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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).

Mature hepatocytes 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 *Bam*HI-*Hin*dIII 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 pΔE1sp1B (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 pJM17 (26), containing the full human

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[1] The abbreviations used are: C/EBPs, CCAAT/enhancer-binding proteins; m.o.i., multiplicity of infection.

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adenovirus serotype 5 genome, into HEK293 cells using Lipofectamine (Life Technologies, Inc.). As described previously (25, 27), homologous recombination between pCEBPα and pJM17 replaced portions of the adenovirus E1 regions with the 2200-base pair gene insertion to make AdCEBPα. We confirmed successful insertion of the C/EBPα gene cassette by restriction enzyme digestion and by Northern and Western blot analyses as described below. The Adβgal virus containing the *Escherichia coli lacZ* gene with the human cytomegalovirus immediate early promoter was provided by G. Wilkinson (Department of Medicine, University of Wales College of Medicine, Cardiff, CF4 4XX, United Kingdom).

For large-scale preparations, 9–18 T-150 flasks of HEK293 cells were infected with AdCEBPα or Adβgal 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 0.05% glutaraldehyde for 5 min at room temperature and stained with 5-bromo-4-chloro-3-indoyl β-D-galactoside solution. The cells were lysed by freeze-thawing, and the supernatant was purified on a CsCl step cushion 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. Janice 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 containing varying concentrations (10–100 m.o.i.) of AdCEBPα or Adβgal for 2 h. After replacing this medium with Dulbecco's modified Eagle's medium containing 4% fetal calf serum and 0.2 μM dexamethasone, cells were cultured for an additional 1–3 days. To measure treatment-related differences in proliferative activity, 5 μCi of [^{3}H]thymidine was added to cultures 2 h before harvesting, and the tritium-specific activity of DNA was determined. For experiments performed at the restrictive temperature, cultures were grown on plastic dishes coated with type I collagen at 33 °C until 70–80% confluent and then treated with AdCEBPα or Adβgal (10–100 m.o.i.) in Dulbecco's modified Eagle's medium containing 2% fetal calf serum for 2 h at 37 °C. This medium was replaced with serum-free Chee medium with 1% dimethyl sulfoxide, ITS (insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml)), and 1 μM dexamethasone, and cells were cultured at 39 °C for an additional 3 days. Culture under the latter conditions leads to significantly decreased proliferative activity of uninfected RALA255 cells by day 3. In some experiments, Chee medium was replaced with growth medium (Dulbecco's modified Eagle's medium containing ITS, 20 ng/ml epidermal growth factor, Ham's F-12 nutrient mixture (Sigma), 1 mM pyruvic acid, 260 μM L-proline, and 24.5 mM Hepes) at the beginning of day 3.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from cultures according to the protocol of Chomczynski and Sacchi (29). Samples (20 μg of RNA/lane) were loaded onto denaturing agarose gels, separated by electrophoresis, and transferred to nylon membranes as described previously (30). After rinsing membranes with methylene blue dye to ensure equivalency of RNA loading and transfer, membranes were hybridized at 42 °C overnight with ^{32}P-labeled cDNAs for C/EBPα, C/EBPβ, albumin, c-*myc*, or 18 S RNA. cDNAs for C/EBPα and C/EBPβ were donated by M. D. Lane (23). The albumin cDNA was provided by D. A. Shafritz (Albert Einstein College of Medicine, New York) (31), and Dr. Chi Dang (Johns Hopkins University) provided the c-*myc* cDNA (32). The probe for 18 S RNA was obtained from Oncogene Science Inc. (Manhasset, NY). Blots were washed under moderately stringent conditions (30) and then exposed to film. Autoradiographs from triplicate experiments were analyzed by scanning laser densitometry. To quantify treatment-related differences in mRNA expression, results on each radiograph were normalized to 18 S RNA expression at the same time point, and differences in normalized mRNA levels were evaluated by analysis of variance.

Protein Extraction and Western Blot Analysis—Whole cell protein was extracted by boiling cell pellets in 60 mM Tris and 1% SDS for 8 min. Nuclear proteins were extracted from parallel cultures according to the method of Ossipow *et al.* (33) as described previously (34). The protein concentrations in whole cell and nuclear extracts were determined (34). Samples (200 μg of whole cell protein/lane or 40 μg of nuclear protein/lane) in Laemmli sample buffer were loaded onto SDS-12.5% polyacrylamide gels, separated by electrophoresis, and transferred to nylon membranes (34). After staining the gel and membrane with Coomassie Blue dye to ensure equivalency of protein loading and transfer, immunoblot analysis was performed (34). Blots were incubated with primary antiserum (1:1000 dilution) for C/EBPα or C/EBPβ (gifts from M. D. Lane) (35), and immunoreactive bands were demonstrated by the chemiluminescence detection system (Amersham Corp.). Results of blots from triplicate experiments were analyzed by densitometry (Molecular Dynamics, Inc., Sunnyvale, CA). Densitometric data were evaluated by analysis of variance.

Gel Mobility and Supershift Experiments—Nuclear protein samples (8 μg of protein each) in storage buffer (20 mmol/liter Hepes (pH 7.4), 5 mmol/liter dithiothreitol, 1 mmol/liter $MgCl_2$, 60 mM KCl, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 40 μg/ml bestatin, 0.1 mM phenylmethylsulfonyl fluoride, 50 μmol/liter sodium vanadate, 20 mmol/liter β-glycerophosphate, and 10 mmol/liter *p*-nitrophenyl phosphate), the [γ-^{32}P]ATP-labeled double-stranded oligonucleotide fragment containing the C/EBP-binding site in the albumin enhancer (5′-ATCATGATTTTGTAATGGGGTAG-3′), and poly(dI·dC) (1.5 μg/ml) were incubated with or without antisera (1 μl of each antiserum/reaction) to various C/EBP isoforms at room temperature for 20 min. Reaction mixtures were suspended in 20% glycerol with 0.1% bromphenol blue and xylene cyanol FF, loaded onto 6% acrylamide gels, and separated by electrophoresis in 0.5 × Tris borate/EDTA buffer at 200 V for 2 h (34). Complexes were detected by exposing the dried gels to film.

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, 37), differences in albumin mRNA expression are not associated with comparable differences in C/EBP mRNA expression (Fig. 1). Very little C/EBPα mRNA is detected during culture at either 33 or 39 °C. Similarly, stable low levels of C/EBPδ mRNA are detected only after prolonged exposure (21 days) of blots to film. C/EBPβ message 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/EBPα 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) (36). 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/EBPδ antibodies result in a small supershifted band, and anti-C/EBPβ 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-

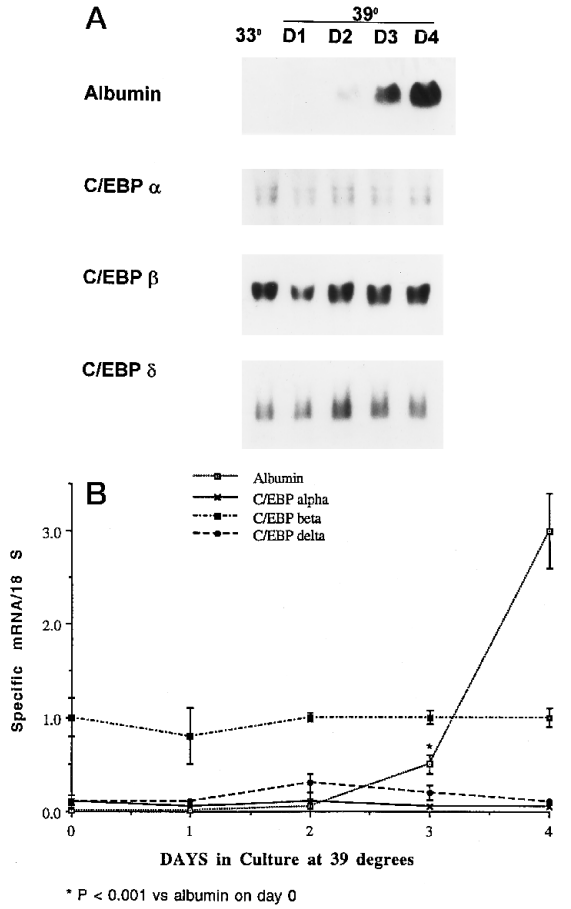

FIG. 1. **Temperature-dependent differences in mRNA levels of several hepatocyte genes in RALA255 cells.** Shown are the results of Northern blot analysis of total RNA (20 μg/lane) harvested from cells grown to 70–80% confluence at 33 °C or similarly confluent cultures maintained for varying periods of time (1–4 days (*D1–D4*)) at 39 °C. *A*, the autoradiographs shown were obtained by repeated hybridizations of a single blot. Results are representative of triplicate experiments. *B*, the effect of temperature on albumin mRNA levels persists when mRNA levels are normalized for lane-lane differences in 18 S RNA levels on each of three different immunoblots (*, $p < 0.001$ for day 0 *versus* days 3 and 4). Similar analysis confirms that expression of the C/EBP mRNAs is temperature-insensitive.

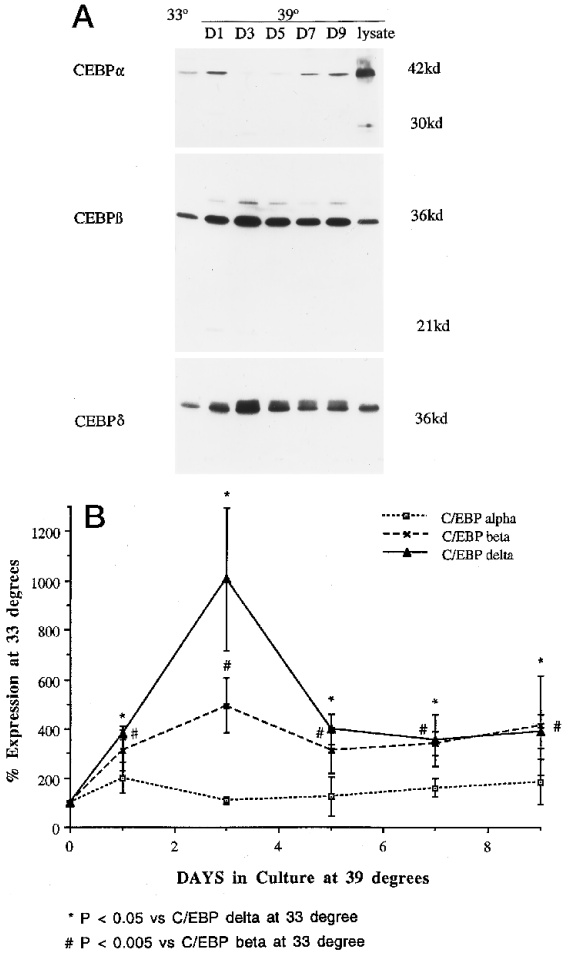

FIG. 2. **Temperature-dependent differences in C/EBP protein concentrations in RALA255 cells.** Nuclear extracts were prepared from cells grown to 70–80% confluence at 33 °C or from similarly confluent cultures maintained for varying periods of time (1–9 days) at 39 °C, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nylon membranes. *A*, C/EBP expression was analyzed by immunoblotting (40 μg of nuclear protein/lane). The *last lane* is a standard and contains reticulocyte-translated C/EBP protein (40 μg/lane) that was prepared by incubating rabbit reticulocytes with RNA prepared from the respective cDNAs for C/EBPα, C/EBPβ, and C/EBPδ (44). *B*, triplicate immunoblots were performed and analyzed by scanning laser densitometry. Levels of each C/EBP isoform at the various time points during culture at 39 °C were normalized for the expression of that isoform in 33 °C nuclear extracts on the same blot. Results were analyzed by analysis of variance (*, $p < 0.05$ *versus* C/EBPβ expression at 33 °C; #, $p < 0.05$ *versus* C/EBPδ expression at 33 °C).

tivity 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 overexpression 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

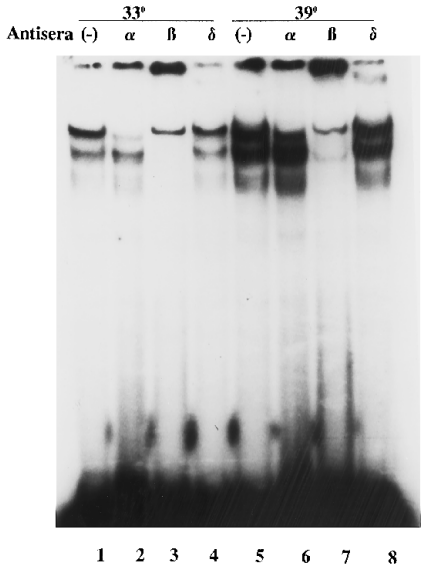

FIG. 3. **Temperature-dependent differences in C/EBP binding activity for the albumin enhancer in RALA255 cells.** Gel mobility shift assays were performed using 23-base pair oligonucleotides that included the C/EBP-binding site in the albumin enhancer plus nuclear extracts (8 μg of protein/lane) harvested from cells grown to 70–80% confluence at 33 °C or from similarly confluent cultures that had been maintained at 39 °C for 3 days. Antisera specific for different C/EBP isoforms were added to some reaction mixtures (*lanes 2–4* and *6–8*) to determine which isoforms were binding to C/EBP sites under the different culture conditions.

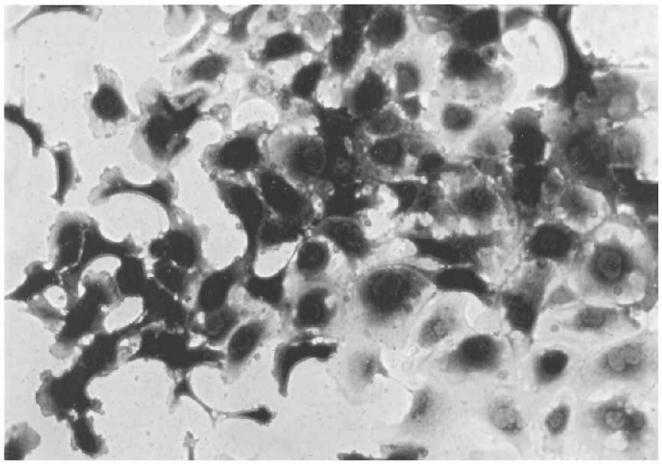

FIG. 4. **β-Galactosidase expression in RALA255 cultures 1 day after infection with Adβgal.** Infection with Adβgal (m.o.i. = 25) at 33 °C for 2 h resulted in expression of the heterologous gene in 100% of the cells 1 day later. Similar results were obtained when cultures were infected at 39 °C. Heterologous gene expression persisted for at least 9 days.

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)

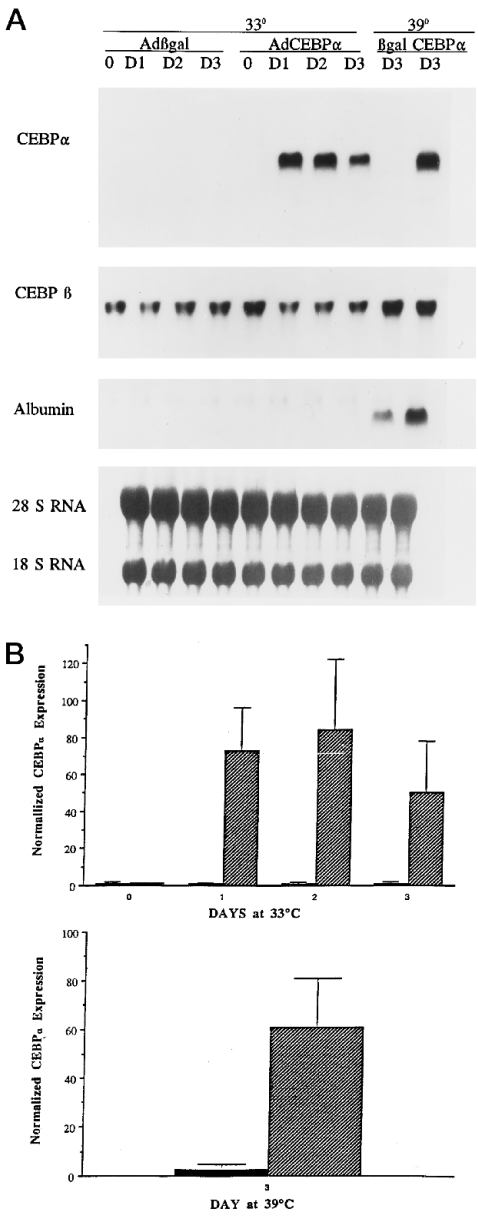

FIG. 5. **Effect of adenovirus-mediated gene transfer on RALA255 cell expression of several hepatocyte mRNAs.** 30–40% confluent cultures of RALA255 were infected with either AdCEBPα or Adβgal (m.o.i. = 25) for 2 h at 33 °C and then maintained at this temperature for an additional 1–3 days (*D1–D3*). In parallel experiments, 70–80% confluent cultures of RALA255 cells were infected with the same vectors for 2 h at 37 °C and then shifted to 39 °C and maintained at this temperature for 3 days. By day 3 at 39 °C, T antigen had been degraded, and albumin mRNA levels were increased in uninfected cultures (see Fig. 1). *A*, total RNA was harvested from cultures at various time points, and treatment-related differences in mRNA levels of C/EBPα, C/EBPβ, and albumin were evaluated by Northern blot analysis (20 μg of RNA/lane). The autoradiographs shown were obtained by repeated hybridization of a single blot. The *bottom panel* illustrates the same blot after staining with methylene blue. This, coupled with normalization of results to 18 S RNA expression at the same time points (data not shown), confirms that differences in C/EBPα and albumin expression are not due to uneven RNA loading or transfer. The results shown are representative of triplicate experiments. *B*, densitometric analysis of these results and those from two other blots confirms that infection with AdCEBPα increases C/EBPα mRNA levels 50–80-fold and albumin mRNA levels 4.5–7-fold ($p < 0.01$ for AdCEBPα at days 1–3 at 33 °C and day 3 at 39 °C *versus* Adβgal at the same time points). ■, Adβgal; ▨, AdCEBPα.

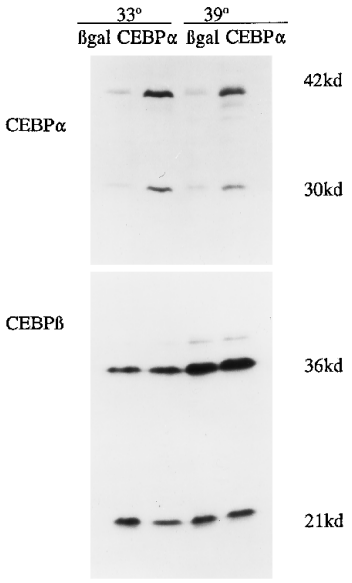

FIG. 6. **Effect of adenovirus-mediated gene transfer on RALA255 cell expression of C/EBPα and C/EBPβ proteins.** Cultures were infected with either AdCEBPα (*CEBPα*) or Adβgal (*βgal*) as described in the legend to Fig. 5 and harvested after either 2 days of culture at 33 °C or 3 days of culture at 39 °C. Whole cell and nuclear proteins were isolated as described under "Experimental Procedures." Immunoblot data from whole cell extracts (200 μg of protein/lane) are shown. Similar results were obtained when nuclear extracts were analyzed. Results shown are representative of triplicate experiments in which protein expression was analyzed during 3 days of culture at 33 °C and during days 3–6 of culture at 39 °C. At every time point evaluated, C/EBPα protein concentrations were greater in AdCEBPα-infected cultures than in parallel controls ($p < 0.01$).

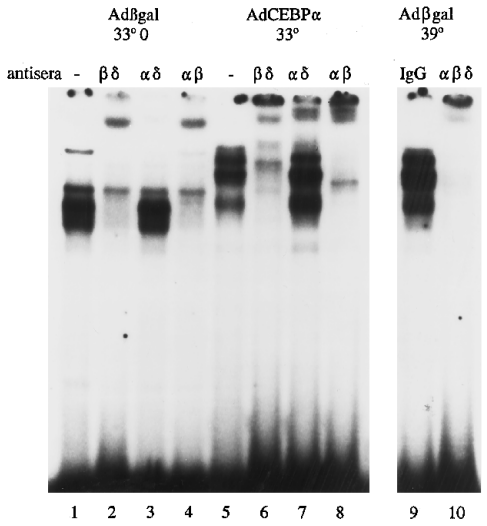

FIG. 7. **Effect of adenovirus-mediated gene transfer on C/EBP binding activity of RALA255 cells.** Gel mobility and supershift assays were performed using the 23-base pair oligonucleotide described in the legend to Fig. 3 plus nuclear extracts (8 μg of protein/lane) from cultures infected with either AdCEBPα or Adβgal. *Lanes 1–8* illustrate results in extracts harvested 3 days after infection at 33 °C. *Lanes 9* and *10* show results obtained when identical assays were performed with extracts harvested from Adβgal-infected cultures maintained at 39 °C for 3 days. These are included as a control to demonstrate that addition of a mixture of C/EBPα, C/EBPβ, and C/EBPδ antisera (total volume of 3 μl) virtually eliminates complex formation with the same probe (*lane 10*) but that addition of an equal volume of preimmune serum (IgG) has little effect.

indicate that the fastest moving residual bands contain C/EBPβ. Presumably, the residual slow moving bands in *lane 7* also contain C/EBPβ since addition of antibodies to all three C/EBP isoforms totally eliminates complex formation with this

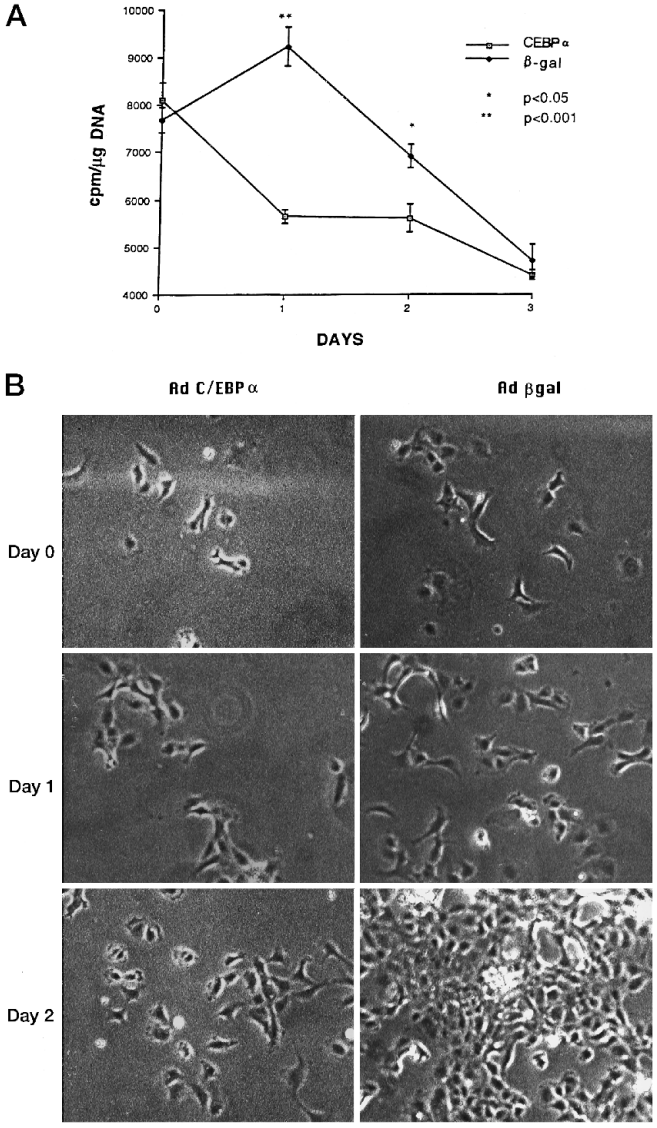

FIG. 8. **Effect of adenovirus-mediated gene transfer on T antigen-driven proliferation of RALA255 cells at 33 °C.** *A*, RALA255 cells were plated at low density (~10% confluence) and infected with either AdCEBPα (*CEBPα*) or Adβgal (*βgal*). Photomicrographs were taken at the time of infection (day 0) and 1 or 2 days postinfection. These results are representative of those obtained in three other experiments and demonstrate the antiproliferative effects of AdCEBPα. *B*, 30–40% confluent cultures were infected with either AdCEBPα or Adβgal as described above. One, two, or three days later, triplicate cultures were incubated for 2 h in medium containing [^{3}H]thymidine and then immediately harvested. DNA was isolated, and treatment-related effects on proliferation were measured by comparing differences in the rate of incorporation of [^{3}H]thymidine. Results of four separate experiments are shown.

probe (*lane 10*). Taken together, these experiments indicate that, although infection of cultures with AdCEBPα results in increased C/EBPα mRNA, increased C/EBPα protein, and increased C/EBPα DNA binding activity, this is not sufficient to result in increased expression of albumin, a C/EBP-regulated gene, at 33 °C. Similarly, C/EBPβ binding activity is not associated with albumin gene expression at this temperature.

Since C/EBPα has been identified as a growth arrest gene in adipocytes, studies were carried out to determine if overexpression of C/EBPα could influence the proliferative activity of hepatocytes. As shown in Fig. 8*A*, infection with AdCEBPα inhibits proliferation in cultures maintained at 33 °C. Differences in the rate of [^{3}H]thymidine incorporation are very obvi-

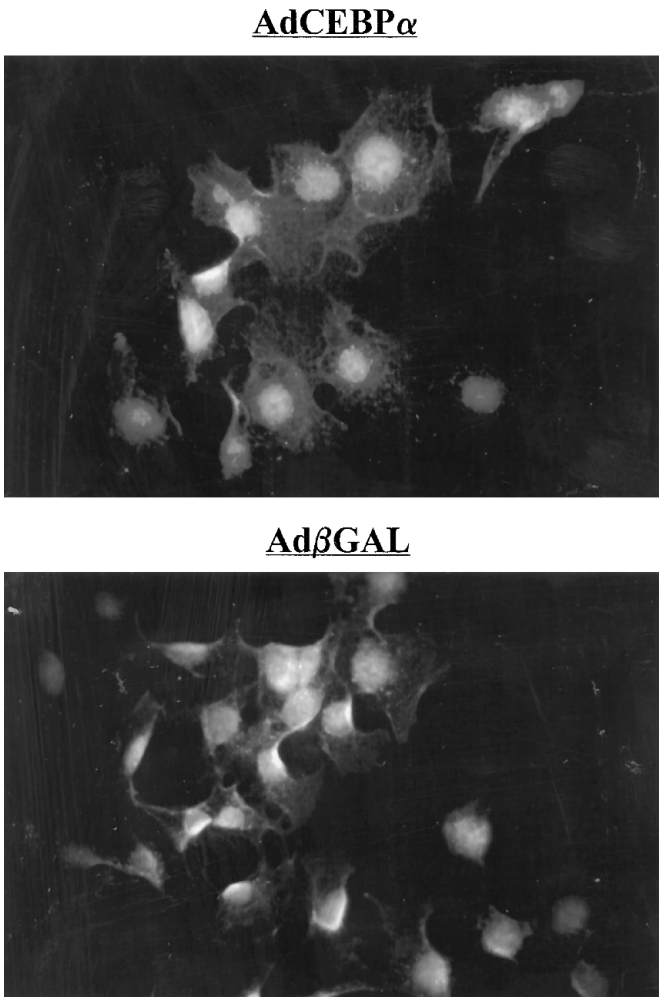

FIG. 9. **Effect of adenovirus-mediated gene transfer on T antigen expression in RALA255 cultures at 33 °C.** Immunocytochemistry was used to evaluate the expression of T antigen in AdCEBPα- and Adβgal-infected cultures. These experiments were performed in parallel with those described in the legend to Fig. 8. Monoclonal antibodies to SV40 T antigen were generous gifts of Thomas Kelly (Johns Hopkins University). Immunohistochemical evaluation of T antigen expression was performed as described by Hayward *et al.* (61). T antigen expression in cultures maintained at 33 °C for 1 day after infection is shown. Identical results were obtained in cultures maintained for up to 3 days at 33 °C.

ous after 1 day of culture, when the concentrations of DNA are not different statistically in the two treatment groups, but become less apparent over time, as DNA concentrations increase in the cultures treated with Adβgal (Fig. 8*B*). Infection with AdCEBPα does not overtly influence the expression of T antigen (Fig. 9), which is thought to drive proliferation in cultures maintained at 33 °C (28).

When nearly confluent RALA255 cells are infected with AdCEBPα or Adβgal and cultures are shifted to 39 °C, T antigen expression disappears after 2 days (data not shown) (28), C/EBPβ is induced (Fig. 6), and proliferative activity declines in both groups (from 4413 ± 176 to 179 ± 2 and from 5198 ± 160 to 169 ± 11 cpm/μg DNA, respectively, by the third day of culture at 39 °C). As shown in Fig. 10, changing these cultures (which do not express T antigen) to growth factor-enriched medium increases steady-state levels of c-*myc* 3–5-fold in Adβgal-infected cultures ($p < 0.01$ *versus* Adβgal cultures without mitogens). AdCEBPα-mediated expression of C/EBPα blunts this mitogenic induction of c-*myc* by at least 50%, such that c-*myc* mRNA levels are increased <1.5-fold (range of 0.9–1.5-fold greater than without mitogens; p = not significant).

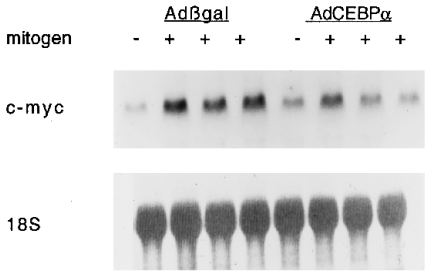

FIG. 10. **Effect of adenovirus-mediated gene transfer on mitogenic induction of c-*myc* in RALA255 cells at 39 °C.** 70–80% confluent cultures were infected with either AdCEBPα or Adβgal at 37 °C for 2 h and then shifted to 39 °C and maintained under conditions that supported differentiated gene expression. By the third day of culture under these conditions, T antigen had been degraded so that proliferative activity was very low in both groups. Growth factor-enriched medium was added to cultures at the beginning of day 3, and cultures were harvested at various times, ranging from 1 h to 3 days thereafter. Total RNA was isolated, and treatment-related differences in c-*myc* expression were evaluated by Northern blot analysis (20 μg of RNA/lane) in triplicate experiments. *Upper panel*, c-*myc* expression in representative Adβgal- or AdCEBPα-infected cells after 3 days of culture at 39 °C before (*first* and *fifth lanes*) or 3 h after (*second* through *fourth* and *sixth* through *eighth lanes*) addition of mitogens, when c-*myc* is maximally induced. *Bottom panel*, 18 S RNA on the same blot. Densitometry of triplicate autoradiographs and normalization of c-*myc* expression to 18 S RNA levels at the same time point indicated that c-*myc* mRNA levels were similar in AdCEBPα- and Adβgal-infected cultures before addition of growth factors. Mitogens increased c-*myc* mRNA levels 4.3 ± 1-fold in Adβgal-infected cultures ($p < 0.05$), but did not alter c-*myc* mRNA levels in AdCEBPα-infected cultures (c-*myc* mRNA levels after mitogens = 1.1 ± 0.3-fold greater than before mitogens; p = not significant). Thus, c-*myc* mRNA levels were at least 50% lower in AdCEBPα-infected cultures than in Adβgal-infected cultures at any time point evaluated after addition of mitogens ($p < 0.05$ for AdCEBPα *versus* Adβgal).

Results of triplicate experiments indicate that, after treatment with growth factors, levels of c-*myc* mRNA are significantly lower in AdCEBPα-infected cultures than in Adβgal-infected controls ($p < 0.05$).

DISCUSSION

C/EBPα 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/EBPα, and increased C/EBPα expression accompanies hormonal induction of differentiation in these cells (5, 10, 23, 39, 40). Transfection of adipoblasts with inducible expression vectors for C/EBPα 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/EBPα antisense RNA blocks expression of C/EBPα and inhibits the induction of adipocyte-specific genes (41, 42). However, whether C/EBPα regulates proliferation and differentiated gene expression in other highly differentiated cells is less clear. Indeed, in some cells, including myeloid precursors, C/EBPα expression actually declines during terminal differentiation (43).

The adult liver expresses more C/EBPα than any other tissue (10), yet the physiological role of C/EBPα as a regulator of the hepatocyte phenotype remains undefined. In rats, hepatic expression of C/EBPα 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/EBPα knockout mice, and these animals express many, albeit not all, hepatocyte-specific genes (45). In the adult, hepatic concentrations of C/EBPα mRNA and protein remain relatively stable during

liver regeneration after 70% partial hepatectomy (21, 30, 46, 47), although both the rate of hepatocyte proliferation and the transcription of some hepatocyte-specific genes increase significantly (48). Finally, in hepatoma cell lines and hepatocyte-derived hybridomas, loss of C/EBPα does not extinguish the hepatocyte phenotype (49, 50). Thus, although C/EBPα has a clear antiproliferative effect in adipocytes, its role in the liver appears more complex.

It is conceivable that, in hepatocytes, identification of potentially important C/EBPα actions may be obscured by non-α C/EBP isoforms. Several metabolic and inflammatory mediators induce expression of C/EBPβ and C/EBPδ 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/EBPα. Work in neoplastic hepatocyte lines and primary hepatocytes has not resolved this dilemma because these systems express relatively high levels of C/EBPβ, but very little C/EBPα endogenously (21, 46). Furthermore, standard transfection techniques transfer exogenous C/EBPα 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/EBPα knockout mice offer exciting opportunities to study C/EBP interactions, progress in this model has been slow because the α-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/EBPα inhibits proliferation in hepatocytes, as it does in adipocytes. In hepatocytes, following infection with AdCEBPα, the antiproliferative actions of C/EBPα are particularly apparent when C/EBPβ expression is low (at 33 °C). Indeed, at this temperature, overexpression of C/EBPα is sufficient to inhibit proliferation driven by T antigen. Immunohistochemistry indicates that T antigen expression persists in cells that overexpress C/EBPα. Although this technique is not sensitive enough to detect small treatment-related differences in T antigen levels, our results suggest that C/EBPα 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 G_1 to S phase transition (51, 52), this implies that C/EBPα may retard cell cycle progression. Present evidence that mitogenic induction of c-*myc* expression is inhibited by overexpression of C/EBPα in hepatocytes lacking functional T antigen further supports this theory and suggests that C/EBPα may regulate entry into early G_1 phase. Since c-*myc* is expressed in mid-G_1 (53), this could explain why C/EBPα 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/EBPα (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/EBPα concentrations are increased in cells expressing high levels of C/EBPβ at 39 °C. Since overexpression of C/EBPα 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/EBPα on albumin expression are a nonspecific reflection of its ability to block hepatocyte proliferation. Furthermore, extracts obtained from uninfected or Adβgal-infected RALA255 cells grown at 33 °C demonstrate some binding activity for C/EBP sites in gel mobility shift assays, and this is mainly due to C/EBPβ. However, although complex formation between C/EBPα and C/EBP sites is greater in 33 °C cultures infected with AdCEBPα, 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/EBPα 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/EBPβ and C/EBPα can each *trans*-activate the albumin gene (6, 59, 60). However, our findings indicate that albumin transcription is greater when cells express more C/EBPα.

In summary, efficient adenovirus-mediated introduction of the mouse C/EBPα gene into cultures of a rat hepatocyte-derived cell line under conditions that variably induce C/EBPβ expression has clarified the functions of these C/EBP isoforms. Proliferative activity is inhibited by overexpression of C/EBPα. Hence, C/EBPα functions as a growth arrest gene in hepatocytes. In nonproliferating hepatocytes, C/EBPα 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/EBPβ) is capable of supporting hepatocyte-specific gene transcription when C/EBPα expression declines. Thus, induction of C/EBPβ 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/EBPα and C/EBPβ 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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