Research Article

CCAAT-Enhancer Binding Protein: A Component of a Differentiation Switch

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The CCAAT-enhancer binding protein (C/EBP) has now been found to promote the terminal differentiation of adipocytes. During the normal course of adipogenesis, C/EBP expression is restricted to a terminal phase wherein proliferative growth is arrested, and specialized cell phenotype is first manifested. A conditional form of C/EBP was developed, making it feasible to test its capacity to regulate the differentiation of cultured adipocytes. Premature expression of C/EBP in adipoblasts caused a direct cessation of mitotic growth. Moreover, when abetted by the effects of three adipogenic hormones, C/EBP promoted terminal cell differentiation. Since C/EBP is expressed in a variety of tissues, it may have a fundamental role in regulating the balance between cell growth and differentiation in higher animals.

THE FORMATION OF DIFFERENTIATED CELL TYPES IN HIGHER animals is often coupled to the cessation of mitotic cell growth. Some tissues, including various components of the mammalian brain, reach a post-mitotic state shortly after birth. Other tissues, exemplified by the epithelial lining of the gut, contain proliferative stem cells that, throughout adult life, are continuously converted to the post-mitotic, terminally differentiated state. In either case, the ultimate, specialized properties of an organ or tissue layer can be traced through distinctive stages of mitotic growth and terminal differentiation.

The balance between cell proliferation and differentiation has long been recognized as a fundamental problem in biology. However, the molecular processes that govern this balance are poorly understood. For example, it is not known how stem cell populations are conserved so that they maintain the appropriate ratio between renewing and differentiating cells. Nor is it known how acquisition of the terminally differentiated state is linked to growth arrest.

We have now identified a transcription factor that may participate in growth arrest and terminal cell differentiation. This factor, termed CCAAT-enhancer binding protein (C/EBP), binds DNA in a sequence-specific manner (*1*). The protein has been purified (*2*), and its encoding gene has been cloned (*3*). Such reagents have led to specific antibody and nucleic acid hybridization probes, allowing detection of C/EBP in various tissues including liver, lung, adipose, gut, and placenta (*4*).

Several lines of evidence indicate that C/EBP functions exclusively in terminally differentiated, growth-arrested cells. In adult liver, C/EBP is restricted to the nuclei of mature hepatocytes (*4*). By contrast, rapidly proliferating hepatoma cells, whether derived from human or rodent liver, express only a small fraction of the C/EBP characteristic of normal hepatocytes (*5*). Western (immuno) and Northern (RNA) blot assays have shown that C/EBP is also restricted to the terminally differentiated cells of adipose tissue. Cultured 3T3-L1 adipoblasts do not express C/EBP during proliferative growth, yet exhibit a marked induction of this transcription factor when stimulated to differentiate into mature adipocytes (*4, 6*).

The correlation between C/EBP expression and terminal cell differentiation implies two potential regulatory roles—execution of specialized phenotype and acquisition of the growth-arrested state. Initial evidence consistent with C/EBP's proposed role in elaborating cell specialization has come from studies on the serum albumin gene (*5*), which is expressed exclusively in hepatocytes, and several adipose-specific genes that are expressed only upon terminal differentiation (*6–8*). Each of these putative target genes contains an avid C/EBP binding site that mediates activation by C/EBP as tested in transient transfection assays. The hypothetical role of C/EBP in growth arrest has heretofore not been studied.

We have investigated the capacity of ectopically expressed C/EBP to stimulate the differentiation of cultured adipoblasts. Since C/EBP is not expressed in proliferating adipoblasts, yet is believed to induce gene expression in terminally differentiated adipocytes, we sought to ascertain whether its premature expression might promote the terminal step of adipogenesis.

All initial attempts to derive adipoblast lines expressing functional C/EBP failed. Such efforts involved the co-transfection of 3T3-L1 adipoblasts with two plasmids, one encoding a neomycin-resistance marker (*9*), and another encoding native C/EBP (*5*). Inclusion of the C/EBP expression vector consistently led to a decrease in the number of neomycin-resistant colonies relative to transfection with the resistance marker alone. More importantly, immunological tests of more than 100 colonies that emerged from drug selection and were propagated as continuous cultures proved to be uniformly negative for C/EBP expression.

Having consistently failed to establish a C/EBP-expressing adipoblast cell line, we attempted to develop a means of expressing C/EBP conditionally. The adenovirus E1A and cMyc transforming proteins, when fused to the ligand binding domain of a steroid hormone receptor, have been shown to become regulated by the corresponding hormone (*10, 11*). We followed this strategy and prepared expression vectors encoding fusion proteins (*12*) in which C/EBP was linked through its carboxyl-terminal alanine to the hormone binding domain of either the glucocorticoid receptor (*13, 14*) or the estrogen receptor (*15, 16*).

Transient trans-activation assays were used to test whether these

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vectors might express a hormone-regulated form of C/EBP. Cultured hepatoma cells were cotransfected with a reporter plasmid consisting of the mouse albumin promoter linked to the herpesvirus thymidine kinase gene (*17*), along with an expression vector encoding native C/EBP, or derivatives fused to the hormone binding domain of either the glucocorticoid receptor (C/EBP-GR) or the estrogen receptor (C/EBP-ER). The results (Fig. 1A) demonstrate

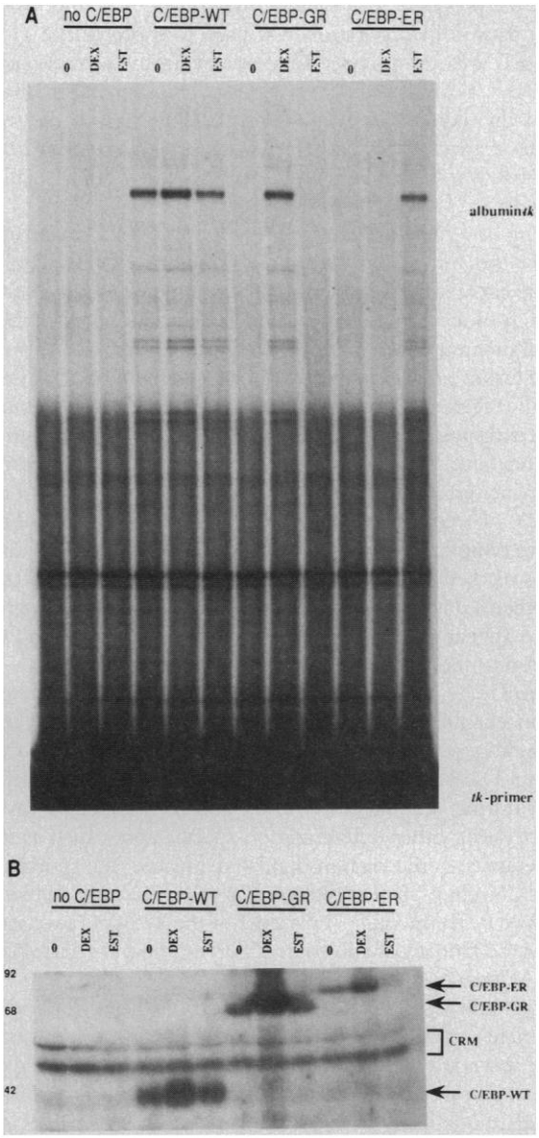

Fig. 1. Steroid-hormone regulated forms of C/EBP. (**A**) Primer extension analysis used to monitor trans-activation by various forms of C/EBP. A reporter plasmid containing the albumin promoter linked to the herpesvirus *tk* gene (*17*) was transfected into HepG2 hepatoma cells alone (no C/EBP) or together with plasmids expressing native C/EBP (C/EBP-WT), a C/EBP–glucocorticoid receptor fusion protein (C/EBP-GR), or a C/EBP–estrogen receptor fusion protein (C/EBP-ER). One day after transfection, culture media was changed and modified, as indicated, by the addition of 10^{-8}M dexamethasone (DEX) or 10^{-8}M β-estradiol (EST). Total RNA was harvested and primer extension analyses were performed as previously described (*5*). The position of the primer (*tk*-primer) and the primer extension product (albumin-*tk*) are indicated. (**B**) Western blot analysis used to quantify expression of C/EBP and C/EBP fusion proteins in transfected hepatoma cells. Protein was harvested from duplicate plates manipulated as in A, sized on an 8% polyacrylamide electrophoresis gel and Western blotted using an antiserum specific to C/EBP (*3*). The positions of C/EBP-WT, C/EBP-GR, C/EBP-ER, and cross-reacting material (CRM) are indicated. The positions of molecular size markers (in kilodaltons) are indicated on the left of the autoradiogram.

that activation of the albumin-TK reporter gene by native C/EBP is largely unaffected by steroid hormones. In marked contrast, activation by C/EBP-GR only occurs in the presence of dexamethasone. Likewise, activation by C/EBP-ER requires β-estradiol.

Western blots of duplicate cell cultures (Fig. 1B) demonstrated expression of the expected forms of C/EBP. When cultures were treated with dexamethasone, expression of each protein was consistently increased. We provisionally attribute this hormone-specified variation to the MSV (murine sarcoma virus), LTR (long terminal repeat) that was used to promote transcription from each expression vector. Transcription from the MSV LTR is known to be inducible by dexamethasone (*18*). Although the Western blot assays shown in (Fig. 1B) reveal considerable variation in protein concentration among the various experimental conditions, trans-activation levels fluctuated according to hormonal conditions rather than absolute protein level (Fig. 1A).

To prepare a stable cell line expressing the C/EBP-ER fusion protein, its encoding plasmid was co-transfected into 3T3-L1 adipoblasts along with a plasmid encoding the neomycin resistance marker. Cells were selected in phenol red–free medium that had been stripped of estrogen (*11*). We chose C/EBP-ER rather than C/EBP-GR because dexamethasone is itself growth inhibitory to adipoblasts (*20*), whereas β-estradiol is not (*19*). Protein extracts were prepared from individual clones and analyzed by Western blotting for the presence of the C/EBP-ER fusion protein. Several clones expressing C/EBP-ER were obtained and one was selected for further analysis. In addition, a neomycin-resistant clone that did not express the fusion protein was propagated to serve as an experimental control.

Terminal differentiation of 3T3-L1 cells results in distinct morphological changes, including the appearance of fat droplets. We initially asked whether hormone-mediated activation of C/EBP-ER was sufficient to cause terminal differentiation of adipoblasts. Both confluent and sub-confluent cultures of C/EBP-ER expressing adipoblasts were treated with 10^{-8}M β-estradiol. No overt signs of terminal differentiation were observed. Although estrogen stimulation of C/EBP-ER expressing cells was insufficient to directly execute terminal differentiation, addition of β-estradiol to proliferating cells caused an abrupt cessation of mitotic growth. The growth-inhibitory effect of estrogen was specific to the cell line that expressed C/EBP-ER (Fig. 2). Administration of estrogen to the control cell line did not affect cell growth. Three additional cell lines that express C/EBP-ER also became growth-arrested on exposure to estrogen, whereas mitotic growth of seven neomycin-resistant lines that did not express C/EBP-ER was unaffected (*19*).

The strict correlation between C/EBP-ER expression and estrogen-mediated growth inhibition pointed to the involvement of the fusion protein in growth arrest. We provisionally attribute the antiproliferative effects of the C/EBP-ER fusion protein to the C/EBP component of the chimeric protein rather than to the estrogen receptor ligand binding domain. This conclusion is supported by the prior observations of Yamamoto, Bishop, and colleagues who found that an analogously constructed Myc-ER fusion protein causes enhanced cell proliferation in response to estrogen (*11*). Since deregulated expression of Myc protein is understood to promote cell proliferation (*21*), it would appear to constitute the growth-promoting component of the Myc-ER fusion. We likewise believe that C/EBP is the growth inhibitory component of the C/EBP-ER fusion protein.

The antiproliferative effects of the C/EBP–ER fusion protein are reversible. Estrogen arrested cells were capable of repopulating the culture dish following estrogen removal (Fig. 2). Such observations indicate that the fusion protein arrests mitotic proliferation, rather than simply causing cell death. To further address the growth state

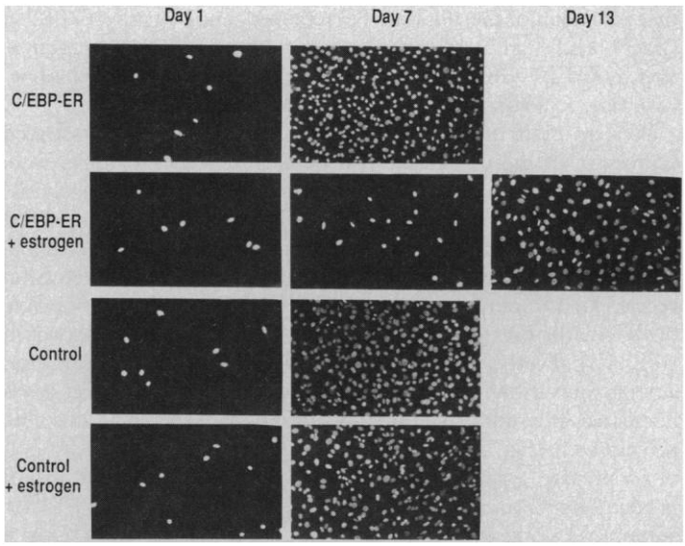

Fig. 2. Estrogen-mediated growth inhibition of 3T3-L1 cells expressing C/EBP-ER. Control 3T3-L1 cells and 3T3-L1 cells that express C/EBP-ER were seeded at low density on acid-etched cover slips. One day later, 10^{-8}M β-estradiol was added. Cover slips were harvested one and seven days after addition of estrogen. A sample of C/EBP-ER cells was cultured an additional 6 days in the absence of estrogen subsequent to 7 days incubation in the presence of 10^{-8}M β-estradiol (day 13). The cells were fixed, stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and photographed under ultraviolet illumination as reported (*5*). Cells were propagated in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10 percent calf serum (Gibco) in the presence of G418 sulfate (300 μg/ml active drug). β-Estradiol (Sigma) was dissolved in ethanol and added as a 100× stock.

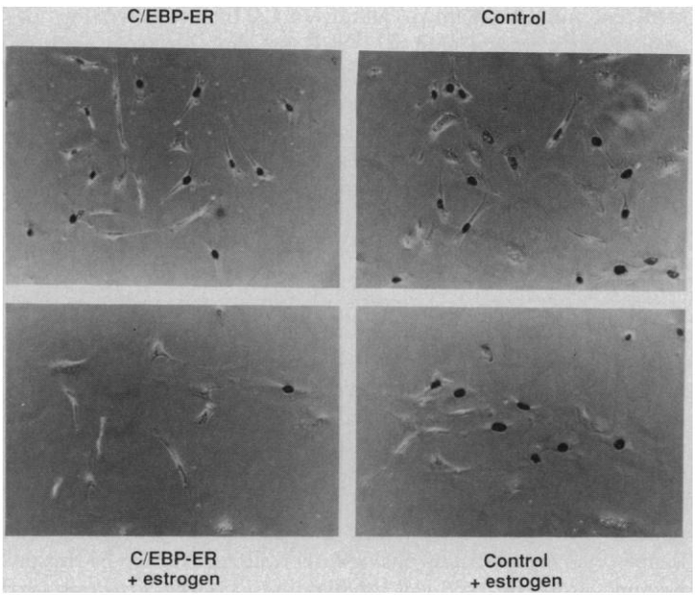

Fig. 3. Estrogen-mediated inhibition of DNA synthesis in 3T3-L1 cells expressing C/EBP-ER. Experimental and control 3T3-L1 cells were propagated on acid-etched cover slips at low density in the presence or absence of 10^{-8}M β-estradiol. Twenty-four hours after addition of estrogen, tritiated thymidine (5 μ Ci; 129 Ci/mmol) was added to each culture. Cover slips were harvested 1 hour later. Emulsion autoradiography was performed, and the cells were subsequently photographed by phase contrast optics. The fraction of 500 cells scoring positive for uptake of labeled precursor were: C/EBP-ER, 44 percent; C/EBP-ER plus estrogen, 13 percent; control, 39 percent; control plus estrogen, 40 percent. The fraction of 500 C/EBP-ER cells scoring positive for uptake of labeled precursor when continuously labeled for 24 hours after the removal of estrogen was 91 percent.

resulting from estrogen treatment of C/EBP-ER expressing cells, we monitored the incorporation of tritiated thymidine in the presence and absence of β-estradiol. Cells were labeled for a 1-hour interval, fixed, and monitored for thymidine incorporation by autoradiography (*22*). The C/EBP-ER expressing cells exposed to β-estradiol exhibited a significant reduction in the number of cells that incorporate label relative to untreated and control cells (Fig. 3). Estrogen affected only the C/EBP-ER expressing cell line, decreasing by 70 percent the proportion of cells synthesizing DNA (Fig. 3, legend). Finally, tritium incorporation was used to monitor the growth of C/EBP-ER expressing cells after release from hormone-mediated growth arrest. More than 90 percent of arrested cells incorporated tritiated thymidine within 24 hours after hormone was removed. Such observations substantiate the interpretation that the activity of C/EBP-ER was not detrimental to cell viability, but instead caused growth arrest.

Having observed that the C/EBP-ER fusion protein substantially impedes the mitotic growth of adipoblasts, we reexamined its potential effect on differentiation. Although derepression of C/EBP-ER by β-estradiol was alone insufficient to catalyze the overt terminal differentiation of adipocytes, we considered the possibility that the fusion protein might accelerate differentiation in association with other factors. One of the standard procedures for converting 3T3-L1 adipoblasts to differentiated adipocytes involves growth to confluency and subsequent culture medium supplementation with fetal bovine serum, insulin, dexamethasone, and methylisobutylxanthine (*23*). Three days later, dexamethasone and methylisobutylxanthine are removed. Although morphological evidence of differentiation, as marked by the accumulation of fat droplets, is not apparent for another 3 days, mRNA's characteristic of the differentiated state begin to appear soon after removal of dexamethasone and methylisobutylxanthine.

The mRNA's that are induced during terminal adipocyte differentiation encode lipogenic enzymes, transport proteins, and fatty acid binding proteins (*24*). As mentioned previously, the gene encoding C/EBP is also regulated in a differentiation-dependent manner during adipogenesis (*4, 6*) and its product is capable of trans-activating other differentiation-specific genes such as stearoyl-CoA desaturase, the insulin-regulated glucose transporter, and a fatty acid binding protein termed 422 (*6–8*). Using a hybridization probe specific to the mRNA product of the 422 gene, we set out to monitor the kinetics of adipocyte differentiation in control cells and in cells expressing C/EBP-ER.

Control and C/EBP-ER–expressing adipoblasts were induced to differentiate in the aforementioned manner in the continuous presence or absence of β-estradiol. Cultures were harvested daily and RNA was prepared for Northern blot analysis (*25*). The 422 mRNA increased dramatically in the estrogen-treated C/EBP-ER cells on the day after dexamethasone and methylisobutylxanthine were removed, 4 days after the addition of estrogen and the induction of differentiation (Fig. 4). A gradual increase in 422 mRNA, typical of parental 3T3-L1 cells, was observed for the C/EBP-ER cells that were not treated with estrogen, as well as for control cells, regardless of the presence of estrogen.

The results outlined in the preceding paragraph indicate that premature expression of C/EBP accentuates the expression of 422 mRNA. This result is consistent with earlier experiments that demonstrated C/EBP-mediated trans-activation of the 422 gene in transiently transfected adipoblasts (*6*). Indeed, they extend previous observations by providing evidence that C/EBP can activate the 422 gene that is endogenous to the adipocyte chromosome complement. The requirement for differentiation media to facilitate accumulation of 422 mRNA in estrogen-stimulated C/EBP-ER cells points to the involvement of additional components in the differentiation pro-

gram, perhaps regulated by the sequential addition and removal of dexamethasone and methylisobutylxanthine.

It may be important to point out that the C/EBP fusion protein and native C/EBP share a common dimerization interface (the leucine zipper). It is therefore possible that heteromeric complexes form during the latter stages of the differentiation program when endogenous C/EBP first begins to be expressed (*4*). It is also possible that the endogenous C/EBP gene is the target for activation by C/EBP-ER. Thus, although we can safely conclude that the C/EBP-ER fusion protein is capable of specifying growth arrest in the absence of endogenous C/EBP, its stimulatory role in terminal differentiation may be somehow channeled through the native transcription factor.

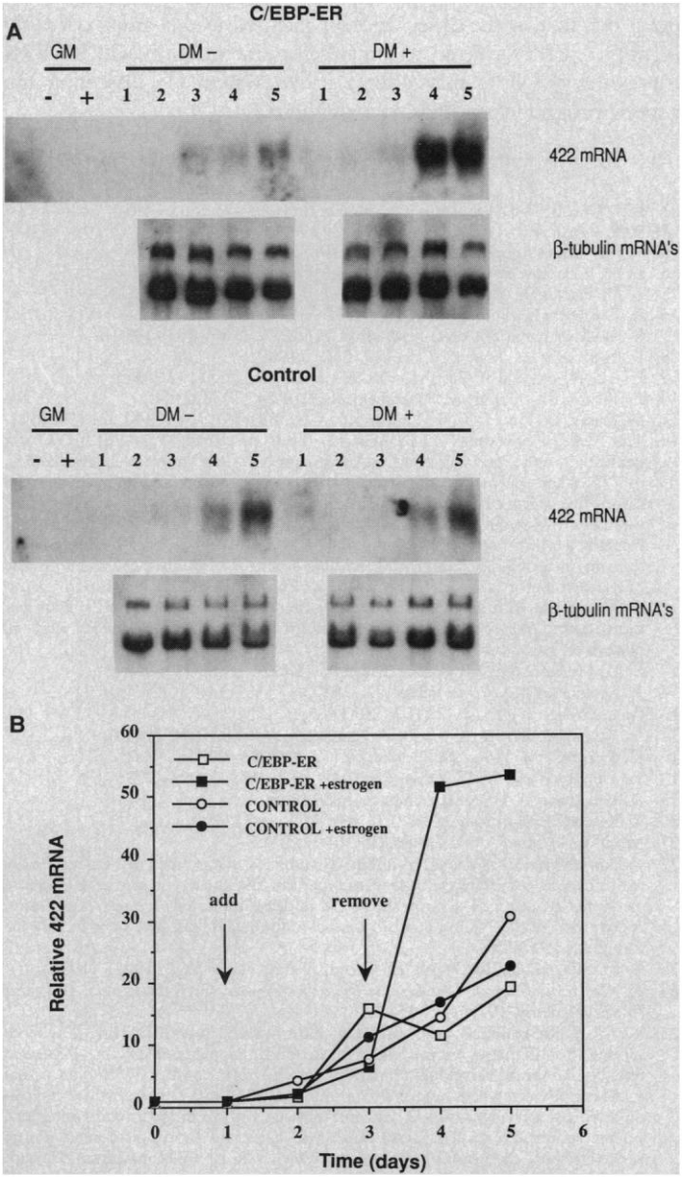

Fig. 4. Premature expression of C/EBP accentuates the accumulation of an adipocyte-specific mRNA. (**A**) Northern (RNA) blot analysis of 422 gene expression in control 3T3-L1 cells and those expressing C/EBP-ER fusion protein. Total RNA was harvested from control and experimental cells after propagation in growth media (GM) in the absence (−) or presence (+) of 10^{-8}M β-estradiol for 48 hours. In addition, cultures of experimental and control cells were treated with differentiation media (DM) to effect the conversion of adipoblasts to adipocytes (*23*) either in the absence (−) or presence (+) of 10^{-8}M β-estradiol. Total RNA was harvested each day for 5 days (1 to 5) after the administration of differentiation media and β-estradiol. As a control for the quantity of RNA placed in each lane, (i) a duplicate filter of RNA from days 2 to 5 was probed with a cDNA to β-tubulin (*35*) (shown below the 422 mRNA bands) and (ii) 28*S* and 18*S* ribosomal RNA's were visualized by ethidium bromide staining of duplicate gels. (**B**) Quantitation of the relative concentration of 422 mRNA as function of adipocyte differentiation. The band intensities in (A) were quantified by densitometry. Relative mRNA concentration for C/EBP-ER plus (■) and minus estrogen (□) and control cells plus (●) and minus (○) estrogen are displayed relative to the time course of adipocyte differentiation. After confluence (day 0), adipoblasts were exposed to culture. medium supplemented with fetal bovine serum, insulin, dexamethasone, and methylisobutylxanthine. Two days later, media was changed to remove dexamethasone and methylisobutylxanthine (remove).

The discovery that C/EBP can arrest cell growth and accentuate terminal differentiation prompted an analysis of the functional components of the protein required for each activity. In two earlier studies, we investigated the polypeptide components of C/EBP required for activation of a differentiation-specific gene (*5, 26*). In these studies, we identified four parts of C/EBP critical for activation of the serum albumin gene (Fig. 5). These include the subunit dimerization domain (termed the leucine zipper), the DNA contact surface (termed the basic region), and two "activation" domains (termed AR1 and AR2) located within a 120–amino acid segment of the protein.

To assess the polypeptide components of C/EBP required for growth inhibition, expression vectors encoding variants of C/EBP defective in each of the aforementioned parts of the protein were tested in a colony suppression assay. A 10-cm culture of thymidine kinase (TK)–deficient mouse fibroblasts was transfected with 150 ng of a plasmid containing the herpesvirus TK gene. Selection in medium containing hypoxanthine, aminopterin, and thymidine (HAT) (*27*), yielded 250 to 500 HAT-resistant colonies. Inclusion of an expression vector encoding native C/EBP caused a dramatic reduction in the number of transformant colonies (Table 1). In contrast, vectors encoding forms of C/EBP defective in the leucine zipper or basic region failed to cause a substantial reduction in colony formation. Removal of either AR1 or AR2 did not impair the colony suppression activity of C/EBP. However, a mutant form lacking both activating regions failed to suppress colony formation.

The results of the colony suppression assays (Table 1) indicate that defective forms of C/EBP can be expressed in proliferating mouse fibroblasts, but that intact C/EBP cannot. Evidence supportive of this conclusion came from immunological assays that tested for the presence of C/EBP or C/EBP variants in transformant colonies that emerged after HAT selection. No C/EBP could be detected in any of 27 colonies that survived HAT selection after co-transfection with the expression vector encoding intact C/EBP (Table 1). In contrast, C/EBP variants defective in gene activation (LZ^-, BR^-, and $AR1^-/AR2^-$) could readily be detected in most of the HAT-resistant colonies tested. C/EBP variants that are partially compromised in gene activation ($AR1^-$ and $AR2^-$) were detected in roughly half of the colonies tested (Table 1). These same experiments have been repeated with similar results on 3T3-L1 adipoblasts and 10T1/2 mouse fibroblasts (*19*).

The observations outlined above provide evidence that C/EBP plays a fundamental regulatory role in the terminal differentiation of adipocytes. Premature expression of C/EBP, in the form of a conditionally active fusion protein, has been shown to arrest mitotic growth, and furthermore, in collaboration with a set of three hormonal factors, C/EBP stimulates the program of adipocyte differentiation.

There are several reasons to suspect that C/EBP may catalyze differentiation in tissues other than adipose. First and foremost is the fact that C/EBP is present in the terminally differentiated cells of other tissues including liver (*4*), intestine, and skin (*28*). Second, evidence has already been presented that C/EBP is capable of trans-activating the serum albumin gene, which is expressed exclu-

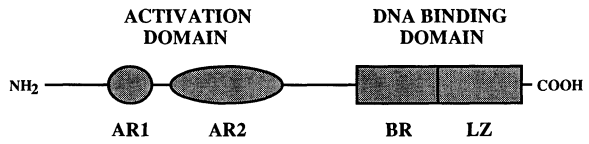

Fig. 5. Functional components of C/EBP. The DNA binding domain of C/EBP contains a dimerization interface termed the leucine zipper (LZ), and a DNA contact surface termed the basic region (BR) (*37*). Two activating regions, termed AR1 and AR2, are located on the amino-terminal side of the DNA binding domain (*26*).

Table 1. Colony suppression test of various forms of C/EBP. Mouse L cells were transfected with 150 ng of *tk* plasmid alone or with a tenfold molar excess of plasmid expressing various forms of C/EBP (*36*). Transformant colonies were selected in medium supplemented with hypoxanthine, aminopterin and thymidine (HAT) (*23*). The number of HAT-resistant colonies formed represents the average of two experiments. Randomly selected, individual cell clones were isolated, propagated as continuous cultures, and analyzed by immuno (Western) blotting for the presence of immunoreactive material of the molecular weight expected for each C/EBP derivative. NA, not applicable.

Plasmid	Colonies formed (*N*)	Fraction expressing immunoreactive material
tk alone	351	NA
C/EBP-WT	10	0/27
LZ^-	171	9/13
BR^-	180	9/10
$AR1^-$	8	6/12
$AR2^-$	18	7/12
$AR1^-/AR2^-$	234	9/9

sively in terminally differentiated hepatocytes. Finally, the growth inhibitory properties of C/EBP have been observed in a number of different cell types, including pluripotent 10T1/2 cells, which are capable of differentiating into a variety of specialized cell types (*29*), and TK-deficient mouse fibroblasts, which are not known to be capable of assuming a terminally differentiated state in culture.

It may be of value to compare and contrast the properties of C/EBP with MyoD, a DNA binding protein that is a potent myogenic factor (*30*). Both proteins exert an antiproliferative effect when introduced into dividing cells [(*31*), and our data]. MyoD is able to specify, on its own, a distinctive differentiation program (*30*). Such is not the case for C/EBP. When expressed prematurely in cultured adipoblasts, C/EBP fails to induce terminal differentiation. Only after adipoblasts are exposed to a set of hormonal inducers is C/EBP able to catalyze the differentiation process. Since we believe that C/EBP promotes differentiation in a variety of tissues, it is not surprising that this single transcription factor is incapable of independent specification of a distinctive, differentiated phenotype. We instead consider C/EBP as an auxiliary factor that, at the appropriate time, assists in the execution of otherwise predetermined differentiation programs.

We have tentatively identified two roles played by C/EBP in the differentiation process—activation of genes that endow a differentiated cell with its specialized phenotype, and acquisition of the growth-arrested state. Interpreted most literally, our experiments suggest that the state of growth arrest resulting from C/EBP expression can be achieved without display of specialized, terminally differentiated phenotype. If so, we speculate that C/EBP may regulate a genetic program of growth arrest distinguishable from its auxiliary role in terminal cell specialization. The mechanism of C/EBP action may, however, be similar in both roles. Because of this possibility, we point out the fact that growth arrest by C/EBP requires the same polypeptide components necessary for transcriptional activation. Perhaps C/EBP collaborates with ubiquitous transcription factors to suppress mitotic growth and with differentiation-specific factors to elicit specialized phenotypes. Finally, we point out that, if the observations presented in this study correctly identify C/EBP's role in terminal cell differentiation, C/EBP-ER–expressing cell lines may offer a useful system for dissecting the genetic program that leads to growth arrest.

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36. The plasmids expressing mutant forms of C/EBP have been described previously [LZ^- = L12V, BR^- = BR3, $AR1^-$ = Δ3, $AR2^-$ = Δ5–9, $AR1^-/AR2^-$ = Δ3–9 (*5, 26*)].
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