Adenovirus-mediated Transfer of CCAAT/Enhancer-binding Protein-α Identifies a Dominant Antiproliferative Role for This Isoform in Hepatocytes*

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CCAAT/enhancer-binding protein (C/EBP) isoforms are thought to be important regulators of the hepatocyte phenotype. However, the specific physiological roles of different isoforms are poorly understood because hepatocytes express multiple C/EBPs, and various isoforms have overlapping functions. To identify the functions of C/EBPα in mature hepatocytes, replication-defective adenovirus vectors were used to efficiently and homogeneously overexpress the mouse C/EBPα gene in a SV40 virus-conditionally transformed rat hepatocyte line that can be induced to express C/EBPβ and C/EBPδ but that has little endogenous C/EBPα expression. Hepatocytes were infected with a recombinant adenovirus vector carrying the cDNA for C/EBPα driven by Rous sarcoma virus promoter elements (AdCEBPα) or a similar vector carrying the Escherichia coli lacZ gene (Adβgal). Staining for β-galactosidase demonstrated an infection efficiency of 100% at a multiplicity of infection of 25 plaque-forming units/cell and persistence of foreign gene expression for at least 9 days. Cultures infected with AdCEBPα had 50-fold higher levels of C/EBPα mRNA and protein than those infected with Adβgal, but similar expression of C/EBPβ. Infection with AdCEBPα inhibited proliferation in cells expressing little C/EBPβ, even when proliferation was driven by the SV40 transforming antigen, and also blunted mitogenic induction of the c-myc proto-oncogene in nontransformed cells with high levels of C/EBPβ. Although overexpression of C/EBPα consistently increased C/EBPα DNA binding activity, it was not sufficient for albumin expression. Infection with AdCEBPα only increased albumin mRNA levels in nontransformed cells that also expressed relatively high levels of C/EBPβ. Thus, in hepatocytes, C/EBPα has a dominant antiproliferative function, but must interact with other factors to regulate hepatocyte-specific gene expression.

CCAAT/enhancer-binding proteins (C/EBPs)1 are members of the basic leucine zipper superfamily of transcription factors. To date, five distinct genes that encode different C/EBP isoforms have been identified (1-9). These genes vary in their pattern of tissue distribution. For example, C/EBPα is expressed predominately in tissues that regulate metabolic homeostasis (e.g. liver, fat, and intestine), while C/EBPβ and C/EBPδ are more ubiquitous (10–13). Work in several cell lines indicates that, although they bind to similar regulatory elements, unique C/EBP isoforms differ in their ability to activate various tissue-specific promoters. In cells that express more than one C/EBP isoform, different C/EBPs interact to regulate gene expression by forming homo- and heterodimers (14, 15). Since the regulatory regions of many tissue-specific genes contain C/EBP-binding sites, such cells are able to variably titrate the transcriptional rates of a wide array of genes by modifying the relative activities of different C/EBP isoforms (5, 12, 16, 17).

Matheiptocytes express at least three C/EBP isoforms (α, β, and δ) that interact to regulate their phenotype (12). However, since these different C/EBP isoforms have overlapping functions (14), the specific physiological role of each isoform in these cells is not well understood. Attempts to modulate the relative levels of these isoforms in vitro and to examine the resulting phenotype have been thwarted, in part, because the transfection efficiency of both primary adult and neoplastic hepatocytes is relatively poor, in the range of 20–30% (18–20). Furthermore, basal expression of C/EBPα is low in these cell lines (21), unlike in mature hepatocytes, where C/EBPα is strongly and constitutively expressed (10, 11). To overcome these experimental difficulties and to clarify the role of C/EBPα in mature hepatocytes, replication-defective adenovirus vectors were used to efficiently overexpress the mouse C/EBPα gene in a conditionally transformed rat hepatocyte line that can be induced to express C/EBPβ and C/EBPδ but that has little endogenous C/EBPα expression. In vitro adenoviral gene transfer has a significant advantage over traditional transient transfection strategies in that up to 100% of the targeted cells can be infected (22). The subsequent analysis reflects changes in a homogeneous population of cells and indicates that C/EBPα has a dominant antiproliferative function in hepatocytes, but must interact with other factors to regulate the expression of hepatocyte-specific genes.

EXPERIMENTAL PROCEDURES

Adenovirus Vector Preparation—The coding sequence for the mouse adipose C/EBPα gene was provided by M. D. Lane (Johns Hopkins University, Baltimore) (23). A 2200-base pair BamHI-HindIII fragment containing the entire coding sequence for the C/EBPα gene was excised from pBluescript (24) and subcloned into the vector pE1RSV (25), a modified version of pE1sp18 (obtained from F. Graham, McMaster University, Hamilton, Ontario, Canada) that incorporated the Rous sarcoma virus long terminal repeat promoter, a multiple cloning site, and the polyadenylation signal from bovine growth hormone between adenovirus E1 recombination sequences. This plasmid, denoted pCEBPα, was cotransfected with pJ M17 (26), containing the full human...
adeno virus serotype 5 genome, into HEK 293 cells using Lipofectamine (Life Technologies, Inc.). As described previously (25, 27), homologous recombination between pCEBPα and pJ M17 replaced portions of the adenovirus E1 regions with the 2200-base pair gene insertion to make AdC EBPα. We confirmed successful insertion of the C/EBPα gene cassette by restriction enzyme digestion and by Northern and Western blot analyses described below. The Adenovirus CEBPα and pJM17 plasmids each contain a unique restriction fragment with a rat CDNA, and the C/EBPα gene was confirmed by sequencing. The C/EBPα gene was inserted into the Adenovirus by homologous recombination. The Adenovirus was purified by cesium chloride—iodixanol gradient centrifugation.

For large-scale preparations, 9–18 T-150 flasks of HEK 293 cells were infected with AdC EBPα or AdJagilal at a multiplicity of infection (m.o.i.) of 1–5 plaque-forming units/cell. For detection of β-galactosidase activity, cells were fixed with 0.5% glutaraldehyde for 5 min at room temperature and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside solution. The cells were lysed by freeze-thawing, and the supernatant was purified on a C18 step column and dialyzed against phosphate-buffered saline as described previously (25).

Cell Culture Experiments—A hepatocyte line (RALA255-10G) derived from adult rat hepatocytes that were conditionally transformed with mutant SV40 virus containing a temperature-sensitive T antigen was obtained from Dr. J. ance Chou (National Institutes of Health, Bethesda, MD). At 33 °C (permissive temperature), T antigen is expressed, and these cells actively proliferate, but do not express several hepatocyte-specific genes, including albumin. At 37–39 °C (restrictive temperature), T antigen is degraded in RALA255 cells, so they are no longer transformed, and their proliferative activity decreases. This is accompanied by increased transcription of albumin and several other hepatocyte-specific genes (28). For experiments performed at 33 °C, cultures were grown on plastic dishes to 30–40% confluence and then exposed to Dulbecco’s modified Eagle’s medium, 2% fetal calf serum, and growth medium (Dulbecco’s modified Eagle’s medium containing ITS, dimethyl sulfoxide, and insulin) for 2 h at 37 °C.

For experiments performed at the restrictive temperature, cultures were grown on plastic dishes containing 4% fetal calf serum and 0.2 μM deoxymethasone, cells were cultured for an additional 1–3 days. To measure treatment-related differences in proliferative activity, 5 μCi of [3H]thymidine was added to cultures 2 h before harvesting, and the tritiated thymidine was measured in TCA-precipitable material (29). For treatments related to the activation of C/EBPα and C/EBPβ, cultures were treated with 0.1 μM of the activator (30). The activator was added to cultures 2 h before harvesting, and the C/EBPα and C/EBPβ isoforms were measured.

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RESULTS
RALA255 Characterization—As expected, uninfected RALA255 cells under the influence of T antigen do not express detectable levels of albumin mRNA at 33 °C, but gradually accumulate albumin message during culture at 39 °C (Fig. 1). Although others have reported that these condition-dependent differences in albumin expression are transcriptionally regulated and reflect differences in the protection of several DNase-sensitive sites in regulatory elements of the albumin gene (36), differences in albumin mRNA expression are not associated with comparable differences in C/EBPα or C/EBPβ expression (Fig. 1). Very little C/EBPα mRNA is detected during culture at either 33 or 39 °C. Similarly, stable low levels of C/EBPβ mRNA are detected only after prolonged exposure (21 days) of cells to 39 °C. C/EBPβ mRNA is more readily detected at both temperatures, but mRNA levels increase <2-fold when cultures are shifted from 33 to 39 °C.

In contrast to the relatively temperature-insensitive message levels, immunoblot analysis reveals that levels of certain C/EBP isoforms increase at 39 °C (Fig. 2). Although C/EBPβ expression varies <2-fold, the nuclear concentrations of C/EBPβ and C/EBPδ increase 5–10-fold when cultures are shifted to the higher temperature (p < 0.05 for expression at 33 °C versus expression during days 1–9 at 39 °C). These increases in C/EBPβ and C/EBPδ expression are paralleled by increases in DNA binding activity of these isoforms in gel mobility shift experiments with an oligonucleotide probe that contains the C/EBP-binding site in the albumin gene (Fig. 3). Complex formation with this oligonucleotide is completely eliminated when a mixture of antibodies to C/EBPα, C/EBPβ, and C/EBPδ is added to the reaction mixture, but is unaltered by preimmune sera (see Fig. 7, lanes 9 and 10), confirming previous reports (36) that this probe specifically identifies C/EBP binding activity in liver nuclear extracts.

Total C/EBP binding activity is increased in nuclear extracts prepared from 39 °C cultures compared with extracts from 33 °C cultures. In experiments with extracts obtained from 39 °C cultures, addition of anti-C/EBPα antibodies has little effect on complex formation; anti-C/EBPβ antibodies result in a small supershifted band, and anti-C/EBPδ antibodies disrupt most of the protein-probe complexes. Thus, almost all of the increased binding activity at 39 °C is secondary to C/EBPβ. Because C/EBPβ accounts for most of the C/EBP binding ac-
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Activity in these cells at both 33 and 39°C, subsequent experiments focused on identifying effects of C/EBPα gene transfer on the expression and function of the C/EBPβ isoform.

Adenovirus-mediated Gene Transfer—Infection of RALA255 cultures with Adβgal (m.o.i. = 25) results in expression of the heterologous gene in virtually 100% of the cells within 1 day (Fig. 4). Following exposure to AdCEBPα (m.o.i. = 25), steady-state levels of C/EBPα mRNA are >50-fold higher than in cells treated with Adβgal. Increased expression of C/EBPα mRNA occurs both during culture at 33°C, when T antigen is actively expressed, and after 2 days of culture at 39°C, when T antigen has been completely degraded. In contrast, C/EBPβ mRNA levels are similar in the two groups at both temperatures (Fig. 5). Infection with AdCEBPα increases albumin mRNA levels at 39°C (p < 0.01 versus Adβgal control on the third day of culture at 39°C), but not in cultures maintained at 33°C. However, as shown in a representative immunoblot (Fig. 6), the concentration of C/EBPα protein is increased in AdCEBPα-infected cultures maintained at either 33 or 39°C compared with parallel cultures treated with Adβgal. Triplicate experiments demonstrate that both whole cell and nuclear concentrations of C/EBPα protein are greater in AdCEBPα-infected cultures than in Adβgal-infected cultures at every time point evaluated (i.e. during the first 3 days of culture at 33°C and on the third day of culture at 39°C). Evidence that the treatment-related differences in C/EBPα expression (illustrated in Fig. 6) are not merely artifacts produced by lane-lane differences in the amount of protein on the blot is provided in Fig. 6 (lower panel). When this representative blot was reprobed to search for treatment-related differences in C/EBPβ expression, the results demonstrated that C/EBPβ protein concentrations are similar in Adβgal- and AdCEBPα-infected cultures. Thus, the results of Northern and Western blot analyses are consistent with each other and demonstrate that adenovirus-mediated transfer of C/EBPα results in upexpression of this C/EBP isoform, but does not alter the expression of C/EBPβ, a closely related transcription factor.

Virus-mediated overexpression of C/EBPα also has functional relevance. Gel mobility and supershift experiments demonstrate increased complex formation between C/EBPα and the C/EBP-binding site in the albumin enhancer in extracts from...
AdCEBPα-treated cultures compared with Adβgal-infected cultures at both 33 and 39 °C. As shown in Fig. 7, complex formation is qualitatively different in extracts obtained from 33 °C cultures that overexpress C/EBPα (lane 5) compared with extracts obtained from parallel Adβgal-infected cultures (lane 1). Comparison of the residual bands that are left in lanes 2 and 6 (after addition of anti-C/EBPβ and anti-C/EBPδ antibodies) demonstrates more residual slow migrating bands that contain C/EBPα in lane 6 (from AdCEBPα cultures) than in lane 2 (from Adβgal cultures). Comparison of lane 2 with lane 4 and of lane 6 with lane 8 confirms that the slow migrating bands contain C/EBPα because they disappear when antibodies to C/EBPα are added to the reaction mixture. Data shown in lanes 3 and 7 (after addition of antibodies to C/EBPα and C/EBPδ)
indicate that the fastest moving residual bands contain C/EBP\(\beta\). Presumably, the residual slow moving bands in lane 7 also contain C/EBP\(\beta\) since addition of antibodies to all three C/EBP isoforms totally eliminates complex formation with this probe (lane 10). Taken together, these experiments indicate that, although infection of cultures with AdC/EBP\(\alpha\) results in increased C/EBP\(\alpha\) mRNA, increased C/EBP\(\alpha\) protein, and increased C/EBP\(\alpha\) DNA binding activity, this is not sufficient to result in increased expression of albumin, a C/EBP-regulated gene, at 33 °C. Similarly, C/EBP\(\beta\) binding activity is not associated with albumin gene expression at this temperature.

Since C/EBP\(\alpha\) has been identified as a growth arrest gene in adipocytes, studies were carried out to determine if overexpression of C/EBP\(\alpha\) could influence the proliferative activity of hepatocytes. As shown in Fig. 8A, infection with AdC/EBP\(\alpha\) inhibits proliferation in cultures maintained at 33 °C. Differences in the rate of \(^{3}H\)thymidine incorporation are very obvi-
Results of triplicate experiments indicate that, after treatment with growth factors, levels of c-myc mRNA are significantly lower in AdCEBP<sub>α</sub>-infected cultures than in AdβGAL-infected controls (p < 0.05).

**DISCUSSION**

C/EBP<sub>α</sub> has been incriminated as a component of a “differentiation-proliferation switch” based on work in adipocyte cell lines (15, 38). Proliferating undifferentiated adipoblasts do not express C/EBP<sub>α</sub>, and increased C/EBP<sub>α</sub> expression accompanies hormonal induction of differentiation in these cells (5, 10, 23, 39, 40). Transfection of adipoblasts with inducible expression vectors for C/EBP<sub>α</sub> arrests their proliferation and promotes differentiation in the presence of adipogenic hormones (15). In addition, during hormonally induced differentiation of preadipocytes, plasmid vector-directed expression of C/EBP<sub>α</sub> antisense RNA blocks expression of C/EBP<sub>α</sub> and inhibits the induction of adipocyte-specific genes (41, 42). However, whether C/EBP<sub>α</sub> regulates proliferation and differentiated gene expression in other highly differentiated cells is less clear. Indeed, in some cells, including myeloid precursors, C/EBP<sub>α</sub> expression actually declines during terminal differentiation (43).

The adult liver expresses more C/EBP<sub>α</sub> than any other tissue (10), yet the physiological role of C/EBP<sub>α</sub> as a regulator of the hepatocyte phenotype remains undefined. In rats, hepatic expression of C/EBP<sub>α</sub> increases during development and is greater postnatally than earlier during embryogenesis (10, 11, 44). However, hepatocytes continue to proliferate actively for the first several weeks after birth (44). Furthermore, at birth, liver morphology appears normal in C/EBP<sub>α</sub> knockout mice, and these animals express many, albeit not all, hepatocyte-specific genes (45). In the adult, hepatic concentrations of C/EBP<sub>α</sub> mRNA and protein remain relatively stable during
It is conceivable that, in hepatocytes, identification of potentially important C/EBP \( \alpha \) actions may be obscured by non-\( \alpha \) C/EBP isoforms. Several metabolic and inflammatory mediators induce expression of C/EBP\( \beta \) and C/EBP\( \delta \) in hepatocytes, and it has been postulated that the resulting changes in C/EBP dimerization increase the transcription of some genes while decreasing the trans-activation of others (6, 8, 12, 16, 17, 44). However, since the differences among C/EBP isoform transcriptional activities appear more qualitative than quantitative (14), it has been difficult to attribute unique functions to C/EBP\( \alpha \). Work in neoplastic hepatocyte lines and primary hepatocytes has not resolved this dilemma because these systems express relatively high levels of C/EBP\( \beta \), but very little C/EBP\( \alpha \) endogenously (21, 46). Furthermore, standard transfection techniques transfer exogenous C/EBP\( \alpha \) to < 20% of the cultured cells (18–20), making it difficult to identify treatment-related differences because of low signal-to-noise ratios. While recently developed C/EBP\( \alpha \) knockout mice offer exciting opportunities to study C/EBP interactions, progress in this model has been slow because the \( \alpha \)-null phenotype is lethal within a few days of birth (45).

We have exploited the high efficiency of adenovirus-mediated gene transfer (22, 25) to demonstrate that C/EBP\( \alpha \) inhibits proliferation in hepatocytes, as it does in adipocytes. In hepatocytes, following infection with AdCEBP\( \alpha \), the antiproliferative actions of C/EBP\( \alpha \) are particularly apparent when C/EBP\( \beta \) expression is low (at 33 °C). Indeed, at this temperature, overexpression of C/EBP\( \beta \) is sufficient to inhibit proliferation driven by T antigen. Immunohistochemistry indicates that T antigen expression persists in cells that express C/EBP\( \alpha \). Although this technique is not sensitive enough to detect small treatment-related differences in T antigen levels, our results suggest that C/EBP\( \alpha \) may interfere with the function of this transforming protein. Given evidence that the mechanism of action of T antigen involves alteration of events that regulate G1 to S phase transition (51, 52), this implies that C/EBP\( \alpha \) may retard cell cycle progression. Present evidence that mitogenic induction of c-myc expression is inhibited by overexpression of C/EBP\( \alpha \) in hepatocytes lacking functional T antigen further supports this theory and suggests that C/EBP\( \alpha \) may regulate entry into early G1 phase. Since c-myc is expressed in mid-G1 (53), this could explain why C/EBP\( \alpha \) and c-myc are considered opposing elements in a differentiation-proliferation switch that regulates the phenotype of transformed adipocytes (15, 54). Although additional experiments are necessary to confirm this theory, it is tempting to speculate that, in mature hepatocytes, high constitutive expression of C/EBP\( \alpha \) (46, 47, 55) may help to maintain these cells in their normal growth-arrested state.

Our results also provide direct evidence that interactions among C/EBP isoforms modulate differentiated gene expression in hepatocytes by demonstrating that albumin mRNA levels rise when C/EBP\( \alpha \) concentrations are increased in cells expressing high levels of C/EBP\( \beta \) at 39 °C. Since overexpression of C/EBP\( \alpha \) does not increase albumin mRNA levels at 33 °C, despite inhibiting proliferative activity at this temperature, and some albumin mRNA is expressed in uninfected RALA255 cells at 39 °C, it is unlikely the effects of C/EBP\( \alpha \) on albumin expression are a nonspecific reflection of its ability to block hepatocyte proliferation. Furthermore, extracts obtained from uninfected or Ad\( \alpha \)gal-infected RALA255 cells grown at 33 °C demonstrate some binding activity for C/EBP\( \alpha \) sites in gel mobility shift assays, and this is mainly due to C/EBP\( \beta \). However, since complex formation between C/EBP\( \alpha \) and C/EBP\( \beta \) sites is greater in 33°C cultures infected with AdCEBP\( \alpha \), albumin expression is not increased.

Liu et al. (37) reported that at least five distinct DNase-sensitive sites in the albumin enhancer element are differentially protected in RALA255-like hepatocytes grown at permissive and restrictive temperatures. Our data suggest that some of the factors that bind to these other sites may be differentially expressed in RALA255 cells cultured at the two temperatures. Since multiple trans-acting factors interact to promote transcription of the albumin gene (56, 57), this may explain why overexpression of C/EBP\( \alpha \) increases albumin expression only at the restrictive temperature. Adult hepatocytes in the healthy liver normally express the full complement of trans-acting factors necessary to activate albumin transcription (58). In these cells, C/EBP\( \beta \) and C/EBP\( \alpha \) can each trans-activate the albumin gene (6, 59, 60). However, our findings indicate that albumin transcription is greater when cells express more C/EBP\( \alpha \).

In summary, efficient adenovirus-mediated introduction of the mouse C/EBP\( \alpha \) gene into cultures of a rat hepatocyte-derived cell line under conditions that variably induce C/EBP\( \beta \) expression has clarified the functions of these C/EBP isoforms. Proliferative activity is inhibited by overexpression of C/EBP\( \alpha \). Hence, C/EBP\( \alpha \) functions as a growth arrest gene in hepatocytes. In nonproliferating hepatocytes, C/EBP\( \alpha \) also cooperates with other factors to regulate the expression of certain hepatocyte-specific genes, such as albumin. Of note, however, another C/EBP isoform (C/EBP\( \beta \)) is capable of supporting hepatocyte-specific gene transcription when C/EBP\( \alpha \) expression declines. Thus, induction of C/EBP\( \beta \) may be one mechanism whereby adult hepatocytes maintain their differentiated phenotype while proliferating. Taken together, the results of our gene transfer experiments help to explain the significance of the reciprocal variations in C/EBP\( \alpha \) and C/EBP\( \beta \) DNA binding activity that have been noted during liver regeneration (30, 46, 47) and suggest that C/EBP isoforms interact to regulate the hepatocyte phenotype during a physiological growth response.

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