were isolated and nuclear extracts were prepared using 1  $\times$  NUN buffer [22] containing 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM Hepes (pH 7.9), and 1 mM DTT. Protein concentration was determined by the Bradford method (Bio-Rad). EMSA was performed essentially as described [23,24] with the following modifications. Reaction mixtures containing ~0.25 ng of the appropriate <sup>32</sup>P-labeled oligonucleotide probe, 2  $\mu$ g poly[d(I-C)], and 10  $\mu$ g of nuclear extract protein in 30  $\mu$ l buffer (10 mM Hepes, 0.1 mM EDTA, 5% glycerol, 100 mM NaCl, 0.3 M urea, and 0.3% NP-40) were incubated on ice for 15 min, at room temperature for 15 min. Proteins and labeled oligonucleotide probes were separated electrophoretically on 5% polyacrylamide gels 0.5  $\times$  TBE [44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA (pH 8.3)]. For supershift experiments, 1  $\mu$ l of antiserum (~5  $\mu$ g of IgG protein) was added to the reaction mixture before the addition of labeled probe. The labeled oligonucleotide probes included double-stranded oligonucleotides corresponding to: (1) the C/EBP regulatory element in the C/EBP $\alpha$  promoter, (–191) GCGTTGCGCCACGATCTCTC (–172) [15]; and (2) PPAR response element (ARE7) [25] in 422/aP2 promoter, 5'-TTTGCTTCTTACTGGATCAGAGTTCAC-3'.

**Immunoblotting.** To follow changes in the levels of C/EBP $\beta$ , C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 proteins after induction of differentiation, 2-day postconfluent (day 0) 3T3-L1 preadipocytes were treated with MDI in 10% FBS as described as above. At various times thereafter, cell monolayers (6-cm dishes) were washed once with cold phosphate-buffered saline (PBS) (pH 7.4) and then scraped into lysis buffer containing 1% SDS and 60 mM Tris-HCl (pH 6.8). Lysates were heated at 100 °C for 10 min, clarified by centrifugation, and then equal amounts of protein were subjected to SDS-PAGE and immunoblotted with antibodies to C/

EBP $\beta$ , C/EBP $\alpha$ , PPAR $\gamma$  or 422/aP2 (C/EBP $\beta$ , C/EBP $\alpha$ , and 422/aP2 antibodies were prepared in this laboratory [7]; PPAR $\gamma$  antibody was provided by Dr. Mitchell Lazar (University of Pennsylvania, Philadelphia).

**Chromatin immunoprecipitation analysis.** Chromatin immunoprecipitation (ChIP) analysis was performed essentially as described in the protocol of ChIP assay kit (Upstate Biotechnology). Briefly, 3T3-L1 preadipocytes were maintained and induced to differentiate as above. At various times thereafter, cells were cross-linked with 1% formaldehyde in PBS buffer. The cross-linked cells were harvested, lysed in SDS lysis buffer, and sonicated. After incubation with salmon sperm DNA-protein A at 4 °C overnight, the DNA-protein complexes in the supernatant were immunoprecipitated with antibodies against C/EBP $\beta$ , or C/EBP $\alpha$  or PPAR $\gamma$  and the immune complexes recovered by adding protein A-agarose. After washing the DNA-protein complexes with saline, DNA was extracted with phenol/chloroform, precipitated, redissolved, and used as templates for PCR with the following primers. The primers used to amplify the DNA fragments containing were: (A) C/EBP binding site in C/EBP $\alpha$  promoter: 1. TCCCTAGTGTGGCTGGAAG and 2. CAGTAGGATGGTGCCTGCTG; (B) C/EBP binding site in PPAR $\gamma$  promoter: 1. TTCAGATGTGTGATTAGGAG and 2. AGACTTGGTACATTACAAGG; (C) C/EBP binding site in 422/aP2 promoter: 1. CCTCCACAATGAGGCAAATC and 2. CTGAAGTCCAGATAGCTC; and (D) PPAR $\gamma$  binding site in the 422/aP2 promoter: 1. CAAGCATGCGACAAAGGCA and 2. TAGAAGTCGCTCAGGCCACA.

## Results

### Sequential expression of C/EBP $\beta$ , C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 during adipocyte differentiation

Treatment of growth-arrested 3T3-L1 preadipocytes with differentiation inducers triggers the expression of C/EBP $\beta$  followed by the expression of C/EBP $\alpha$  and PPAR $\gamma$  (Fig. 1 and [7,11]). Previous investigations established that expression of C/EBP $\beta$  is rapid occurring within 2–3 h [7,11]. Expression of C/EBP $\beta$  remains

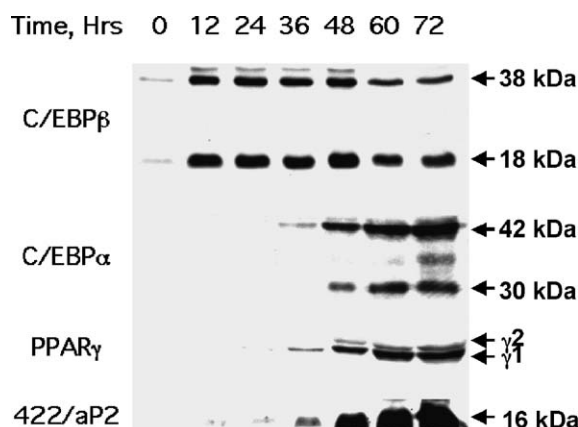


Fig. 1. Expression of C/EBP $\beta$ , C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 during differentiation of 3T3-L1 preadipocytes. Day 0 postconfluent 3T3-L1 preadipocytes were induced to differentiate using the standard differentiation protocol. At different times after induction of differentiation, cell lysates were subjected to SDS-PAGE and Western blotted with antibodies directed against C/EBP $\beta$ , C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2. It should be noted that C/EBP $\beta$  and C/EBP $\alpha$  each have two isoforms translated from the same mRNA.

constant over the next 36 h and then begins to decline (Fig. 1). Both the 38 and 18 kDa isoforms of C/EBP $\beta$ , which are transcription products of the same mRNA [26], exhibit the similar expression kinetics. The expression of C/EBP $\alpha$  and PPAR $\gamma$ , apparent target genes of C/EBP $\beta$ , is delayed for  $\sim$ 36 h and occurs as the expression of C/EBP $\beta$  declines (Fig. 1). The long delay of C/EBP $\alpha$  and PPAR $\gamma$  expression corresponds to a long delay in the acquisition of DNA binding activity by C/EBP $\beta$  [7]. The acquisition of DNA binding activity by C/EBP $\beta$  occurs synchronously with entry of S phase of mitotic clonal expansion and the association of C/EBP $\beta$  with centromeric C/EBP regulatory elements [7]. As the preadipocytes exit mitotic clonal expansion, expression of the 422/aP2 gene, a representative target gene of C/EBP $\alpha$  and PPAR $\gamma$ , is activated (Fig. 1) along with numerous other adipocyte genes [19,25,27].

#### *Sequential binding of C/EBP $\beta$ and C/EBP $\alpha$ to the promoters of C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 genes*

C/EBP $\beta$  was previously shown to activate expression of C/EBP $\alpha$  and PPAR $\gamma$  promoter-reporter genes through C/EBP regulatory elements in their proximal 5'-flanking regions [7,14,16,17]. To determine whether C/EBP $\beta$  and C/EBP $\alpha$  bind to the C/EBP regulatory elements in the promoters of these genes in intact cells, 3T3-L1 preadipocytes were induced to differentiate after which ChIP analyses was performed. ChIP assays were conducted using antibodies against C/EBP $\beta$  or C/EBP $\alpha$  and PCR primers corresponding to DNA sequences containing the C/EBP binding sites in the proximal promoters of the C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 genes. As shown in Fig. 2 C/EBP $\beta$  binds to these gene promoters as early as day 1 after which binding is maintained until at least day 3 following the induction of differentiation. This timing is consistent with the time at which C/EBP $\beta$  is first expressed (Fig. 1). After a lag of  $\sim$ 2 days, i.e., until day 3, C/EBP $\alpha$  is expressed (Fig. 1) at

which point it immediately binds to the C/EBP regulatory elements in the C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 gene promoters (Fig. 2). At day 2, only C/EBP $\beta$  exhibits significant binding to these gene promoters, presumably since expression of C/EBP $\beta$  is still dominant at this point in the differentiation program. However, by day 3 both C/EBP $\beta$  and C/EBP $\alpha$  exhibit binding, in part because expression of C/EBP $\alpha$  has reached its maximum and expression of C/EBP $\beta$  persists.

EMSA experiments were also performed to verify binding by C/EBP $\beta$  and C/EBP $\alpha$ . Thus, nuclear extracts were prepared at each of the time points described above and subjected to EMSA using a labeled C/EBP-binding site probe corresponding to that in the proximal promoter of the C/EBP $\alpha$  gene. As shown in Fig. 3 binding of C/EBP $\beta$  to the C/EBP regulatory element (as evidenced by the supershift with C/EBP $\beta$  antibody) begins on day 1 and persists at a constant level over the entire 3-day period. However, the binding of C/EBP $\alpha$  to the C/EBP element in the promoter of its own gene begins weakly on day 2 and becoming strong on day 3 based on supershifting with C/EBP $\alpha$ -specific antibody. These results are consistent with the ChIP analyses shown in Fig. 2.

#### *Binding of PPAR $\gamma$ to the promoter of the 422/aP2 gene*

It was previously shown that PPAR $\gamma$  can activate expression of the promoter of the 422/aP2 gene through a specific regulatory element, i.e., ARE7, during adipogenesis [25,27]. ChIP analysis verifies that PPAR $\gamma$  binds to this regulatory element in intact 3T3-L1 preadipocytes, binding activity becoming evident on day 2 and continuing through day 3 (Fig. 3A) concomitant with the expression of PPAR $\gamma$  (Fig. 1). EMSA verified the binding of PPAR $\gamma$  to this regulatory element in the 422/aP2 gene promoter with the same kinetics. Specificity is indicated by the supershift of the PPAR $\gamma$ -oligonucleotide complex with specific PPAR $\gamma$  antibody, but not by preimmune antibody (Fig. 3B).

Antibody	2% Input				Preim				C/EBP $\beta$				C/EBP $\alpha$			
Time, Day	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
C/EBP $\alpha$																
PPAR $\gamma$																
422/aP2																

Fig. 2. Sequential binding of C/EBP $\beta$  and C/EBP $\alpha$  to the C/EBP regulatory elements in the promoters of the C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 genes in intact 3T3-L1 cells determined by ChIP analysis. At different times after induction of differentiation, about  $1 \times 10^7$  cells were cross-linked with formaldehyde, the DNA fragmented and the chromatin-associated DNA immunoprecipitated with preimmune serum (PreIm) or antibodies against C/EBP $\beta$  or C/EBP $\alpha$ . PCR amplification of the DNA fragments was conducted with specific primers flanking the C/EBP regulatory element in each of the C/EBP $\alpha$ , PPAR $\gamma$ , and 422/aP2 gene promoters. In all cases, only the expected  $\sim$ 200 bp PCR product was observed.

## Discussion

To initiate differentiation of 3T3-L1 preadipocytes, the cells must be growth-arrested at the time of treatment with differentiation inducers. Upon induction, the cells reenter the cell cycle synchronously traversing the G<sub>1</sub>-S checkpoint at  $\sim$ 14 h [7]. After completing 2 rounds of division, i.e., mitotic clonal expansion, the cells progress to the terminal differentiation phase during which adipocyte genes are expressed producing the differentiated phenotype. The timing of these events is critical. Expression of C/EBP $\alpha$  must not occur prior to mitotic clonal expansion, since C/EBP $\alpha$  is anti-mitotic and its premature expression would block mitotic clonal

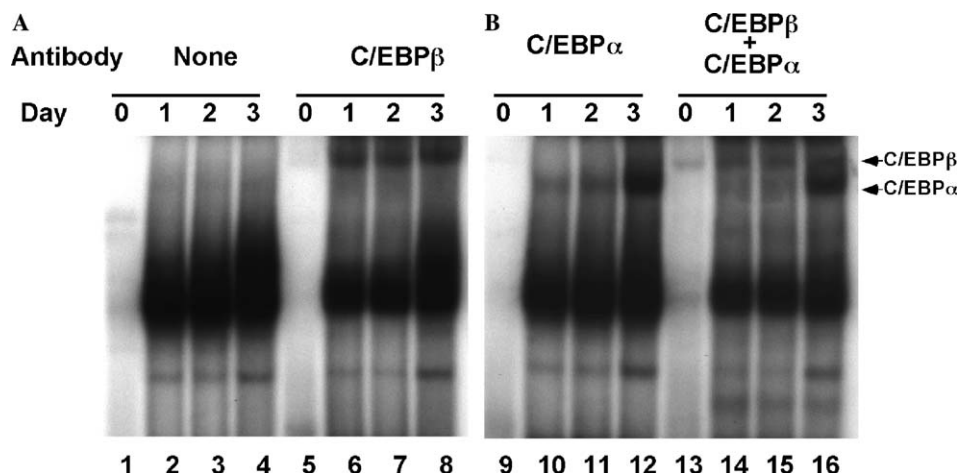


Fig. 3. Sequential binding of C/EBPβ and C/EBPα to the C/EBP regulatory elements in the promoters of the C/EBPα, PPARγ, and 422/aP2 genes determined by EMSA. Day 0 postconfluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes using the standard differentiation protocol. Each day after induction nuclear extracts were prepared and subjected to EMSA with labeled oligonucleotide corresponding to the C/EBP binding site in C/EBPα gene promoter as probe (lanes 1–4). Supershift experiments were performed with antibodies directed against: C/EBPβ (lanes 5–8), C/EBPα (lanes 9–12), and C/EBPβ plus C/EBPα (lanes 13–16).

expansion, which is essential for subsequent steps in the differentiation program [9,10,12].

It has been established that expression of C/EBPβ occurs early in the program and is a prerequisite for mitotic clonal expansion [12,13]. Moreover, C/EBPβ is thought to transcriptionally activate the expression of C/EBPα and PPARγ, transcription factors that activate expression of adipocyte genes that give rise to the adipocyte phenotype. While the kinetics of expression of members of the C/EBP family and of PPARγ has been reported [7,11], it has not been proven that these transcription factors actually interact with the appropriate gene promoters in the correct order in intact preadipocytes. The present study tracked the expression and acquisition of DNA binding activity both by EMSA in vitro and by ChIP analysis in intact preadipocytes. Based on these analyses, the following conclusions can be made.

As judged by immunoblotting, C/EBPβ is rapidly ( $\leq 24$  h) expressed, followed ( $\sim 36$ – $48$  h after induction) by C/EBPα and PPARγ and a representative adipocyte gene, 422/aP2 (Fig. 1). By 24 h after the induction of differentiation, C/EBPβ has acquired DNA binding activity as judged by EMSA (Fig. 3) and interacts with/binds to C/EBP regulatory elements in the promoters of the C/EBPα and PPARγ and 422/aP2 genes in intact preadipocytes as judged by ChIP analysis (Fig. 2). By day 2/3 following induction of differentiation, both C/EBPα and PPARγ have acquired DNA binding activity as judged by EMSA (Figs. 3 and 4B), and C/EBPα interacts with the C/EBP regulatory elements in its own promoter, the PPARγ promoter, and the 422/aP2 gene promoter (Fig. 2) as judged by ChIP analysis. By day 2/3 PPARγ interacts with the C/EBP regulatory element in the 422/aP2 gene promoter (Fig. 4). Together these findings provide strong evidence for a cascade of transcriptional activation

involving C/EBPβ, C/EBPα, and PPARγ that is initiated by C/EBPβ.

The long lag in the acquisition of DNA binding activity by C/EBPβ delays the expression of C/EBPα,

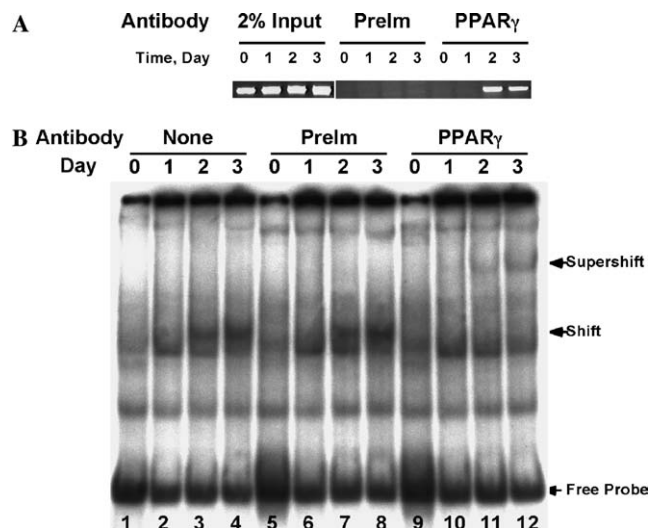


Fig. 4. Binding of PPARγ to the promoter of the 422/aP2 gene promoter. (A) ChIP analysis: at different times after induction of differentiation, about  $1 \times 10^7$  cells were cross-linked with formaldehyde, DNA fragmented, and chromatin-associated DNA immunoprecipitated with preimmune serum (Prelm) or antibodies against PPARγ. PCR amplifications of the DNA fragments were conducted with specific primers flanking the PPARγ regulatory element (ARE7) in the promoter of 422/aP2 gene. In all cases, only the expected  $\sim 200$  bp PCR product was observed. (B) EMSA analysis: day 0 postconfluent 3T3-L1 preadipocytes were induced to differentiate using the standard differentiation protocol. Each day after induction nuclear extracts were prepared and subjected to EMSA with labeled ARE7 as described in Materials and methods. Supershift experiments were performed with preimmune (lanes 5–8) or antibody directed against PPARγ (lanes 9–12).

which is anti-mitotic. This sequence of events guarantees that mitotic clonal expansion, a requirement for terminal differentiation, is completed before C/EBP $\alpha$  is expressed. Furthermore, since the promoters of both the C/EBP $\alpha$  and PPAR $\gamma$  genes possess C/EBP regulatory elements, we suggest that once expressed, C/EBP $\alpha$  (acting in a positive feedback loop) is responsible for maintaining the expression of both C/EBP $\alpha$  and PPAR $\gamma$  and thereby, the differentiated state.

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