Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells

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The CCAAT/enhancer-binding protein α (C/EBP α) has been implicated in the regulation of adipoblast differentiation. In this study we investigate the potential of C/EBP α to promote the adipogenic program in a variety of fibroblastic cells. Transduction of the C/EBP α gene into eight mouse fibroblastic cell lines by retroviruses and DNA transfection generates adipocyte colonies at variable frequencies. The most dramatic results are obtained with NIH-3T3 cells, in which the percentage of G418-resistant colonies that exhibit the adipocyte morphology is reproducibly >50% when the C/EBP α gene is transduced by retroviruses. The ability to promote the adipogenic program requires the potent transcriptional activation domain of C/EBP α and is not observed with C/EBP β . Paradoxically, in spite of its antimitogenic effects, clonal cell lines that stably express high amounts of C/EBP α can readily be generated. Stable expression of C/EBP α in BALB/c-3T3 cells dramatically enhances their ability to terminally differentiate into adipocytes. The results demonstrate that C/EBP α can efficiently promote the adipogenic program in a variety of mouse fibroblastic cells, including those that have little or no spontaneous capacity to undergo adipogenesis.

[Key Words: C/EBP α ; leucine zipper; differentiation; adipogenesis; retroviruses]

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The CCAAT/enhancer-binding protein α (C/EBP α) is the prototype of the leucine zipper proteins and belongs to a broader class of transcription factors that regulate cell growth and differentiation (Landschulz et al. 1988; Vasseur-Cognet and Lane 1993). Several lines of evidence indicate that C/EBP α regulates two aspects of terminal differentiation: induction of differentiation-specific genes and cessation of mitotic growth. C/EBP α is dramatically induced when 3T3-L1 adipoblasts commit to the differentiation pathway, and it is highly expressed in the mature, postmitotic adipocyte (Birkenmeier et al. 1989; Christy et al. 1989). Induction of C/EBP α is necessary for 3T3-L1 adipogenesis (Samuelsson et al. 1991; Lin and Lane 1992). C/EBP α binds to and stimulates transcription from genes that are abundantly expressed in the mature adipocyte (Christy et al. 1989; Kaestner et al. 1990). Many of these genes code for proteins that function in fat metabolism. Similarly, C/EBP α is also highly expressed in the mature hepatocyte and stimulates the transcription of liver-specific genes (Costa et al. 1988; Friedman et al. 1989; Park et al. 1990). It is likely that C/EBP α plays an important role in the development and function of fat and liver tissue.

Two previous studies that examined whether ectopic expression of C/EBP α could promote the adipogenic program in 3T3-L1 adipoblasts gave disparate results (Umek et al. 1991; Freytag and Geddes 1992). One study showed that conditional activation of a C/EBP α -estrogen receptor fusion protein suppressed 3T3-L1 adipoblast growth; however, it was not sufficient for overt differentiation (Umek et al. 1991). In contrast, a second study showed that ectopic expression of authentic C/EBP α in 3T3-L1 adipoblasts induced adipogenesis in ~12% of G418-resistant colonies (Freytag and Geddes 1992). That none of the adipocyte colonies expanded beyond 200 cells nor could be propagated as clonal lines indicated that such colonies were comprised of mature, postmitotic adipocytes. Thus, although both studies indicated that expression of C/EBP α suppressed 3T3-L1 adipoblast growth, there was disagreement with regard to the ability of C/EBP α to promote the adipogenic program.

In an attempt to clarify this issue, we examined whether C/EBP α could promote the adipogenic program in fibroblastic cells that have little or no spontaneous capacity to undergo adipogenesis. We demonstrate here that ectopic expression of C/EBP α can efficiently promote the adipogenic program in a variety of mouse fibroblastic cells. The results support the conclusion that

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C/EBP α is a key component of the adipocyte differentiation control mechanism.

Results

Ectopic expression of C/EBP α promotes the adipogenic program in mouse fibroblastic cells

To explore further the potential of the C/EBPs to promote the adipogenic program, three C/EBP genes were introduced into a variety of fibroblastic cells by retroviral infection and DNA transfection. The pWZLneo-based retroviral vectors used (Fig. 1) contain an internal ribosome entry site (IRES) from the encephalomyocarditis virus that allows for translation of the neomycin and C/EBP gene products from the same RNA transcript (Boris-Lawrie and Temin 1993). This novel feature makes it likely that cells that acquire resistance to G418 also express the C/EBP gene product, a situation that is less likely when the neomycin and C/EBP gene products are expressed from different RNAs. Transcription of the dicistronic proviral RNA is driven from the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR). WZLneo-C/EBP α codes for authentic rat C/EBP α (42 kD); WZLneo-C/EBP α Δ 1-117 codes for a smaller 30-kD C/EBP α protein that contains the DNA-binding domain but lacks the potent transcriptional activation domain of C/EBP α (Lin et al. 1993; Ossipow et al. 1993); and WZLneo-C/EBP β codes for rat C/EBP β (Cao et al. 1991; Williams et al. 1991). The parental (WZLneo) and C/EBP retroviral vectors were used to infect (transfect) the following fibroblastic cell lines: BALB/c-3T3, C3H10T1/2, NIH-3T3, Swiss 3T3, Swiss 3T3-L1, PA317, ψ -CRE, ψ -CRIP, MRC-5, and WI-38. The first five are well-characterized and widely used mouse fibroblastic cell lines; PA317, ψ -CRE, and ψ -CRIP are retrovirus-packaging cell lines derived from NIH-3T3 cells (Miller and Buttimore 1986; Danos and Mulligan 1988); MRC-5 and WI-38 are human diploid fibroblasts. Two days following retroviral infection (DNA transfection), cells were subcultured and selected in growth medium containing G418 for 2–3 weeks.

Three types of macroscopic G418-resistant colonies were observed in the WZLneo-C/EBP α -infected (transfected) dishes with all eight mouse fibroblastic cell lines. One colony type, which became apparent when the

G418 selection was complete (~5 days), was comprised solely of mature adipocytes. These colonies typically contained <50 cells that had large cytoplasmic fat droplets (Fig. 2A, a). Most of the cells contained a single cytoplasmic fat droplet that filled the entire cell. Because they did not expand beyond 50 cells and could not be propagated as clonal lines, we conclude that these colonies are comprised of mature, postmitotic adipocytes. The second colony type contained a mixture of mature-looking adipocytes and undifferentiated fibroblastic cells (Fig. 2A, b). The mature-looking adipocytes were typically located near the center of the colony, whereas the undifferentiated fibroblastic cells were at the periphery. Although these colonies were often observed within 5 days, they gradually became more prominent over the 2- to 3-week selection period. Essentially all of these colonies could be propagated as clonal cell lines; however, those cells that exhibited the adipocyte morphology gradually lost it during clonal expansion. The third colony type was comprised solely of undifferentiated fibroblastic cells (Fig. 2A, c), and, as expected, these colonies could be propagated as clonal lines. In contrast to WZLneo-C/EBP α , the parental retrovirus (WZLneo) produced only undifferentiated fibroblastic colonies that had the morphology of that shown in Figure 2A(c). We have never observed adipocyte colonies in the control infections (transfections) with any cell line (Table 1). These observations and others (see below and discussion) argue that the emergence of the adipocyte colonies in the WZLneo-C/EBP α -infected (transfected) dishes is not attributable to spontaneous differentiation.

Table 1 summarizes the results of over a dozen experiments. To simplify presentation of a large amount of data, results from retroviral infection and DNA transfection experiments were combined. It should be mentioned here, however, that the adipogenic program could be induced more efficiently (by approximately fivefold) when the WZLneo-C/EBP α vector was introduced into cells by retroviral infection compared with CaPO₄-DNA transfection. Infection of mouse fibroblastic cells with WZLneo-C/EBP α generated large numbers of single, isolated, postmitotic adipocytes, making it difficult to determine the true number of adipocyte colonies. Thus, only macroscopic G418-resistant colonies were scored. Colonies in which >50% of the cells contained lipid

Figure 1. Schematic diagram of WZLneo-based retroviral vectors. (Solid rectangles) Mo-MuLV LTR; the internal ribosome entry site (IRES), C/EBP α , C/EBP α Δ 1-117, C/EBP β , and neomycin genes are indicated. The approximate positions of the first three C/EBP α AUG codons are indicated by the lines above the C/EBP α gene. The region of C/EBP α that was deleted to generate WZLneo-C/EBP α Δ 1-117 is indicated below the box by the bracket. The arrows indicate the transcription initiation site. (B) *Bam*HI, (E) *Eco*RI.

Figure 2. (A) Photomicrographs of the three types of macroscopic G418-resistant colonies generated by infection of mouse fibroblastic cells with WZLneo-C/EBP α . Results for NIH-3T3 cells are shown. (a) Mature, postmitotic adipocyte colony; (b) adipocyte colony containing a mixture of mature-looking adipocytes and undifferentiated fibroblastic cells; (c) undifferentiated fibroblastic colony. Refractile objects in the cytoplasm are lipid droplets. Magnification, 40 $\times$ for a and b; 20 $\times$ for c. Other mouse fibroblastic cell lines gave similar results. (B) Oil Red O staining of dishes following G418 selection for 2 weeks. (Left) NIH-3T3 cells infected with WZLneo-C/EBP α ; (right) NIH-3T3 cells infected with WZLneo. Each dish contained between 100 and 200 macroscopic G418-resistant colonies. (C) Photomicrograph of a mature adipocyte colony 10 days after infection of NIH-3T3 cells with WZLneo-C/EBP α . Cells were stained with Oil Red O. Magnification, 32.5 $\times$.

droplets were considered to be adipocyte colonies. Infection of NIH-3T3 cells with WZLneo-C/EBP α gave striking results. We observed reproducibly that >50% of G418-resistant colonies in the WZLneo-C/EBP α -infected dishes showed lipid droplets that stained positive with Oil Red O (Fig. 2B,C). Such results were obtained by use of a wide range of multiplicity of infection (from 10^{-4} to 1). For reasons unknown, cells had to be subcultured at low density following retroviral infection to obtain such striking results. All seven of the other mouse fibroblastic cell lines produced similar, but less dramatic, results. In these cases, the frequency of adipocyte colonies varied between 1 and 50%. Surprisingly, three mouse fibroblastic lines (NIH-3T3, BALB/c-3T3, and C3H10T1/2) were more adipogenic than the 3T3-L1 ad-

ipoblast line. The low frequency of adipose conversion observed with the three virus-packaging cell lines (PA317, ψ -CRE, and ψ -CRIP) is somewhat paradoxical given that these cells are derived from NIH-3T3 cells. Infection of human diploid fibroblasts (MRC-5 and WI-38) with WZLneo-C/EBP α failed to produce either single, isolated adipocytes or macroscopic adipocyte colonies, even though G418-resistant cells expressed the authentic C/EBP α protein (see Fig. 4, lanes 13 and 15, below). Because single, isolated, postmitotic adipocytes were not included in these analyses, it is likely that the results shown in Table 1 underestimate the true ability of C/EBP α to promote the adipogenic program.

In sharp contrast, infection (transfection) of mouse fibroblastic cells with either WZLneo-C/EBP α Δ 1-117 or

Table 1. Percentage of G418-resistant colonies that exhibited the adipocyte morphology

Cell Line	WZLneo	Retroviral vector		
		C/EBP α	WZLneo-C/EBP α Δ 1-117	C/EBP β ^a
NIH-3T3	0	10-85	0	0
BALB/c-3T3	0	5-50	0	0
C3H10T1/2	0	1-30	0	0
3T3-L1	0	1-15	0	0
Swiss 3T3	0	1-15	0	0
PA317	0	1-5	0	0
ψ CRE	0	1-5	0	0
ψ CRIP	0	1-5	0	0
MRC-5	0	0	0	0
WI-38	0	0	0	0

The numbers represent the percentage of macroscopic G418-resistant colonies that exhibited the adipocyte morphology. Only those colonies in which more than 50% of the cells contained lipid droplets were considered as adipocyte colonies. Results from retroviral infection and DNA transfection experiments were combined. Because the percentage of G418-resistant colonies that exhibited the adipocyte morphology varied from experiment to experiment and was dependent on the method of gene transfer, ranges are given. Other C/EBP α expression vectors (pMSV-C/EBP α and LNL6-based retroviral vectors) were also capable of promoting the adipogenic program in mouse fibroblastic cells. However, when transcription of the neomycin and C/EBP α genes was driven by separate viral promoters (either in the same vector or in separate vectors), the frequency of adipose conversion was 5- to 10-fold less than that observed with WZLneo-C/EBP α (S. Freytag et al., unpubl.). ^aSome of the fibroblastic cell lines express detectable levels of the C/EBP β mRNA and protein. Transduction of WZLneo-C/EBP β resulted in increased expression of C/EBP β (as determined by Western blotting and activation of C/EBP-dependent promoters) for at least 5 days, which is when the mature, postmitotic, adipocyte colonies began to appear in the WZLneo-C/EBP α -infected dishes. However, the increased expression of C/EBP β was transient, as clonal G418-resistant cell lines expressed C/EBP β at a level similar to that observed in parental cells.

WZLneo-C/EBP β failed to produce adipocyte colonies (Table 1), even though G418-resistant cells expressed the expected C/EBP protein (see Fig. 4, below, and Table 1 footnotes). Thus, the ability to promote the adipogenic program requires the potent transcriptional activation domain of C/EBP α and is not a universal property of the C/EBPs.

To demonstrate further that the emergence of the adipocyte colonies was attributable to expression of the exogenous C/EBP α gene and not to spontaneous differentiation, we determined whether the number of macroscopic adipocyte colonies observed correlated with the amount of pWZLneo-C/EBP α DNA used in the transfections. As expected, the number of adipocyte colonies observed increased with increasing amounts of pWZLneo-C/EBP α DNA (Table 2).

Development of the adipocyte morphology requires expression of C/EBP α

We initially attempted to use immunofluorescence to correlate development of the mature adipocyte morphology with expression of C/EBP α in isolated G418-resistant colonies. This method proved unreliable because the large fat droplet often obscured the nucleus. Others have encountered this problem (Weintraub et al. 1989). Therefore, isolated colonies were examined for expression of the proviral WZLneo-C/EBP α RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis as described previously (Freytag and Geddes 1992). Although the primers used in these analyses hybridize to the neomycin gene, they detect the same RNA transcript that generates C/EBP α (because of the dicistronic nature of proviral RNA transcript). [RT-PCR analysis with primers that hybridize to the Mo-MuLV LTR and C/EBP α gene (Freytag and Geddes 1992) proved to be unreliable with the WZLneo-C/EBP α proviral RNA template. This may be attributable to secondary structure in the proviral RNA transcript.] These analyses demonstrated that development of the mature adipocyte morphology required expression of the WZLneo-C/EBP α proviral RNA. Results for NIH-3T3 cells are shown in Figure 3. All 12 of the mature adipocyte colonies examined expressed the WZLneo-C/EBP α proviral RNA [Fig. 3 top, lanes 1-12]. Generation of the WZLneo-C/EBP α RT-PCR product was dependent on the addition of reverse transcriptase (lane 13) and was eliminated by pretreatment of extracts with DNase-free RNase (lane 14) but not RQ1 DNase (lane 15), indicating that it was generated from an RNA template. Similar results were obtained with mature adipocyte colonies generated by WZLneo-C/EBP α infection of other mouse fibroblastic cells. In contrast, no specific RT-PCR product was observed with RNA from uninfected NIH-3T3 cells (lane 18). [Because the PCR primers hybridize to the neomycin gene, we could not use WZLneo-infected cells as controls in these experiments.] To confirm that cells in the mature adipocyte colonies had activated the adipogenic

Table 2. Correlation between the amount of pWZLneo-C/EBP α DNA and adipocyte colony formation in NIH-3T3 cells

Amount of DNA (μ g)			
pWZLneo	pWZLneo-C/EBP α	G418-resistant colonies ^a (no.)	Adipocyte colonies ^a (no.)
30	0	643	0
29	1	657	8
27	3	611	14
20	10	417	34 ^b
0	30	54	29 ^b

^aThe numbers represent the average of three experiments. The variance was ~20%. Only macroscopic adipocyte colonies were counted.

^bThese transfections generated large numbers of single, isolated postmitotic adipocytes, which were not included in the results.

Figure 3. Expression of WZLneo-C/EBP α proviral and aP2 RNA in mature adipocyte colonies. Results for NIH-3T3 cells are shown. (Top) RT-PCR analysis for WZLneo-C/EBP α proviral RNA transcript. (Lanes 1–12) Extracts from mature adipocyte colonies; (lane 13) same as lane 1, but without reverse transcriptase; (lane 14) same as lane 1, but the extract was pretreated with RNase; (lane 15) same as lane 1, but the extract was pretreated with RQ1 DNase; (lane 16) same as lane 1, but with a 10-fold dilution of the extract; (lane 17) same as lane 1; (lane 18) extract from uninfected NIH-3T3 cells. (Bottom) RT-PCR analysis for aP2 RNA transcripts. (Lanes 19–24) Extracts from mature adipocyte colonies; (lanes 25–30) extracts from undifferentiated fibroblastic colonies infected with WZLneo; (lane 31) same as lane 19, but without reverse transcriptase; (lane 32) same as lane 19, but the extract was pretreated with RNase; (lane 33) same as lane 19, but the extract was pretreated with RQ1 DNase.

program, we examined them for expression of aP2 RNA transcripts (Freytag and Geddes 1992). As expected, those colonies that exhibited the mature adipocyte morphology expressed the mature aP2 mRNA [Fig. 3 (bot-

tom), lanes 19–24]. In contrast, colonies infected with the WZLneo retrovirus did not [Fig. 3 (bottom), lanes 25–30].

Because those G418-resistant colonies that contained a mixture of mature-looking adipocytes and undifferentiated fibroblastic cells could be propagated as clonal lines, we could examine them for expression of C/EBP α by Western blotting. Overall, 98% (138 of 141) of clonal lines derived from these adipocyte colonies expressed the authentic 42-kD C/EBP α protein. Surprisingly, many of these lines expressed C/EBP α at a level approaching that observed in rat liver and terminally differentiated 3T3-L1 adipocytes (Fig. 4). In contrast, cell lines derived from control infections (transfections) (Fig. 4, lanes 1,6,9,12,18,21), or those infected with WZLneo-C/EBP α that did not exhibit the adipocyte morphology, either failed to express C/EBP α or expressed a mutant form of the protein (lanes 28–33). Thus, although ectopic expression of C/EBP α can efficiently promote the adipogenic program in mouse fibroblastic cells, cell lines that stably express high amounts of C/EBP α can readily be generated.

The results described above differ dramatically from those obtained previously with the 3T3-L1 adipoblast cell line. All previous attempts to isolate 3T3-L1 adipoblast lines stably expressing C/EBP α have failed (Umek et al. 1991; Freytag and Geddes 1992; Lin et al. 1993). This raised the concern that the C/EBP α protein detected in the Western blots was inactive (because of post-translational modification, complex with another protein, or selection of cell lines that express a mutant C/EBP α protein). To address this possibility, C/EBP α -

Figure 4. Western blot analysis of clonal cell lines for expression of C/EBP α . Soluble nuclear protein from $\sim 10^5$ nuclei was applied to each lane. Cell lines are indicated above the blots. (Top) Transfected cell lines. (Lanes 1,6,9,12) Cells transfected with pWZLneo; (lanes 2,3,5,7,8,10,11) cells transfected with pWZLneo-C/EBP α ; (lane 4) rat liver. (Bottom) Retrovirus-infected cell lines. (Lanes 14,16,18,21,26) Cells infected with WZLneo; (lanes 13,15,19,20,22–24) cells infected with WZLneo-C/EBP α ; (lanes 28–33) NIH-3T3 cells infected with WZLneo-C/EBP α that did not exhibit the adipocyte morphology; (lane 25) NIH-3T3 cells infected with WZLneo-C/EBP α Δ 1–117; (lanes 17,27) rat liver. The arrowhead indicates a larger, presumably mutant, C/EBP α protein. The 42- and 30-kDa C/EBP α proteins are indicated. (RL) Rat liver. Numbers at right indicate molecular mass of prestained protein markers in kilodaltons.

expressing cell lines were examined for the ability to stimulate transcription from a transfected plasmid that contained a C/EBP α -dependent promoter (aP2 promoter; Christy et al. 1989). Results for two NIH-3T3 cell lines that express different amounts of C/EBP α (by about fivefold; see Fig. 4, lanes 7,8) are shown in Figure 5. The wild-type aP2 promoter was 14-fold more active in cells that express high amounts of C/EBP α than a promoter that lacks a functional C/EBP α -binding site (Fig. 5, lanes 1,2). A cell line that expresses lower amounts of C/EBP α showed less activation (fourfold, lanes 3,4), and NIH-3T3 cells, which do not express C/EBP α , showed no activation (lanes 5,6). Thus, the degree of stimulation of the aP2 promoter correlated well with expression of C/EBP α . These results indicate that the C/EBP α protein is active in NIH-3T3 cells. Similar results were obtained with two other cellular promoters that contain C/EBP-binding sites (M-CSF and c-fos promoter), and with other C/EBP α -expressing fibroblastic cell lines (S. Freytag, D. Paielli, and J. Gilbert, unpubl.). Thus, in contrast to 3T3-L1 adipoblasts, fibroblastic cells (NIH-3T3, BALB/c-3T3, C3H10T1/2, PA317, ψ CRE, and ψ CRIP) that stably express high amounts of C/EBP α can be propagated as clonal lines.

Although most of the clonal C/EBP α -expressing cell lines (NIH-3T3, BALB/c-3T3, and C3H10T1/2) exhibited normal growth rates, longer doubling times (by two- to fourfold) were observed for the virus-packaging cell lines and human fibroblasts that expressed high amounts of C/EBP α (S. Freytag et al., unpubl.). Only a small fraction of cells (<1%) in lines that expressed high amounts of C/EBP α contained lipid droplets when proliferating.

Figure 5. Activation of the aP2 promoter in C/EBP α -expressing NIH-3T3 cells. Cells were transiently transfected with an aP2 promoter-CAT plasmid that contained either a wild-type (W) or mutant (M) C/EBP-binding site (Christy et al. 1989). (Lanes 1,2) Clonal NIH-3T3 cell line that expresses high amounts of C/EBP α (see Fig. 4, lane 8); (lanes 3,4) clonal NIH-3T3 cell line that expresses moderate amounts of C/EBP α (see Fig. 4, lane 7); (lanes 5,6) NIH-3T3 cells. The percent conversion (% conv) of the [14 C]chloramphenicol substrate into all acetylated products, after correcting for transfection efficiency, is shown below each lane.

None of the C/EBP α -expressing cell lines accumulated lipid droplets when made quiescent by serum-deprivation or high cell density. Thus, expression of C/EBP α , at the level observed in the stable cell lines, is not sufficient for overt differentiation.

High stable expression of C/EBP α promotes adipogenesis in BALB/c-3T3 cells

Clonal variants of mouse fibroblastic cells that have the proclivity to terminally differentiate into adipocytes have been isolated and characterized. Such clonal variants can either arise spontaneously [3T3-L1 from Swiss 3T3 (Green and Kehinde 1974); BALB/c-T-3T3 from BALB/c-3T3 (Diamond et al. 1977)] or be generated by treatment of cells with agents that alter DNA methylation [TA1 from C3H10T1/2, Chapman et al. 1984]. Although these variant cell lines typically show a low frequency of spontaneous differentiation, they can be induced to differentiate at high frequency by treatment with lipogenic hormones [3T3-L1 (Rubin et al. 1978)], high concentrations of human plasma [BALB/c-T-3T3 (Scott et al. 1982)] or high cell density [TA1 (Chapman et al. 1984)]. To determine whether clonal fibroblastic cell lines that stably express high amounts of C/EBP α could be induced to terminally differentiate, cells were treated with a variety of known differentiation inducers at either low or high cell density. Except for the C/EBP α -expressing BALB/c-3T3 cell lines, none of the clonal lines could be induced to differentiate overtly. BALB/c-3T3 clones that express high amounts of C/EBP α rapidly (within 24 hr) showed signs of overt differentiation when placed in medium containing 10% human plasma. Such cells accumulated numerous cytoplasmic lipid droplets that ultimately coalesced into one large droplet that filled the cytoplasm (Fig. 6b). Over 90% of cells in lines that expressed high amounts of C/EBP α differentiated to this extent. Cell lines that expressed lower amounts of C/EBP α differentiated, although less well (10–30%). The ability to overtly differentiate was unique to C/EBP α -expressing cells, as control and uninfected BALB/c-3T3 cells did not differentiate well (~0.1%) under these conditions (Fig. 6d). Differentiation was not induced by quiescence, as C/EBP α -expressing cells exposed to low concentrations of serum (0.5%) quiesced but did not differentiate.

To confirm that C/EBP α -expressing BALB/c-3T3 cells could activate the genetic program of the mature adipocyte, we examined them for expression of two genes that are markedly induced (endogenous C/EBP α and aP2 genes; Christy et al. 1989) and one that is suppressed [pro α 2(I) collagen; Weiner et al. 1989] during adipogenesis. Expression of the endogenous aP2 and C/EBP α genes was dramatically induced, and that of the pro α 2(I) collagen gene was suppressed, in C/EBP α -expressing cells following exposure to 10% human plasma (Fig. 7, cell line 1.4). In contrast, expression of these genes was unaffected in control cells (cell line 3.5). Cells that overtly differentiated did not replicate DNA when returned to medium that favors growth (S. Freytag, D. Paielli, and J. Gilbert,

Figure 6. Photomicrographs of C/EBP α -expressing and control BALB/c-3T3 cell lines. (a,b) Clonal cell line that expresses high amounts of C/EBP α from a stably transfected pMSV-C/EBP α gene; (c,d) clonal cell line derived from control transfections that does not express C/EBP α . (a,c) Proliferating cells; (b,d) cells exposed to differentiation medium for 9 days. BALB/c-3T3 cells infected with the WZLneo-based retroviral vectors gave similar results. Magnification, 17.5 $\times$ for a,c, and d; 35 $\times$ for b.

unpubl.). Thus, high stable expression of C/EBP α in BALB/c-3T3 cells dramatically enhances their ability to differentiate into mature, postmitotic adipocytes.

Discussion

This study demonstrates clearly that ectopic expression of C/EBP α can promote the adipogenic program in a variety of mouse fibroblastic cells. Transduction of the C/EBP α gene into eight mouse fibroblastic lines [NIH-3T3, BALB/c-3T3, C3H10T1/2, Swiss 3T3, 3T3-L1, PA317, ψ CRE, and ψ CRIP] led to the emergence of adipocyte colonies at variable frequencies. The most dramatic results were obtained with NIH-3T3 cells, in which the percentage of G418-resistant colonies that exhibited the adipocyte morphology was reproducibly >50% when the C/EBP α gene was transduced by retroviruses. Six observations make a compelling argument that the emergence of adipocyte colonies in the WZLneo-C/EBP α -infected dishes is attributable to expression of the introduced C/EBP α gene and not to spontaneous differentiation: (1) Development of the mature adipocyte morphology requires expression of the WZLneo-C/EBP α proviral RNA transcript; (2) 98% of the adipocyte colonies that could be propagated as clonal lines express the 42-kD C/EBP α protein, which, in those cases examined, is biologically active; (3) three WZLneo-based retroviruses that do not encode authentic C/EBP α fail to produce adipocyte colonies; (4) infection of mouse fibroblastic cells with WZLneo-C/EBP α generates adipocyte colonies at a frequency four to seven orders of magnitude above the spontaneous differentiation frequency; (5) the number of adipocyte colonies observed correlates with the amount of pWZLneo-C/EBP α DNA used in transfection experiments; and (6) >50% of G418-resistant colonies generated by WZLneo-C/EBP α infection of NIH-

3T3 cells exhibit the adipocyte morphology. The high frequency at which WZLneo-C/EBP α generates adipocyte colonies in NIH-3T3 cells suggests that expression of C/EBP α may be sufficient to induce the adipogenic program in these cells. It is possible, and likely, that induction of the adipogenic program in mouse fibroblastic cells involves the activity of C/EBP α as well as other C/EBP family members (Cao et al. 1991; Williams et al. 1991).

The precise role of C/EBP α in adipoblast differentiation has been controversial. Two previous studies that examined whether ectopic expression of C/EBP α could promote the adipogenic program in 3T3-L1 adipoblasts gave somewhat conflicting results. Conditional activation of a C/EBP α -estrogen receptor fusion protein in 3T3-L1 adipoblasts suppressed growth; however, it was not sufficient for overt differentiation (Umek et al. 1991). In contrast, transduction of a gene that encodes authentic C/EBP α into 3T3-L1 adipoblasts by CaPO₄-DNA transfection suppressed clonal cell growth and caused ~12% of G418-resistant colonies to develop into mature, postmitotic adipocytes (Freytag and Geddes 1992). Thus, although both studies agreed that C/EBP α has a potent antimitogenic effect in 3T3-L1 adipoblasts, they disagreed with regard to the ability of C/EBP α to promote the adipogenic program. The results presented here confirm and significantly extend our previous findings with 3T3-L1 adipoblasts and support the general conclusion that C/EBP α is an important component of the adipogenic differentiation control mechanism (Umek et al. 1991; Freytag and Geddes 1992). That the potent transcriptional activation domain of C/EBP α is required for its ability to promote the adipogenic program (this study) is consistent with previous observations that mutants of C/EBP α lacking this domain are unable to activate differentiation-specific genes (Friedman et al. 1989; Pei and

Figure 7. Expression of endogenous aP2, C/EBP α , and pro α 2(I) collagen mRNAs in C/EBP α -expressing and control BALB/c-3T3 cell lines. (–) Untreated cells; (+) cells incubated in differentiation medium for 9 days. Cell line 1.4 expresses high amounts of C/EBP α from a stably transfected pMSV-C/EBP α gene; cell line 3.5 was derived from control transfections and does not express C/EBP α . Equal loading among lanes was confirmed by staining gels with ethidium bromide. Positions of the various RNAs are indicated. Arrowheads indicate positions of the 28S and 18S rRNAs.

Shih 1991; Nerlov and Ziff 1994). It is likely that induction of the adipogenic program in mouse fibroblastic cells requires a minimum threshold level of the authentic 42-kD C/EBP α protein. Similar conclusions have been made by Lane and colleagues with an inducible C/EBP α expression system (D. Lane, pers. comm.).

Interestingly, even though C/EBP α was able to induce the adipogenic program in mouse fibroblastic cells, it was unable to do so in human diploid fibroblasts. Although this result might be explained on the basis of species differences (i.e., rat C/EBP α in mouse vs. human cells), we favor an alternative, albeit unproved, hypothesis that the ability of C/EBP α to promote the adipogenic program depends upon the developmental state of the host cell. Whereas all eight mouse fibroblastic cell lines were derived from embryos, the human diploid fibroblasts were derived from fetal lung tissue. It is possible that once a cell has progressed past a certain developmental point and/or has committed to a particular cell lineage, it is refractory to the effects of C/EBP α . Along these lines, we have observed that infection of C2C12 myoblasts with WZLneo-C/EBP α generates adipocyte colonies even though they express high amounts of the myogenic regulator MyoD (S. Freytag, D. Paielli, and J. Gilbert, unpubl.). It will be interesting to determine

whether adipogenesis is possible in C2C12 cells (and other cell types) once the myogenic program is activated (and vice versa).

In spite of its antimitogenic effects (Umek et al. 1991; Freytag and Geddes 1992), fibroblastic cell lines that stably express high amounts of C/EBP α can readily be generated. Although high stable expression of C/EBP α did suppress the growth rate of some fibroblastic cells, this effect was not observed in all cell types examined. It is likely that the effect of C/EBP α on cell growth depends on the particular cellular context and its level of expression. That C/EBP α is abundantly expressed in immature myelomonocytic cells, which are capable of proliferation, is entirely consistent with this view (Scott et al. 1992; S. Freytag et al., unpubl.). None of the cell lines that stably express C/EBP α accumulated lipid droplets when made quiescent by serum deprivation or high cell density. Thus, expression of C/EBP α , at the level observed in the stable cell lines, is not sufficient for overt differentiation. However, BALB/c-3T3 cell lines that stably express high amounts of C/EBP α could be induced to terminally differentiate at high frequency. These observations and others indicate that C/EBP α can direct cells along a specific developmental pathway without suppressing proliferation. It is possible that the involvement of C/EBP α in the regulation of cell specification may not be limited to terminal differentiation.

It is interesting to compare and contrast the properties of the C/EBPs with other families of transcription factors, such as the MyoD family of proteins (Weintraub et al. 1991). The C/EBPs and MyoD-like proteins are related structurally in that they both contain a basic region juxtaposed to a motif [leucine zipper in the C/EBPs, helix-loop-helix (HLH) in the MyoD family] that serves as an interface for subunit dimerization. Both the basic region, which makes contact with DNA, and the dimerization motifs are required for biological activity. The C/EBPs and MyoD-like proteins are related functionally in that they both regulate cell growth and differentiation. Expression of C/EBP α and the MyoD-like proteins in fibroblastic cells suppresses clonal cell growth and promotes terminal differentiation (Davis et al. 1987; Umek et al. 1991; Freytag and Geddes 1992; this study). Nevertheless, in spite of their antimitogenic effects, high expression of these proteins is tolerated in many fibroblastic cell lines (Davis et al. 1987; this study). It is thought that the differentiation-promoting and growth-suppressing effects of the myogenic regulators are suppressed in proliferating cells by the expression of another HLH protein called Id (Benezra et al. 1990; Weintraub et al. 1991). Although negative regulators of the C/EBPs do exist (Ron and Habener 1992; Lin et al. 1993; Ossipow et al. 1993), they are not expressed in proliferating cells. Thus, it is unclear why expression of C/EBP α is tolerated in some cell types but not in others, and why the ability of C/EBP α to promote the adipogenic program varies widely among fibroblastic cells. It is clear from this study and others that proteins of both the leucine zipper and HLH class can function as regulators of cell specification. It is interesting that although fat and muscle can

develop from the same precursor stem cell (Taylor and Jones 1979), they use different classes of sequence-specific DNA-binding proteins to execute their respective developmental program.

An important difference between C/EBP α and the MyoD family of proteins is that expression of C/EBP α is not restricted to one specialized cell type, or cells derived from one germ layer. Whereas the myogenic regulators are expressed only in skeletal muscle (mesoderm), C/EBP α is abundantly expressed in adipose (mesoderm), liver (endoderm), and proliferating myelomonoblasts (mesoderm). The ability to transduce the C/EBP genes by use of retroviruses will enable us to examine whether they regulate the development of other cell lineages.

Materials and methods

Cells and plasmids

The following cell lines were obtained from the American Type Culture Collection: BALB/c-3T3, C3H10T1/2, NIH-3T3, PA317, Swiss 3T3, Swiss 3T3-L1, WI-38, and MRC-5. Cells from several different lots were used to control for lot-to-lot variation. Cells that were maintained on a strict subculturing schedule gave best results. ψ CRE and ψ CRIP cells (Danos and Mulligan 1988) were obtained from R. Mulligan. Cells were grown in Dulbecco's modified Eagle medium supplemented with either 10% fetal calf serum or calf serum as recommended by the supplier. To induce the adipogenic program in C/EBP α -expressing BALB/c-3T3 cells, cells were plated at low density ($\sim 10^3$ cells/cm²) in medium containing 10% calf serum. On the following day, cells were incubated in medium containing 2.0% calf serum, 10% human plasma, and 30 U/ml of heparin for 6–9 days.

The parental retroviral vector, pWZLneo, was obtained from R. Boyce (Massachusetts General Hospital, Boston). pWZLneo-C/EBP α was generated by insertion of a 1.5-kb *Bam*HI–*Bss*HI restriction fragment of pMSV-C/EBP α (Umek et al. 1991), which contains the entire rat C/EBP α -coding sequence, between the *Bam*HI and *Eco*RI sites of pWZLneo. To generate pWZLneo-C/EBP α Δ 1–117, a 340-bp *Bam*HI–*Aat*II restriction fragment of pMSV-C/EBP α , which contains the first two AUG codons, was deleted generating pMSV-C/EBP α Δ 1–117. The 1.2-kb *Bam*HI–*Bss*HI restriction fragment of pMSV-C/EBP α Δ 1–117 containing the deleted C/EBP α gene was then inserted between the *Bam*HI and *Eco*RI sites of pWZLneo. pWZLneo-C/EBP β was generated by insertion of a 1.55-kb *Bam*HI–*Hind*III restriction fragment of pMEXCRP2 (Williams et al. 1991) between the *Bam*HI and *Eco*RI sites of pWZLneo.

Gene transduction

CaPO₄-DNA transfections were performed as described previously (Freytag and Geddes 1992). To generate retrovirus-producing cell lines, packaging cell lines were transfected with 10 μ g of the various retroviral vectors using the CaPO₄-DNA transfection method. Two days following transfection, filtered viral supernatants from the ecotropic packaging cell line (ψ CRE) were used to infect the amphotropic packaging lines (ψ CRIP and PA317) (and vice versa). Two days following infection, cells were selected in G418 (1 mg/ml, 50–60% active; GIBCO) for 10–14 days. Clonal cell lines were isolated and titered on NIH-3T3 cells. Titers of retrovirus-producing cell lines used in this study were 10^5 – 10^6 cfu/ml. In all retroviral infections, recipient

cells (2×10^5 to 5×10^5 cells in a 60-mm-diam. dish) were incubated with retrovirus for 2.5 hr in the presence of 8 μ g/ml of polybrene. Cells were subcultured (1:5) 2 days later in medium containing G418.

General methods

RT-PCR analysis for aP2 mRNA transcripts and Western blotting was performed exactly as described previously (Freytag and Geddes 1992). The following primers, which hybridize to the neomycin gene and generate a 323-bp product, were used in RT-PCR experiments to detect the WZLneo-C/EBP α proviral RNA: 5'-ATCAGGATGATCTGGACGAAGAGC-3' (5' primer); 5'-GTCAAGAAGGCGATAGAAGGCGAT-3' (3' primer). Conditions for PCR were those described previously for pMSV-C/EBP α (Freytag and Geddes 1992). In most experiments, the 3' primer was 5'-end-labeled with [γ -³²P]ATP. CaPO₄-DNA transfection of aP2 promoter-CAT plasmids was performed with 20 μ g of the reporter gene and 5 μ g of pRSV- β gal as an internal control. Cells were harvested 40 hr after addition of the CaPO₄-DNA precipitate. Chloramphenicol acetyltransferase (CAT) and β -galactosidase assays were performed using standard procedures. The percent conversion of the [¹⁴C]chloramphenicol substrate into all acetylated products was determined by use of an AMBIS radioanalytic detector and normalized to β -galactosidase activity. Northern blot analysis of total RNA was performed using standard procedures.

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