Conditional ectopic expression of C/EBP β in NIH-3T3 cells induces PPAR γ and stimulates adipogenesis

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Activation of adipogenesis in 3T3 preadipocytes by exposure to the adipogenic inducers dexamethasone, methylisobutylxanthine, insulin, and fetal bovine serum is accompanied by a transient burst of C/EBP β protein expression that precedes the induction of the fat gene program. In this study we have investigated the role of C/EBP β in initiating the adipogenic program by overexpressing C/EBP β in multipotential NIH-3T3 fibroblasts. Conditional ectopic expression of C/EBP β was accomplished by using an artificial transcriptional regulatory system based on the *Escherichia coli* tetracycline repressor to generate a stable cell line, β 2, that expresses C/EBP β mRNA and protein in a tightly controlled tetracycline dose-dependent manner. Induction of C/EBP β DNA-binding activity in NIH-3T3 β 2 cells exposed to dexamethasone in the presence of insulin and fetal bovine serum activates the expression of an adipocyte-specific nuclear hormone receptor, PPAR γ , that stimulates the conversion of these fibroblasts into committed preadipocytes. Either ectopic expression of C/EBP β or treatment with dexamethasone alone is incapable of inducing PPAR γ expression, but when present together, they have a synergistic effect on the adipogenic program. Exposure of these stimulated cells to a PPAR activator 5,8,11,14-eicosatetraenoic acid (ETYA) results in the accumulation of fat droplets and expression of the adipocyte-enriched genes aP2 and glycerol phosphate dehydrogenase (GPD). The number of β 2 cells that can differentiate into adipocytes is related to the concentration of tetracycline and, therefore, the amount of the exogenous C/EBP β protein expressed. C/EBP β can induce PPAR γ mRNA in the absence of ETYA; however, expression of aP2 mRNA and maximum fat deposition is dependent on the PPAR activator. Our results suggest that enhanced expression of C/EBP β converts multipotential mesenchymal precursor cells into preadipocytes that respond to adipogenic inducers, including dexamethasone and PPAR activators to differentiate into adipocytes.

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The differentiation of multipotential stem cells into functionally distinct cell types within different tissues involves the activation of a regulatory network of nuclear factors and the cessation of cell proliferation. Significant progress toward an understanding of these elaborate processes has been made by investigators studying the in vitro differentiation of mesenchymal cells into fat and muscle. In both cases, families of transcription factors have been identified that play a direct role in regulating the expression of tissue-restricted genes in the different cell types (Tapscott and Weintraub 1991; Vasseur-Cognet and Lane 1993). The advances made in understanding adipogenesis have stemmed from the isolation and characterization of the 3T3-L1 and 3T3-F442A preadipocyte cell lines that were originally derived from

mouse fibroblasts (Green and Kehinde 1974, 1975, 1976). In the case of 3T3-L1 cells, differentiation is induced upon exposure of a confluent population of cells to fetal bovine serum (FBS), dexamethasone (DEX), methylisobutylxanthine (MIX), and insulin. Differentiation of 3T3-F442A cells, however, requires only exposure of confluent cells to FBS and insulin. In both cases, the differentiation is marked by a major change in cell morphology that includes the accumulation of large lipid droplets and the induction of a program of adipocyte-specific genes.

Regulation of this program of gene expression has recently been shown to involve two families of transcription factors, the CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs). C/EBPs are members of a diverse group of nuclear factors that contain a leucine zipper domain required for dimer formation and a basic DNA-binding do-

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main that binds to regulatory elements in the promoters and/or enhancers of target genes. Expression of the C/EBP genes is regulated in such a way that three of the proteins (α , β , and δ) are restricted to a limited number of tissues, most notably fat and liver (Cao et al. 1991; Williams et al. 1991). All three C/EBPs readily form heterodimers with one another, exhibit similar DNA-binding activities, and are capable of *trans*-activating reporter genes that contain C/EBP regulatory elements within their promoters (Cao et al. 1991). The pattern of expression of these genes during *in vitro* adipogenesis is consistent with a role for each protein in controlling the sequential activation of the adipogenic gene program (Cao et al. 1991). Recent studies suggest that C/EBP α has a direct role to play in establishing the quiescent, terminally differentiated state of adipocytes (Samuelsson et al. 1991; Umek et al. 1991; Lin and Lane 1994), and it can also stimulate adipogenesis when overexpressed in various mouse fibroblast cell lines (Freytag et al. 1994). Whether C/EBP α is responsible for initiating adipogenesis is questionable, however, because its expression occurs relatively late in the differentiation process (Vasseur-Cognet and Lane 1993).

A search for an adipocyte-specific transcription factor responsible for activating the adipogenic gene program led Spiegelman and co-workers (Tontonoz et al. 1994a) to identify a tissue-restricted member of the PPAR family of nuclear hormone receptors, PPAR γ 2. As is the case with the C/EBPs, the PPAR proteins consist of a family of related proteins that are expressed to varying extents in different tissues (Kliwer et al. 1994). PPAR α and PPAR γ are produced along with C/EBP α in tissues involved in energy metabolism. PPAR α is expressed abundantly in liver (Isseman and Green 1990; Dreyer et al. 1992; Zhu et al. 1993), whereas PPAR γ expression is most prominent in adipose tissue (Tontonoz et al. 1994a). PPAR δ and NUC1 are both expressed at low levels in many tissues (Schmidt et al. 1992; Chen et al. 1993; Kliwer et al. 1994). PPARs are activated by a structurally diverse group of compounds, the peroxisome proliferators, which include the fibrate class of hypolipidemic drugs, herbicides, phthalate plasticizers, and long-chain fatty acids (Dreyer et al. 1992; Gottlicher et al. 1992; Keller et al. 1993). Activation of these receptors by specific peroxisome proliferators in target tissues such as liver and fat results in the expression of a variety of genes that encode proteins and enzymes involved in regulating lipid metabolism (Dreyer et al. 1992; Keller et al. 1993; Tontonoz et al. 1994b). Activation of PPAR α in the liver and kidney causes a dramatic increase in the size and number of peroxisomes, thereby enhancing the capacity of these tissues to metabolize long-chain fatty acids through an increased production of enzymes that function in the β -oxidation cycle (Dreyer et al. 1992). The high level of PPAR γ expression in adipose tissue and its early induction during the differentiation of 3T3-L1 and 3T3-F442A preadipocytes suggest that this factor may initiate fat gene expression (Chawla et al. 1994; Tontonoz et al. 1994a). A recent study demonstrates that retroviral expression of PPAR γ 2 in NIH-3T3 mouse fi-

broblasts promotes adipocyte differentiation in a manner that is dependent upon the concentration of PPAR activators in the culture medium (Tontonoz et al. 1994b). Furthermore, the extent of this differentiation is enhanced severalfold when C/EBP α is expressed together with PPAR γ 2, suggesting that both factors cooperate to induce the expression of adipocyte-specific genes. In fact, at least two genes expressed in fat, aP2 and PEPCK, contain regulatory elements within their promoters or enhancers that respond to *trans*-activation by C/EBP α and PPAR γ (Christy et al. 1989; Park et al. 1990; Tontonoz et al. 1994a, 1995).

It appears, therefore, that PPAR γ and C/EBP α are both capable of inducing fat-specific gene expression in multipotential precursor cells such as NIH-3T3 fibroblasts, but it is not known whether one of these factors is responsible for inducing the expression of the other. It is unlikely that C/EBP α regulates PPAR γ , because it is expressed at least 1 day after the induction of PPAR γ during the differentiation of 3T3 preadipocytes (Tontonoz et al. 1994b). It is more likely that PPAR γ regulates C/EBP α . If this is the situation, then an important question is, Which transcription factors activate PPAR γ ? The early transient activation of C/EBP β and C/EBP δ in 3T3-L1 cells in response to DEX and MIX (Cao et al. 1991) suggests that these factors may provide this important function of activating PPAR γ . To address this question, we have developed an inducible expression system to facilitate the production of different amounts of C/EBPs in NIH-3T3 cells in response to an extracellular regulator. During the final stages of this work, a study by McKnight and co-workers (Yeh et al. 1995) demonstrated that retrovirus-mediated overexpression of C/EBP β in NIH-3T3 cells can induce adipogenesis in the presence of adipogenic inducers. This observation was in conflict with earlier investigations by Freytag et al. (1994) who reported that C/EBP β was not capable of converting fibroblasts into fat cells. In the present study we demonstrate that conditional ectopic expression of C/EBP β in NIH-3T3 cells activates the synthesis of PPAR γ mRNA and subsequent exposure of these cells to PPAR activators stimulates their conversion into adipocytes.

Results

Conditional expression of C/EBP β using the TET activator expression system

Previous investigations have shown that PPAR γ and C/EBP β mRNAs are induced severalfold during the initial period following the differentiation of 3T3 preadipocytes into adipocytes (Cao et al. 1991; Tontonoz et al. 1994a). It appears, however, that the peak of expression of C/EBP β protein precedes the activation and accumulation of PPAR γ mRNA. These data are consistent with our hypothesis that C/EBP β is involved in activating PPAR γ expression during fat cell differentiation. To test this idea, we established a stable line of NIH-3T3 cells that produce controlled amounts of C/EBP β protein from an inducible expression vector, and we have deter-

mined that C/EBP β can stimulate adipogenesis by inducing PPAR γ expression. To conditionally express C/EBP β proteins in NIH-3T3 cells, we employed an artificial transcriptional regulatory system developed by Gossen and Bujard [1992] that uses the bacterial tetracycline-resistant operator/repressor (Materials and methods). C/EBP β cDNA was subcloned into the expression vector, pUHD10-3, downstream of a synthetic promoter composed of tandem repeats of the tetracycline operator and a CMV promoter. Activation of this minimal promoter is dependent on coexpression of an artificial *trans*-activator (tTA) composed of the tetracycline repressor fused to the activation domain of the herpes simplex virus transcriptional activator VP16. The presence of tetracycline prevents the binding of this *trans*-activator to the operator and, therefore, inhibits the expression of C/EBP β mRNA (Fig. 1A). The plasmid, pUHD15-1, expressing tTA was transfected together with the C/EBP β expression vector and a neomycin-resistant plasmid. Conditional expression of C/EBP β mRNA in stably transfected cells was assessed by Northern blot analysis of total RNA isolated from G418-resistant colonies cultured in the presence or absence of tetracycline. One clone, referred to as β 2, which produced enhanced levels of C/EBP β mRNA in response to culture medium lacking tetracycline, was selected for further analysis. Cell lines expressing abundant quantities of tTA *trans*-activator alone were selected as controls.

The Northern blot analysis in Figure 1B shows a modest level of C/EBP β mRNA expression in cells grown in the presence of tetracycline (lane 1). Removal of the drug results in a gradual but extensive induction of the exogenous mRNA over a 48-hr period. The size of this mRNA is larger than the endogenous species as shown by its migration to a slightly higher position in the gel. This is attributable to the transcription of part of the expression vector that contains the SV40 polyadenylation element giving rise to an additional $\sim$ 100 nucleotides in the exogenous mRNA. Addition of tetracycline to these stimulated cells results in a rapid decrease of C/EBP β mRNA levels within the initial 4 hr after treatment, indicating that the exogenous C/EBP β mRNA species is very short lived. Western blot analysis of total cell lysates obtained from β 2 cells maintained for several days in the presence or absence of tetracycline was performed with an anti-C/EBP β antibody. Figure 1C shows that β 2 cells grown in the presence of tetracycline and the NIH-3T3 cells expressing the tet activator alone (tTA cells) contained detectable quantities of the 32-kD C/EBP β (LAP) polypeptide but significantly lower amounts of the 21-kD C/EBP β (LIP) protein. Culture of the β 2 cells in the absence of tetracycline resulted in an increase by severalfold of both C/EBP β polypeptides, indicating that the ectopically expressed C/EBP β mRNA (see Fig. 1A) is translated correctly in this 3T3 fibroblast clone.

Figure 1. Inducible expression of C/EBP β in NIH-3T3 cells. (A) An illustration of the tetracycline activator expression system. (B) Kinetics of activation or inhibition of C/EBP β mRNA expression in β 2 cells in response to removal or addition of tetracycline. β 2 cells were maintained in growth medium containing 1 μ g/ml of tetracycline. When the cells reached $\sim$ 50% confluence (time 0 hr, lane 1), tetracycline was removed and the cultures were incubated for the indicated times (0–48 hr, lanes 2–6). At 48 hr, cells were reexposed to the antibiotic (1 μ g/ml) and cultured for an additional time period as shown (lanes 7–12). Twenty micrograms of total RNA isolated from each time point was analyzed by Northern blot hybridization for expression of the C/EBP β mRNA. (C) Western blot analysis of C/EBP β protein expression in β 2 cells in the presence or absence of tetracycline for 48 hr. The tTA cells were maintained in tetracycline-free medium. Forty micrograms of nuclear protein extracted from both cell lines was examined by Western blot analysis using anti-C/EBP β antibody as described in Materials and methods. (NL) Normal adult rat liver nuclear proteins.

The DNA-binding activity of C/EBP polypeptides in 3T3-L1 preadipocytes and NIH-3T3 fibroblasts

Preadipocytes have been shown to express at least three isoforms of C/EBP, α , β , and δ , at specific times during their differentiation into adipocytes. Each of these C/EBP polypeptides can associate with the others to form homo- and heterodimeric complexes that bind to DNA with different affinities (Cao et al. 1991). To identify the complexes expressed during the early phase of preadipocyte differentiation and to compare them with C/EBP complexes in the β 2 cells, we performed a series of gel mobility supershift assays. Figure 2A (lane 1) shows that 3T3-L1 preadipocytes exposed to the adipogenic inducers DEX, MIX, insulin, and FBS for 2 days result in the expression of a heterogeneous group of C/EBP complexes that are capable of binding to a consensus C/EBP binding site. At 4 days following induction of differentiation, the abundance of these complexes decreases quite significantly (lane 7). Supershift analysis with antibodies against the three C/EBP isoforms, α , β , and δ , shows that the major C/EBP binding activity ex-

pressed at day 2 consists of complexes containing C/EBP β (Fig. 2A, lane 3). Other reports have demonstrated that C/EBP δ polypeptides are also abundantly expressed at this stage of the differentiation, but the supershift assay shown in lane 4 suggests that these proteins do not constitute a significant proportion of the total DNA-binding activity of the C/EBP complexes. The data in lane 2 shows a low but detectable level of C/EBP α binding activity at 2 days post induction. It is worth noting that the anti-C/EBP β antibody supershifts almost all of the C/EBP complexes (lane 3) except two low-abundance species that appear to correspond to $\delta\delta$ homodimers (the lower species) and $\alpha\alpha$ homodimers (the upper species). Specifically, the lower species in lane 3 can be supershifted when the 2-day nuclear extracts are treated with anti-C/EBP β and anti-C/EBP δ antibodies together (lane 5), and the upper species is shifted when anti-C/EBP α antibody is included with the other two antibodies (lane 6). By day 4 of differentiation, the binding activity of both the C/EBP β and C/EBP δ complexes drop severalfold (cf. lane 9 with lane 3 and lane 10 with lane 4). In fact, it appears that the total C/EBP binding

Figure 2. Analysis of C/EBP DNA-binding activity in 3T3-L1 preadipocytes, β 2, and tTA fibroblasts. (A) 3T3-L1 cells were cultured and induced to differentiate as described in Materials and methods. Nuclear proteins were extracted at day 2 and day 4 following exposure to differentiation medium. Ten micrograms of each extract was subjected to EMSA using a ^{32} P-labeled C/EBP oligonucleotide as probe. For the supershift assay, each extract was incubated with the appropriate antibodies (anti-C/EBP α , anti-C/EBP β , anti-C/EBP δ , or the combinations of these three as indicated) for 2 hr at room temperature prior to addition of the radiolabeled probe. (B) The fibroblast cell lines (β 2 and tTA) were cultured in the presence or absence of tetracycline as described in the legend to Fig. 1. Nuclear proteins were extracted, and equal amounts (10 μ g) of each extract were subjected to EMSA as described in A.

activity expressed at this stage of adipogenesis is accounted for by complexes primarily involving C/EBP α (lane 8). Figure 2B shows that nuclear extracts isolated from NIH-3T3 cells, either β 2 cells maintained in tetracycline (lane 3) or fibroblasts transfected with the tTA expression vector alone (lane 11), have low but measurable levels of C/EBP binding activity when compared with differentiating preadipocytes (Fig. 2A) or normal rat liver (Fig. 2B, lane 1). Most of this activity in the 3T3 cells is attributable to C/EBP β polypeptides (Fig. 2B, lanes 5,12). Culture of the β 2 cells in the absence of tetracycline for 48 hr results in a severalfold induction of total C/EBP binding activity (Fig. 2B, lane 7), which can be attributed to the overexpressed C/EBP β proteins because the anti-C/EBP β antibody supershifts almost all of the C/EBP complexes produced in these cells (lane 9). There is very little C/EBP δ DNA-binding activity and no C/EBP α activity.

Conditional expression of C/EBP β in NIH-3T3 cells stimulates adipogenic conversion

To investigate the ability of C/EBP β protein to induce PPAR γ in NIH-3T3 cells and subsequently to stimulate adipogenesis, we cultured subconfluent β 2 cells for 48 hr in the absence of tetracycline to elevate C/EBP β expression to maximum levels during which time the culture reached confluence. Exposure of these cells to the adipogenic inducers DEX, MIX, insulin, and FBS for 48 hr, followed by maintenance in FBS containing insulin and the PPAR activator 5,8,11,14-eicosatetraenoic acid (ETYA), caused a morphological differentiation within 6–10 days after confluence. At this time, 25%–30% of the fibroblasts had differentiated into large round cells containing several small lipid droplets or a few large droplets (Fig. 3). No evidence of differentiation was observed in the cultures maintained in the presence of tetracycline, suggesting that activation of the adipogenic program is dependent upon the expression of C/EBP β protein. Similarly, culture of the control tTA cells in medium lacking tetracycline was incapable of stimulating lipid droplet formation even when cells were exposed to the adipogenic inducers (data not shown).

To determine whether the morphological differentiation seen in Figure 3 was attributable to the activation of the adipogenic gene program, we performed a Northern blot analysis of total RNA isolated from β 2 cells cultured in the presence or absence of tetracycline for the indicated days following exposure to the adipogenic inducers outlined above including ETYA. These data shown in Figure 4 demonstrate a significant increase in mRNAs coding for PPAR γ and the differentiation-linked adipocyte genes aP2 and GPD. The PPAR γ mRNA was detected first 24–48 hr after confluence, and its expression continued to rise, reaching an abundant plateau between 4 and 10 days. Induction of aP2 and GPD mRNAs lagged behind PPAR γ mRNA by 2 days because they were not detected until 4 days after treatment. PPAR γ and aP2 mRNAs reached a level of expression ~20%–30% of that measured in fully differentiated 3T3-L1 cells; this level

Figure 3. Conditional expression of C/EBP β in NIH-3T3 β 2 cells stimulates adipogenic conversion. β 2 cells were cultured in the presence (1 μ g/ml; bottom) or absence of tetracycline (top) for 48 hr, induced to differentiate for 9 days, and stained with Oil Red O as described in Materials and methods. Original magnification, 20 $\times$.

is consistent with the extent of conversion of these NIH-3T3 (β 2) fibroblasts into adipocytes. GPD mRNA, however, reached significantly lower levels of expression than expected, and C/EBP α mRNA was not detected during the 10-day culture period. Culture of β 2 cells in tetracycline prevents the activation of the adipogenic genes as indicated by a complete absence of PPAR γ , aP2, and GPD mRNAs throughout the differentiation period. C/EBP δ mRNA expression is transiently induced in β 2 cells maintained in tetracycline in response to the adipogenic inducers. In fact, overexpression of C/EBP β appears to prevent this induction and results in a gradual decrease of C/EBP δ mRNA as observed during the differentiation of 3T3-L1 cells. As expected, the tTA control cells failed to express the adipose genes following exposure to the adipogenic inducers in the absence of tetracycline (lanes 15,16).

During the initial isolation of the β 2 cells, we identified other clones that expressed C/EBP β mRNA at levels significantly higher than those present in nontransfected NIH-3T3 cells but lower than levels expressed by the β 2 cells. In addition, some clones that were transfected with

Figure 4. Ectopic induction of C/EBP β in NIH-3T3 β 2 cells activates the adipogenic gene program. β 2 cells were cultured in growth medium containing tetracycline (1 μ g/ml). Two days before the cells reached confluence, fresh medium was added so that one-half of the population was maintained in tetracycline (β 2 + tetracycline), whereas the other half was deprived of the antibiotic (β 2 – tetracycline). tTA (tTA-T) and 3T3-L1 (L1) cells were maintained in tetracycline-free media. At day 0 (confluence), all four populations of cells (β 2 + / – tetracycline; tTA-T and L1) were exposed to the inducers of adipogenesis as described in Materials and methods. Twenty micrograms of total RNA was isolated from the various cells at the indicated days (L1 preadipocytes were differentiated for 10 days) and analyzed on Northern blots using ^{32} P-labeled cDNA probes corresponding to PPAR γ , aP2, GPD, C/EBP α , C/EBP β , and C/EBP δ mRNAs.

both the tTA and C/EBP β expression vectors still only expressed the normal 3T3 basal levels of C/EBP β mRNA. For these reasons and the fact that mRNA and protein expression of the β 2 cells was tightly controlled by tetracycline, we decided to use the β 2 cell line to define the role of C/EBP β in regulating adipogenesis. It was important, however, to demonstrate that the induction of adipogenesis in these β 2 cells was owing to the overexpressed C/EBP β polypeptides and not to clonal artifact selected for during the establishment of these stable transfectants. In the experiment shown in Figure 5, a selection of these other clones were analyzed for their level of C/EBP DNA-binding activity and their ability to differentiate into adipocytes. Figure 5A shows that the clones either expressed elevated levels of C/EBP β DNA-binding activity (β 4, β 7, β 5, and β 2) when compared with NIH-3T3 cells that were not transfected with the C/EBP β expression vector (T1 and T2), or they did not show elevated levels (β 8, β 11, and β 13). Exposure of all these cell lines to the adipogenic inducers resulted in a pattern of gene expression that correlated with the enhanced C/EBP β DNA-binding activity measured in each nuclear extract. Specifically, the positive clones (β 4, β 7, β 5, and β 2) that showed an elevated level of C/EBP β binding activity were all capable of responding to the adipogenic inducers by expressing both PPAR γ and aP2 mRNAs (Fig. 5B). It is interesting that the level of expression of these adipose-specific mRNAs correlates closely with the level of C/EBP β DNA-binding activity within each cell line. The negative clones (β 8, β 11, β 9, β 13, T1, and T2) that did not show any significant enrichment in C/EBP β binding activity also did not express

Figure 5. Variation in the level of C/EBP β expression and the extent of adipogenic conversion in additional stable transfectants of NIH-3T3 cells. (A) Analysis of the binding activity of C/EBP β in G418-resistant cell lines generated by transfection with the tTA and C/EBP β expression vectors together (β clones) or the tTA vector alone (T1, T2). All the cell lines were cultured in tetracycline-free media to confluence and then induced to differentiate as described in Materials and methods. Nuclear proteins of each clone were extracted 6 days after confluence. Ten micrograms of each extract was subjected to supershift EMSA using a ^{32} P-labeled C/EBP oligonucleotide as probe and an anti-C/EBP β antibody. (B) Expression of PPAR γ and aP2 mRNAs in β clones and tTA cell lines. All clones were cultured and differentiated as in A. Twenty micrograms of total RNA was isolated from each clone at 6 days following induction to differentiate and was analyzed by Northern blot using ^{32} P-labeled cDNA probes corresponding to PPAR γ , aP2, and C/EBP δ mRNAs.

the adipogenic gene program when exposed to differentiation medium.

The extent of the differentiation of NIH-3T3 cells into adipocytes is dependent on the amount of C/EBP β protein

The data shown in Figure 5 suggest that the extent of induction of the adipose-specific genes may be related directly to the amount of C/EBP β protein expressed in these fibroblasts. To test this hypothesis, we propagated the β 2 cells in tetracycline, then split the population into fresh medium containing varying concentrations of tetracycline. Nuclear extracts were prepared after a 48-hr culture period and analyzed by both Western blot and supershift electrophoretic mobility-shift assay (EMSA) using an anti-C/EBP β antibody. Figure 6A shows that there is a dose-dependent increase in both forms of the C/EBP β protein (LAP-32kD and LIP-21kD) as the concentration of tetracycline is attenuated. At concentrations between 50 ng/ml and 1000 ng/ml, there are low amounts of LAP polypeptides but virtually undetectable quantities of the LIP polypeptides as observed previously in Figure 1C. Induction of C/EBP β protein synthesis occurs when β 2 cells are exposed to doses of 10 ng/ml and below, reaching a maximum level of expression at 0–1.0 ng/ml. The anti-C/EBP β supershift EMSA shown in Figure 6B demonstrates that the increased expression of these C/EBP β polypeptides results in a corresponding increase in DNA-binding activity. To determine whether this tetracycline dose-dependent induction of

the C/EBP β polypeptides corresponds to an increase in the percentage of differentiated preadipocytes, we cultured β 2 cells in different amounts of tetracycline and exposed them to differentiation medium containing 50 μ M ETYA. Figure 7A shows a direct relationship between the percentage of cells containing fat droplets and the concentration of tetracycline in the media. Tetracycline at 1000 ng/ml completely inhibited adipogenesis, whereas cells cultured in concentrations lower than 50 ng/ml began to show signs of differentiation as revealed by the presence of Oil Red O-stained fat droplets. The dose dependence of this response is shown in Figure 7B in which the percentage of cells staining positive with Oil Red O at the different concentrations of the antibiotic are graphed. It is interesting that although at 50 ng/ml we were unable to detect an enhanced expression of C/EBP β proteins (see Fig. 6), the Oil Red O stain was capable of detecting a small percentage of cells that had differentiated into adipocytes. As the concentration of the antibiotic is decreased, there is a corresponding increase in the percentage of cells expressing fat droplets, reaching a maximum of 25%–30% conversion at 0.1 ng/ml. Figure 7C shows that this pattern of morphological differentiation correlates with a tetracycline dose-dependent induction of PPAR γ and aP2 mRNAs. It is noteworthy to mention that there is no significant change in the expression of C/EBP δ mRNA at all concentrations of tetracycline at this stage of the differentiation process.

Induction of PPAR γ expression in NIH-3T3 cells is dependent on DEX as well as enhanced C/EBP β DNA-binding activity

During our initial investigations, we observed that the differentiation of tetracycline-deficient β 2 cells into adipocytes was dependent on exposure to differentiation medium containing DEX, MIX, insulin, and FBS (data not shown). To determine which of these adipogenic inducers was required to activate the adipose-specific genes, we cultured β 2 cells in medium containing calf serum in the presence or absence of tetracycline and allowed them to grow to confluence (~2 days). At this time (day 0), the tetracycline-deficient cells were exposed to FBS containing insulin alone (I), insulin + DEX (ID), insulin + MIX (IM), or all three inducers together (DIM). Cells maintained in tetracycline were also exposed to DIM. After 2 days of treatment, the media of all the cultures was changed to insulin and FBS. Four days later, total RNA was isolated and analyzed by Northern blot hybridization. Figure 8A demonstrates that DEX is the major activator of adipogenesis in these tetracycline-deficient cells because the only inducers that could stimulate PPAR γ and aP2 expression was ID and DIM (lanes 2,4). Insulin alone or insulin + MIX had virtually no effect on the expression of these adipose-specific genes. As shown in Figure 4 above, if cells are exposed to DIM in the presence of tetracycline, which prevents the ectopic expression of C/EBP β protein, the adipogenic program is not activated (Fig. 8A, lane 5). Figure 8B further demonstrates that PPAR γ mRNA is induced within 12–24 hr

Figure 6. Tetracycline dose-dependent expression of C/EBP β in β 2 cells. Equal amounts of nuclear extract isolated from β 2 cells cultured in the indicated concentrations of tetracycline was subjected to Western blot analysis (A) and supershift EMSA (B) as described in Materials and methods. (NL) Normal adult rat liver nuclear extract. (L1) Nuclear extracts from preadipocytes cultured for 2 days in differentiation medium.

Figure 7. Differentiation of NIH-3T3 β 2 cells into adipocytes is directly related to the amount of the C/EBP β protein expressed. (A) Tetracycline dose-dependent conversion of β 2 cells into Oil Red O positive adipocytes. β 2 cells were induced to differentiate as described in Materials and methods in differentiation medium containing different concentrations of tetracycline (as indicated at the top) and 50 μ M ETYA. Cells were fixed and stained with Oil Red O at 9 days postconfluence. Magnification, 20 $\times$. (B) Quantitation of the percentage of β 2 cells that are stained with Oil Red O for each concentration of tetracycline. Two hundred cells in 10 random fields were counted. (C) Tetracycline dose-dependent induction of PPAR γ and aP2 mRNAs. Total RNA was isolated from β 2 cells cultured for 8 days in differentiation medium containing the indicated concentrations of tetracycline and analyzed by Northern blot as described in Materials and methods.

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Figure 8. Induction of PPAR γ expression in NIH-3T3 β 2 cells is dependent on DEX as well as enhanced C/EBP δ expression. (A) β 2 cells were cultured in growth medium containing tetracycline (1 μ g/ml). Two days before the cells reached confluence, fresh medium was added so that one of the cultures was maintained in tetracycline (+), whereas others were deprived of the antibiotic (-). At day 0 (confluence), cells were induced to differentiate by addition of fresh medium containing various adipogenic inducers as indicated: (I), insulin alone; (ID) insulin plus DEX; (IM) insulin plus MIX; and (DIM) insulin, DEX, and MIX. After 48 hr, the media was changed to DMEM containing 10% FBS and 5 μ g/ml of insulin, and cells were maintained in this medium for an additional 4 days (6 days of differentiation). Twenty micrograms of total RNA from each condition was analyzed by Northern blot using ^{32} P-labeled cDNA probes as indicated. (B) β 2 cells were cultured in the presence or absence of tetracycline until they reached confluence (0 hr). Cells were then induced to differentiate by addition of fresh medium containing 5 μ g/ml of insulin and 1 μ M of DEX. After 48 hr, the medium was replaced with DMEM containing 10% FBS and 5 μ g/ml of insulin. Cells were refed every 48 hr with this medium. Twenty micrograms of total RNA from cells at each time point was subjected to Northern blot analysis using the ^{32} P-labeled cDNA probes as indicated.

following exposure of tetracycline-deficient β 2 cells to DEX. Maximum levels of PPAR γ expression are reached by 72–96 hr after treatment. Cells maintained in tetracycline are incapable of expressing PPAR γ throughout this time period. It is worth noting that the β 2 cells that have been deprived of tetracycline for 48 hr prior to the start of this experiment (day 0) express detectable amounts of PPAR γ even before they are exposed to DEX. In contrast, the β 2 cells maintained in tetracycline through the entire experimental period and exposed to the glucocorticoid do not express PPAR γ mRNA. These data suggest that C/EBP δ is the principal activator of PPAR γ mRNA expression and DEX significantly enhances this response. It is also important to note that there is no significant change in the expression of C/EBP δ mRNA in the presence or absence of tetracycline.

The C/EBP δ -dependent induction of adipogenesis in NIH-3T3 cells is enhanced by a synthetic PPAR activator, ETYA

The early induction of PPAR γ mRNA following exposure of the tetracycline-deficient β 2 cells to the adipogenic inducers suggests that this fat-specific transcription factor is responsible for inducing the expression of the downstream genes such as aP2 and GPD and stimulating the production of triglycerides. Other studies have shown that adipose differentiation of preadipocyte cell lines and PPAR γ -expressing NIH-3T3 cells is enhanced manyfold by lipophilic activators of PPARs. To deter-

mine the importance of activating PPARs during the adipogenic conversion of the β 2 cells, we initiated differentiation with DEX, MIX, insulin, and FBS as described above (Fig. 4), but during the switch to maintenance medium (FBS, insulin, and ETYA), half the population of cells were deprived of ETYA. Figure 9A shows that culture of tetracycline-deprived β 2 cells in medium containing 50 μ M ETYA induces 25%–30% of the population to convert to fat cells, whereas in the absence of ETYA the level of conversion is greatly reduced (~5% of the population). The Northern blot analysis shown in Figure 9B demonstrates that activation of PPAR γ mRNA by C/EBP δ and the adipogenic inducers (DEX) does not require ETYA because the same level of PPAR γ expression occurs in the absence or presence of the synthetic fatty acid (cf. lane 2 with lane 3). In contrast, aP2 mRNA expression, which has been shown previously to be stimulated by the ectopic production of PPAR γ , is enhanced severalfold by culture of the stimulated β 2 cells in ETYA (20–50 μ M). Furthermore, exposure of the cells to ETYA in the absence of DEX and MIX is incapable of activating both PPAR γ and aP2 mRNA expression (Fig. 9C), demonstrating that ETYA is not an inducer of PPAR γ gene expression in cells (NIH-3T3 fibroblasts) that do not express PPAR γ .

Discussion

These investigations provide evidence that C/EBP δ plays an important regulatory role during the early stages of adipogenesis by activating the expression of adipo-

Figure 9. Effects of ETYA on C/EBP β -dependent induction of adipogenesis in NIH-3T3 β 2 cells. (A) The conversion of NIH-3T3 β 2 cells into adipocytes by ectopic expression of C/EBP β is enhanced by ETYA. β 2 cells were induced to differentiate by exposure to 1 μ M DEX, 0.5 mM MIX, 10% FBS, and 5 μ g/ml of insulin for 48 hr and were then maintained in either differentiation medium (DMEM containing 5 μ g/ml of insulin and 10% FBS) alone (*top*) or differentiation medium plus 50 μ M of ETYA (*bottom*) for 9 days. Cells were stained with Oil Red O to indicate the accumulation of fat droplets. (B) Expression of aP2 mRNA in β 2 cells that express abundant amounts of PPAR γ mRNA is dependent on ETYA. β 2 cells cultured in the presence (+) or absence (-) of tetracycline were induced to differentiate as described above and were maintained for 4 days in differentiation medium containing insulin and the indicated amounts of ETYA (0, 20, and 50 μ M). Total RNA (20 μ g) from each condition was subjected to Northern blot analysis using the ^{32} P-labeled cDNA probes as indicated. (C) ETYA alone is not capable of activating PPAR γ expression in tetracycline-deficient β 2 cells. β 2 cells were cultured in tetracycline-free medium to confluence. Cells were then incubated in medium containing insulin plus ETYA or insulin, DEX, and MIX. After 48 hr, both populations of cells were exposed to differentiation medium containing insulin and 50 μ M of ETYA and maintained in this medium for an additional 4 days. Twenty micrograms of total RNA from each condition was analyzed by Northern blot.

cyte-restricted transcription factor PPAR γ . We demonstrate that ectopic expression of C/EBP β in NIH-3T3 (β 2, β 4, β 5, β 7) cells in the presence of DEX, MIX, insulin, and FBS induces PPAR γ production that, in the presence of the PPAR activator ETYA, initiates the adipogenic gene program and converts these fibroblasts into adipocytes. This event is directly related to the enhanced levels of C/EBP β protein because treatment of the β 2 cells with the adipogenic inducers in the presence of tetracycline, which blocks the ectopic expression of C/EBP β , does not initiate differentiation. The inhibitory action of the tetracycline is not the result of some unrelated effect on the differentiation process, because exposure of tTA cells (control fibroblasts) to the adipogenic inducers in the absence of the antibiotic is not capable of activating fat cell differentiation. In addition, the β 4 cell line, in which the C/EBP β is constitutively overexpressed, will undergo adipogenesis when exposed to differentiation medium containing tetracycline.

The earliest response to the enhanced expression of C/EBP β in NIH-3T3 cells is the increase in PPAR γ mRNA. This event precedes the appearance of aP2 and GPD mRNAs and the accumulation of fat droplets. Furthermore, this C/EBP β -dependent induction of PPAR γ gene expression occurs in the absence of ETYA, whereas expression of aP2 mRNA and maximum fat deposition are dependent on an exogenous activator of PPAR γ . These data suggest that an important role of C/EBP β is to induce PPAR γ that is then responsible for activating the gene program involved in synthesizing and depositing triglycerides. C/EBP β may also play an additional role in these β 2 cells by synergizing with PPAR γ to promote the transcription of the downstream target genes. In fact, many adipocyte-enriched genes contain binding sites for C/EBP (Vasseur-Cognet and Lane 1993), and at least two of them, aP2 and PEPCK, also contain DR-1 hormone response elements that can interact with PPAR γ (Tontonoz et al. 1994a, 1995). In this regard, our

preliminary data (not shown) suggest that C/EBP β may function to maintain expression of the adipocyte genes at the later stages of the differentiation process because addition of tetracycline to β 2 cells (to inhibit C/EBP β) after PPAR γ has reached a maximum level of expression (day 4) reduces aP2 expression.

Enhanced expression of C/EBP β alone is not sufficient to activate adipogenesis in the NIH-3T3 β 2 cell line because exposure to DEX in the presence of insulin and FBS is also required. What role DEX and the other inducers is playing is not known, but information may be gained from investigations on the mechanisms that regulate the early phase of 3T3-L1 preadipocyte differentiation. Studies by Cao et al. (1991) show that DEX enhances C/EBP δ mRNA expression, whereas MIX is responsible for inducing C/EBP β mRNA production in the preadipocytes. It is unlikely that these effectors are involved in stimulating either C/EBP β or C/EBP δ expression in the NIH-3T3 β 2 cells because C/EBP β , of course, is activated by removal of tetracycline, whereas C/EBP δ appears to decrease somewhat soon after the differentiation process has commenced (Fig. 4). These observations suggest that DEX must be fulfilling a function other than enhancing expression of the C/EBP δ proteins during the differentiation of the β cells. Other investigations (Lemberger et al. 1994) have demonstrated that glucocorticoids can activate PPAR α gene expression in primary cultures of rat hepatocytes, suggesting that the PPAR genes may contain glucocorticoid response elements in their promoters and/or enhancers. The role of insulin and FBS in promoting adipogenesis in both 3T3-L1 and β 2 cells is unclear; one possibility is that they promote the translation of the induced adipogenic mRNAs.

The mechanism by which C/EBP β induces PPAR γ mRNA is not known, although, on the basis of studies analyzing the regulation of other C/EBP-responsive genes, it seems reasonable to propose that PPAR γ gene transcription is likewise regulated by binding of various C/EBPs to corresponding binding sites within the promoter and/or enhancer of the PPAR γ gene. In fact, we have shown that ectopic expression of C/EBP α in NIH-3T3 cells can also induce expression of the PPAR γ gene and stimulate adipogenesis (Z. Wu et al., unpubl.). The data shown in Figure 2 suggest that the C/EBP complexes responsible for inducing PPAR γ expression and stimulating adipogenesis in the 3T3-L1 and β 2 cells consist primarily of C/EBP β homodimers.

It is possible that the forced expression of C/EBP β homodimers in the NIH-3T3 cells and the hormonal induction of these and other complexes in 3T3-L1 cells (Fig. 2A) result in somewhat different programs of gene expression. In this regard, it is interesting that C/EBP α gene expression is not induced by C/EBP β in the β 2 cells. This observation is consistent with that of McKnight and co-workers (Yeh et al. 1995) who have recently found that constitutive, retrovirus expression of C/EBP β in NIH-3T3 cells induces fat droplet accumulation and aP2 expression without enhancing C/EBP α expression. The promoter of the mouse C/EBP α gene contains a C/EBP regulatory element that is capable of ac-

tivating C/EBP α transcription in transfection studies in which C/EBP α and C/EBP β expression vectors are coexpressed along with C/EBP α promoter/CAT reporter gene constructs (Christy et al. 1991; Rana et al. 1995). It appears, therefore, that C/EBP β may be necessary but not sufficient to induce C/EBP α transcription during adipogenesis. Other factors may include C/EBP δ that, as discussed above, appears to contribute only minimally to the formation of functional C/EBP complexes during the early stages of the differentiation of β 2 cells into adipocytes. In addition, the sustained high levels of C/EBP β homodimers expressed in the differentiating β 2 cells may in some way prevent the activation of C/EBP α transcription even though other fat-enriched genes that contain C/EBP-binding sites are activated. In fact, during the normal differentiation of 3T3-L1 cells there is a several-fold decrease in the abundance of the C/EBP β proteins prior to the induction of C/EBP α gene expression (Cao et al. 1991; Fig. 2A). The C/EBP β polypeptide referred to as LIP, which is a potent inhibitor of C/EBP β (LAP) activity (Descombes and Schibler 1991), is also abundantly expressed in β 2 cells following removal of tetracycline (Fig. 1C). It is possible, therefore, that LIP attenuates the activity of LAP during the induction of adipogenesis in β 2 cells by reducing LAP's ability to *trans*-activate some of the adipocyte-enriched genes including C/EBP α . This may be one reason why only 25%–30% of the β 2 cell population differentiate into adipocytes. C/EBP α may also be regulated by PPAR γ , and the C/EBP α gene may resemble other downstream genes such as aP2 and PEPCK by possessing DR-1 hormone response elements. If this is the case, then it is conceivable that activation of C/EBP α by PPAR γ may depend on a PPAR activator that is more potent than the ETYA used in these studies.

As stated at the outset, 3T3 preadipocyte cell lines were originally derived from mouse fibroblasts by selecting colonies that could differentiate into fat cells when exposed to the appropriate medium (Green and Kehinde 1974, 1975, 1976). An important question in light of the present studies is, Which molecular mechanisms are responsible for committing multipotential fibroblast cells to the adipogenic lineage? It is very apparent that NIH-3T3 mouse fibroblasts cannot simply be induced to differentiate into adipocytes by addition of DEX, MIX, insulin, and FBS, suggesting that the signaling pathways that respond to these effectors do not converge on fat-specific genes in these nonadipogenic cells. Our data show that enhancing the production of C/EBP β polypeptides can convert a fibroblast into a preadipocyte when exposed to these adipogenic inducers and suggests that the mechanism responsible for commitment to the adipogenic lineage involves the induction of C/EBP β gene transcription and translation. It is interesting that elevation of cAMP levels by treatment with MIX in 3T3-L1 preadipocytes causes a severalfold increase in C/EBP β gene expression and, therefore, facilitates induction of PPAR γ mRNA. Similar treatment of normal, untransfected NIH-3T3 cells with MIX has little or no effect on C/EBP β production, and the cells are incapable of converting into adipocytes. It is important to note that 3T3-

F442A preadipocytes do not require MIX or DEX to differentiate into adipocytes. It is possible that these cells are slightly further along in the differentiation process than 3T3-L1 cells, and, therefore, they may constitutively express abundant amounts of C/EBP β and C/EBP δ mRNAs. They may then only require insulin and/or FBS to enhance synthesis of the corresponding proteins to activate the fat gene program including PPAR γ expression. It is unclear whether the mechanisms responsible for inducing C/EBP β expression in preadipocytes are specific to the adipogenic lineage. The mechanisms that activate PPAR γ expression appear not to be adipocyte specific because they can operate in NIH-3T3 cells, unless induction of C/EBP β proteins in these fibroblasts converts them to preadipocytes prior to the increase in PPAR γ expression. An additional component of the commitment process may include the production and/or utilization of PPAR activators. It is interesting that differentiation of β 2 fibroblasts into adipocytes after the induction of PPAR γ by C/EBP β requires stimulation with ETYA to presumably activate transcription of genes containing PPAR response elements. In contrast, 3T3-L1 and 3T3-F442A preadipocytes do not require any additional stimulation from PPAR activators to differentiate into fat cells other than the fatty acids present in the serum (Chawla and Lazar 1994). It is possible that these preadipocytes synthesize a specific ligand for PPAR γ and that this event is adipocyte specific and contributes to the acquisition of the adipogenic lineage.

In light of the observations made in this study, it is interesting to compare this regulatory network of adipogenic transcription factors with those expressed in myogenic cells. In the muscle cells, there are several nuclear regulatory proteins including MyoD, Myf 5, Myogenin, Mrf 4, and myocyte enhancer factor 2 (MEF2) that are all involved in regulating skeletal muscle gene expression. Many of the genes coding for these myogenic factors can be *trans*-activated by the other family members, giving rise to a complex regulatory circuit that controls the expression of muscle-specific genes during myogenesis (Edmondson et al. 1992; Weintraub 1993; Olson and Klein 1994). A similar situation may exist in adipocytes in which PPARs and C/EBPs may represent two arms of a network of regulatory factors that can *trans*-activate each other and, in so doing, amplify the expression of the downstream adipocyte-enriched genes.

Materials and methods

Plasmid constructions

tTA (pUHD15-1) and tetracycline operator–CMV promoter expression (pUHD10-3) plasmids were obtained from H. Bujard of ZFMB, University of Heidelberg, Germany (Gossen and Bujard 1992). The MSV–C/EBP β plasmid was provided by S. McKnight of Tularik, Inc., South San Francisco, California. To construct a tTA-dependent C/EBP β expression vector, the 1.5-kb C/EBP β cDNA from an *EcoRI*–*Bam*HI double digest of the MSV–C/EBP β plasmid was inserted into the *EcoRI*–*Bam*HI sites within the polylinker region of pUHD10-3. Transcription of C/EBP β mRNA from the resultant plasmid, Tet–O–C/EBP β , is driven by

the CMV promoter containing seven repeats of the *tet* operator upstream of the C/EBP β cDNA in response to coexpression of tTA from the pUHD15-1 plasmid.

Cell culture and transfections

3T3-L1 preadipocytes were maintained in growth medium consisting of Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD) containing 10% normal calf serum (Intergen, Co., Purchase, NY) and were induced to differentiate as described previously by Student et al. (1980). This involved growing cells to confluence that was considered day 0 of the differentiation program. At this stage, cells were exposed to fresh DMEM containing 10% FBS, 1 μ M DEX, 0.5 mM MIX, and 10 μ g/ml of insulin for 48 hr to initiate adipogenesis. The medium was then replaced with DMEM containing 10% FBS and 2.5 μ g/ml of insulin, and cells were refed every other day. The NIH-3T3 mouse fibroblasts were maintained in DMEM with 10% FBS. To establish an inducible C/EBP β expression cell line, the fibroblasts were transfected with pUHD15-1(tTA), Tet–O–C/EBP β and pSV2neo with lipofectAMINE following the protocol provided by the supplier (GIBCO BRL). Briefly, a mixture of 10 μ g of Tet–O–C/EBP β , 10 μ g of pUHD15-1, 1 μ g of pSV2neo plasmids, and 40 μ l of lipofectAMINE in 1.6 ml of DMEM was incubated at room temperature for 30 min and then diluted to 8 ml with DMEM. Cells (1.5×10^6) per 100-mm dish were washed twice with DMEM, and the diluted DNA–lipid mixture was added. After 6 hr of incubation at 37°C, the cells were refed with DMEM supplemented with 10% FBS and 1 μ g/ml of tetracycline and were cultured for an additional 36 hr before 0.4 mg/ml of G418 was added. The transfected cells were selected in DMEM containing 0.4 mg/ml of G418 for 10 to 14 days, and the resistant clones were subsequently isolated and propagated into cell lines. The stable cell lines were maintained in DMEM containing 10% FBS, 1 μ g/ml tetracycline, and 0.2 mg/ml of G418 and were screened by Northern blot analysis to identify a clone (β 2) that expressed abundant amounts of C/EBP β mRNA in response to withdrawal of tetracycline.

To differentiate the β series of stable transfectants into adipocytes, cells were grown to ~50% confluence in DMEM/10% FBS containing 1 μ g/ml of tetracycline. To activate C/EBP β expression, tetracycline was removed from the culture media, and the cells were allowed to grow for an additional 48 hr by which time they had reached confluence. At this stage, referred to as day 0, adipogenesis was induced by addition of fresh medium containing 1 μ M of DEX, 0.5 mM of MIX, and 5 μ g/ml of insulin. After 48 hr, the medium was changed to DMEM containing 10% FBS, 5 μ g/ml of insulin, and 50 μ M of ETYA or as indicated. The cells were refed every 2 days. To confirm the appearance of adipocyte morphology, especially the accumulated fat droplets in the cytoplasm, the cells were fixed by 10% formaldehyde in phosphate-buffered saline and stained with Oil Red O [Preece 1972].

RNA analysis

Total RNA was isolated from the NIH-3T3 β 2 and 3T3-L1 cells by the procedure of Chomczynski and Sacchi (1987). Cells were washed twice with ice-cold phosphate-buffered saline and lysed with Solution D (4M guanidinium thiocyanate, 25 mM sodium citrate at pH 7.0, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol), and the lysate was extracted with acidic phenol/chloroform; this was followed by isopropanol precipitation at -20°C overnight. Twenty micrograms of each RNA sample was analyzed by Northern blot hybridization as described previously [Bond and

Farmer 1983), except the RNA was blotted onto Hybond-N nylon membrane (Amersham). The probes were labeled by random priming using the Klenow fragment of DNA polymerase I (New England Biolabs, Inc., Beverly, MA) and [α^{32} P]dCTP (DuPont, NEN Research Products, Boston, MA). cDNAs used as probes were as follows: rat C/EBP α , C/EBP β , and C/EBP δ (Cao et al. 1991), 422/aP2 (Spiegelman et al. 1983; Bernlohr et al. 1984), glycerol phosphate dehydrogenase (GPD) (Dobson et al. 1987), and PPAR γ (Aperlo et al. 1995).

Western blot (immunoblot) analysis

Nuclei were isolated from normal liver hepatocytes, 3T3-L1, and NIH-3T3 β 2 cells following a procedure described previously (Rana et al. 1994). The cells were washed twice with ice-cold phosphate-buffered saline and lysed with NP-40 lysis buffer (10 mM Tris-HCl at pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40). The cell lysates were then transferred to a conical tube and incubated on ice for 15 min, and nuclei were pelleted by centrifugation at 1000 g for 5 min at 4°C. The pellets were washed again with lysis buffer, resuspended in nuclear suspension buffer [50 mM Tris-HCl at pH 7.5, 10 mM magnesium acetate, 40% glycerol, 1 mM dithiothreitol (DTT)], and stored at -80°C until ready for use. To extract nuclear proteins, the stored nuclei were thawed on ice and harvested by centrifugation at 1000 g for 10 min; protein extraction buffer (0.4 M NaCl, 5 mM EDTA, 10 mM sodium HEPES at pH 7.5, 0.2 mM PMSF, and 1 mM DTT) was added to the nuclear pellet and incubated on ice for 20 min with occasional mixing. The nuclear protein supernatant was recovered by centrifugation for 15 min and stored at -80°C after addition of glycerol to a final concentration of 15%. The protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fourty micrograms of nuclear protein was fractionated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Membranes were incubated in TBST (25 mM Tris-HCl at pH 8.0, 125 mM NaCl, and 0.1% Tween 20) containing 10% nonfat dry milk (Carnation Co., Los Angeles, CA) to block nonspecific binding. The blots were then incubated with primary antibody, a polyclonal anti-C/EBP β antiserum (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in blocking solution (TBST containing 5% milk) for 1 hr at room temperature. After being washed three times with the blocking solution, the blots were incubated with a secondary antibody, horseradish peroxidase-conjugated rabbit antimouse IgG (1:1000 dilution; Sigma Chemical Co., St Louis, MO) for 1 hr. After a final wash with TBST, the blots were developed by the enhanced chemiluminescence system (Amersham Life Sciences, Arlington, IL) and visualized by exposure to autoradiography film.

EMSA

DNA-binding assays were performed as described previously (Rana et al. 1994). Ten micrograms of nuclear extract was incubated with 3 μ g of poly[d(I-C)], 2 μ l of carrier mix (50 mM MgCl₂ and 340 mM KCl), and delta buffer (0.1 mM EDTA, 40 mM KCl, 25 mM HEPES at pH 7.6, 8% Ficoll, and 1 mM DTT) at 4°C for 15 min. The [^{32} P]dCTP-radiolabeled double-stranded oligonucleotide (50,000 cpm) was added to the reaction mixture and incubated for 30 min at 4°C. For the supershift assay, the appropriate antibody was added to the nuclear extract and incubated at room temperature for 1.5 hr before the probe was added. The anti-C/EBP α , C/EBP β and C/EBP δ antibodies were obtained from Santa Cruz Biotechnology. Binding of the oligonu-

cleotide to specific proteins was determined by fractionating the nuclear proteins through a nondenaturing 6% polyacrylamide gel at 150–200 V for 3–5 hr at 4°C in TBE buffer (0.09 M Tris-borate, 0.002 M EDTA at pH 8.0). The gel was dried at 80°C for 1 hr before exposure to autoradiography. The sequence of the C/EBP oligonucleotide used in this study is 5'-gatccGCGT-TGCCGCCACGATG-3' (Christy et al. 1991). The double-stranded oligonucleotide was end-labeled by T4 DNA polynucleotide kinase (New England Biolabs) and [γ - ^{32} P]ATP (Du Pont).

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Note added in proof

Zhu et al. (*Proc. Natl. Acad. Sci.* **92**: 7921–7925, 1995) have recently demonstrated that the promoter of the mouse PPAR γ gene contains two C/EBP sites.

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