Coexistence of C/EBPα, β, Growth-induced Proteins and DNA Synthesis in Hepatocytes during Liver Regeneration

Implications for Maintenance of the Differentiated State during Liver Growth

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Abstract

During the period of rapid cell growth which follows a two-thirds partial hepatectomy, the liver is able to compensate for the acute loss of two-thirds of its mass to maintain serum glucose levels and many of its differentiation-specific functions. However certain hepatic transcription factors, C/EBPα and β, which are important for establishment and maintenance of the differentiated state, have been shown to be antagonistic to cellular proliferation. To study the interplay between differentiation and cell growth in the liver regeneration model of hepatocyte proliferation, we characterized the expression of C/EBPα and β transcription factors throughout the temporal course of liver regeneration. As determined by immunoblot, the level of C/EBPα decreases more than twofold during the mid to late G1 and S phase (8–24 h after hepatectomy) coordinately with a threefold increase in expression of C/EBPβ. Renormalization of the levels of these proteins occurs after the major proliferative phase. This inverse regulation of C/EBPα and β results in up to a sevenfold increase in the β/α DNA binding ratio between 3 and 24 h after hepatectomy that may have an important impact on target gene regulation. However, total C/EBP binding activity in nuclear extracts remains relatively constant during the 7-d period after hepatectomy. By immunohistochemistry, both C/EBPα and β are expressed in virtually all hepatocyte nuclei throughout the liver during the temporal course of liver regeneration, and there is no exclusion of expression from hepatocytes that are expressing immediate-early gene products or undergoing DNA synthesis. The persistent expression of C/EBPα and β isoforms predicts that C/EBP proteins contribute to the function of hepatocytes during physiologic growth and that significant amounts of these proteins do not inhibit progression of hepatocytes into S phase of the cell cycle. (*J. Clin. Invest.* 1995. 96:1351–1365.) Key words: liver regeneration • CAAT enhancer binding proteins • differentiation • transcription factor

Introduction

The liver is one of the few normally quiescent tissues in the adult body that has the capacity to regenerate. In the rat, after a 70% partial hepatectomy virtually all remnant liver cells reenter the cell cycle (1–3). The first round of DNA synthesis occurs at 12–16 h and the liver regains its original mass within 7–10 d. Multiple factors, including circulating hormones, growth factors, and nervous input, appear to participate in the growth response. Although the majority of hepatocytes undergo a proliferative response, the liver retains and even enhances its ability to perform most of the cellular processes required to maintain homeostasis as reflected by its continued synthesis of liver-specific factors including albumin, coagulation factors, and bile (1) and its ability to maintain normal serum glucose levels. In this regard, gluconeogenic genes glucose-6-phosphatase (*G6Pase*)[1] and phosphoenolpyruvate carboxykinase (*PEPCK*) are induced rapidly in the regenerating liver within minutes of partial hepatectomy, thereby preventing the fall of serum glucose that would accompany the acute loss of liver mass (4, 5). Similarly, at the time of birth when the liver is rapidly proliferating, serum glucose must be established and maintained by the neonatal liver. To accomplish this, we hypothesize that during rapid proliferation the hepatocyte must continue to express transcription factors that are important for the regulation of liver-specific genes.

The regenerating hepatocyte enters proliferation accompanied by the expression of immediate-early (protein synthesis independent) genes during the G0 to G1 transition (4, 6–12). Immediate-early genes are transactivated by preexisting transcription factors that are inactive in the normal liver but are activated within minutes of the partial hepatectomy after post-translational modifications. Two such preexisting transcription factors that are activated in the regenerating liver in the absence of de novo protein synthesis include posthepatectomy factor/nuclear factor κB (PHF/NF-κB, a p65/p50 κB site DNA binding complex (13, 14), and Stat3 (15), an IL-6 and EGF inducible factor. Immediate-early genes themselves encode many

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; C/EBP, CAAT enhancer binding protein; G6Pase, glucose-6-phosphatase; HNF, hepatocyte nuclear factor; IGFBP-1, insulin-like growth factor binding protein; NF-κB, nuclear factor κB; PCNA, proliferating cell nuclear antigen; PEPCK, phosphoenolpyruvate carboxykinase; PHF, posthepatectomy factor; SRE, serum response element.

transcription factors, growth factors, and signal transduction molecules. Recent studies indicate that many transcription factors (e.g., CAAT enhancer binding protein [C/EBP], NF-κB) interact to enhance transcription of target genes (16–18), and it seems likely that growth-induced transcription factors and constitutive hepatic transcription factors interact to regulate liver growth and function during liver regeneration.

The mechanisms that result in the maintenance of differentiation-specific cellular processes in the liver during this period of rapid cell growth are not well understood. The hepatocyte nuclear factor (HNF) class of transcription factors, including HNF1,4, and 3α,β, γ which are responsible for regulation of many liver-specific genes, continues to be expressed at normal levels during liver regeneration (19, 20). C/EBPs are also important transcription factors in the differentiated liver. C/EBPα and β are members of a family of bZIP transcription factors that have been implicated in the establishment and maintenance of cellular differentiation (21–23). C/EBPα and β are highly expressed in liver, and their expression increases at the time of birth when the liver is still rapidly proliferating (24, 25). C/EBPα and β are able to activate the transcription of several liver-specific genes associated with the differentiated state including *albumin* (26), alpha-1-*antitrypsin* (27), and *PEPCK* (28). Although C/EBPα is expressed in developing proliferating hepatocytes, in adipocytes C/EBPα may have an antiproliferative function, based on the observation that premature C/EBPα expression in preadipocytes results in cessation of cell growth (29–32). C/EBPβ has been shown to have a similar antiproliferative effect in some hepatoma cell lines (33).

Because of the need to maintain differentiated function during liver regeneration and the proposed negative impact of C/EBPs on proliferation, investigators have attempted to quantitate the level of C/EBPs in liver regeneration. Results are conflicting, with some investigators finding profound decreases in *C/EBPα* mRNA and others seeing less effect (4, 19, 34–36). However, it is the level of active protein that is the most critical in determining the impact of C/EBP isoforms on gene expression. Measurements of C/EBP protein levels in liver regeneration have shown substantial variability and lack of consistency within and between studies allowing no conclusions to be drawn (19, 36). None of these studies have examined the full temporal course of protein expression and DNA binding activity, and more important, they do not assess in situ expression of the C/EBPs to see if zonal expression is observed in proliferating hepatocytes. If zonal expression were observed, then one could hypothesize that hepatocytes maintain differentiated function only at specific times in the cell cycle. Based on earlier studies (34), we hypothesized that C/EBP proteins would be expressed throughout the period of liver regeneration after partial hepatectomy. Here, we demonstrate that C/EBPα and β proteins are present throughout a 7-d period after partial hepatectomy, but the ratio of C/EBPβ to α increases during the first 24 h. Using in situ immunohistochemistry analyses, we show that the expression of both C/EBPα and β is compatible with immediate-early protein expression and progression of hepatocytes into S phase of the cell cycle.

Methods

Animals. For partial hepatectomy, female Fischer rats (160–200 grams) were ether anesthetized and subjected to midventral laparotomy with ~ 70% liver resection (left lateral and median lobes) (37). Methods were approved by Institutional Animal Care and Use Committee. Sham surgeries were performed after ether anesthesia and entailed midventral laparotomy.

Antibodies. Antibodies used were: α-C/EBPα "C103" (raised against intact polypeptide corresponding to C/EBPα open reading frame); α-C/EBPα "α-14" (raised against amino acids 253–265); α-C/EBPα "α-N" (raised against NH_2-terminal portion of C/EBPα polypeptide); α-C/EBPα "α-C104, α-C106" (raised against internal peptide upstream of α-14); α-C/EBPβ (raised against amino acids 258–276); α-proliferating cell nuclear antigen (α-PCNA; Santa Cruz Biotechnology, Santa Cruz, CA); α-c-Jun (27); α-rat albumin (Cappel Laboratories, Cochranville, PA); α-bromodeoxyuridine (α-BrdU; Boehringer-Mannheim, Indianapolis, IN). All C/EBPα and β antibodies were gifts from Steven McKnight with the exception of α-C/EBP "14AA" (Santa Cruz Biotechnology) and α-C/EBPβ "C-19" (Santa Cruz Biotechnology). 14AA and C-19 were raised against the same amino acids as α-C/EBPα "α-14" and α-C/EBPβ, respectively; α-C/EBPα "a-14" [Fig. 1 *A*]; α-C/EBPα "14AA" [Figs. 1 *C*, 3 *A, right panel,* and *C*]; equal mixture of α-C/EBPα "α-N", α-C/EBPα "C104", α-C/EBPα"C106" [Fig. 3 *A, left panel*]; α-C/EBPα "C103" [Figs. 6 and 8]; α-C/EBPβ [Figs. 1 *B*, 3 *B, left panel,* 7, and 8]; α-C/EBPβ "C-19" [Fig. 3 *B, right panel,* and *C*].

Preparation of nuclear extracts and whole nuclei. At indicated times after 70% partial hepatectomy, nuclear extracts and whole nuclei were prepared according to previously published methods (38) with these modifications. Protease inhibitors were added to all solutions: 2 μg/ml antipain, aprotinin, bestatin, and leupeptin (Boehringer Mannheim Corp., Indianapolis, IN). For nuclear extract preparation, pelleted nuclei were resuspended in 50 μl of lysis buffer per gram of liver (20 mM Hepes, pH 7.9/0.2 mM EDTA/420 mM NaCl/1.5 mM $MgCl_2$). The nuclear pellet was lysed with 10 strokes of pestle B in a small glass Dounce homogenizer. The suspension was rotated for 30 min at 4°C and spun at 14,500 rpm for 30 min at 4°C. The supernatant was dialyzed overnight in dialysis buffer (20 mM Hepes, pH 7.6/0.0002 mM EDTA/20% glycerol) and stored in liquid nitrogen. Whole nuclei were prepared by resuspending the nuclear pellet in a nonlysing suspension buffer (39) (100 mM NaCl/10 mM Tris-Cl, pH 7.6/1 mM EDTA). After protein concentration determination (Bio Rad Laboratories, Hercules, CA), an equal volume of 2× SDS loading buffer (100 mM Tris-Cl, pH 6.8/200 mM DTT/4% SDS/0.2% bromphenol blue/20% glycerol) was added. The samples were then boiled for 10 min, sheared three times by passing samples through a 23-gauge needle, aliquoted, and stored at −70°C.

Immunoblots. 20 μg of whole nuclei was electrophoresed on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected by chemiluminescence (Amersham ECL, Arlington Heights, IL) according to the instructions of the manufacturer (40). C/EBPα protein expression was detected using either α-C/EBPα "α-14" (gift from Steven McKnight) (Fig. 1 *A*) or α-C/EBPα "14 AA" (Santa Cruz Biotechnology) (Fig. 1 *C*). Both antibodies were raised against amino acids 253–265 of the C/EBPα polypeptide. C/EBPβ protein expression was detected using α-C/EBPβ (described above). Primary antibodies were diluted 1:1,000 and incubated with the membrane for 1 h at room temperature. Horseradish peroxidase–linked secondary antibody was then added at a dilution of 1:10,000 for 1 h. Densitometric scanning of a separate Coomassie-stained gel was used to normalize amounts of protein loaded in each lane.

Gel mobility shift assays (EMSA). Binding reactions were performed essentially as described (41). All experiments were performed using an excess of probe. 5 μg of extract was incubated with radiolabeled oligonucleotide (1 ng) for 15 min at room temperature in binding buffer (25 mM Hepes, pH 7.9/50 mM KCl/0.1 mM EDTA/1.0 mM DTT/10% glycerol) (29) and electrophoresed on nondenaturing polyacrylamide gels in 0.5× TBE buffer (44 mM Tris/44 mM EDTA/10% glycerol). The gels were dried and exposed to x-ray film. 2 μg of poly (dI-dC) (Sigma Immunochemicals) was used as a nonspecific DNA competitor in each reaction. Nuclear extract dialysis buffer containing

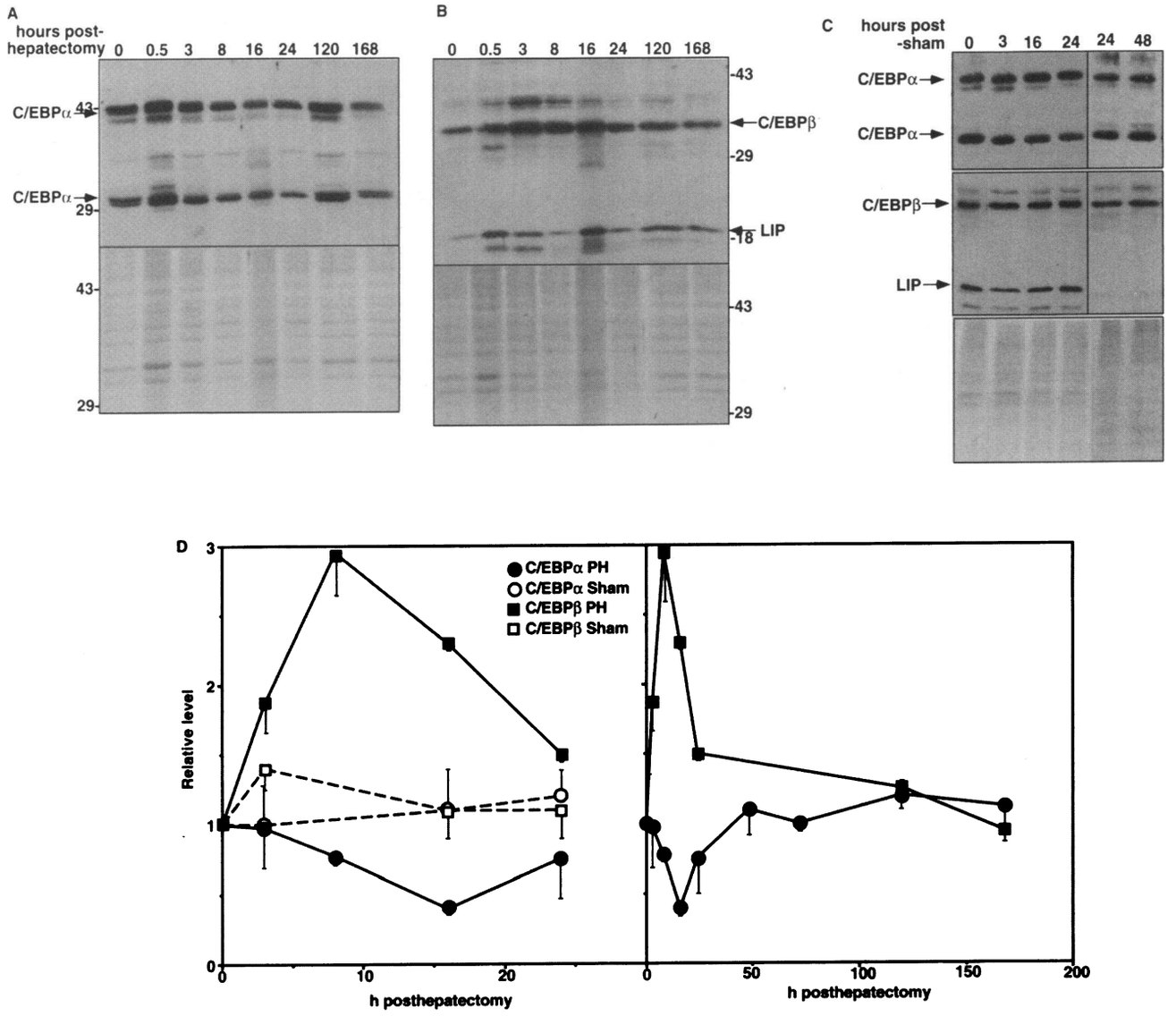

Figure 1. C/EBPα and β proteins are expressed throughout a 168-h period after hepatectomy. (*A*) Representative immunoblot of C/EBPα expression at indicated times after hepatectomy using α-14 antibody. The 42- and 30-kD bands represent C/EBPα alternative translation products. Separate Coomassie-stained gel is shown below. (*B*) Immunoblot of C/EBPβ expression at indicated times after hepatectomy. C/EBPβ and LIP proteins are indicated by arrows. (*C*) Immunoblot comparing protein levels for C/EBPα and β after sham surgery relative to Coomassie-stained gel. The right panel was probed with the Santa Cruz C/EBPβ antibody which does not readily detect LIP. (*D*) Graphic representation of C/EBPα and β protein level as a function of time after hepatectomy or sham surgery. Several C/EBPα (42 kD) and C/EBPβ immunoblots were densitometrically scanned and standard deviations were calculated. The level of protein in normal (0) liver was arbitrarily set to 1.0. Left panel, from 0 to 24 h; right panel, 0–168 h after hepatectomy.

freshly added protease inhibitors (see nuclear extract methods) was added to each reaction to achieve a final reaction volume of 10 μl. The probes used were preannealed high-performance liquid chromatography–purified double-stranded oligonucleotides: C/EBPα/β consensus binding sequence (TACACCATTACACAATTCA) and nuclear factor μE3 (GATCGGTCATGTGGCAAGGCTATTTGGG). The oligonucleotide probes were end labeled with [γ-^{32}P]ATP. Unlabeled oligonucleotide was incubated with extracts for 15 min at room temperature before the addition of the radiolabeled probe in cold competition experiments. Supershift experiments were performed by incubating 1 μl of primary antibody with nuclear extracts in binding buffer for 1 h at 4°C after the addition of labeled oligonucleotide. Primary antibodies used in supershift experiments include α-C/EBPα "14AA" (Santa Cruz Biotechnology) (Fig. 3, *A, right panel,* and *C*); equal mixture of α-C/EBPα "α-N" (raised against NH_2-terminal portion of C/EBPα polypeptide), α-C/EBPα "C104," "C106" (raised against internal peptide upstream of "α-14") (gift of Steven McKnight) (Fig. 3 *A, left panel*); α-C/EBPβ "C-19" (Santa Cruz Biotechnology) (raised against amino acids 258–276) (Fig. 3, *B, right panel,* and *C*).

Liver perfusion. Hepatectomized rats were reanesthetized and ventral laparotomy was performed. Rats whose livers were used for BrdU incorporation experiments received an intraperitoneal injection of BrdU

(Sigma Immunochemicals, St. Louis, MO) at a dose of 50 mg/kg 1 h before killing (42). Normal liver was prepared by subjecting rats directly to laparotomy and subsequent perfusion. The portal vein was cannulated using a 16-gauge angiocath followed by a 5-min PBS perfusion, pH 7.3. Freshly prepared 2% paraformaldehyde/PBS, pH 7.2, (4°C) was then perfused for 10 min at a rate of 15 ml/min. The fixed liver was removed and cut into 5-mm slices with a razor blade followed by a 1-h postfixation in 2% paraformaldehyde/PBS (4°C). The liver slices were paraffin embedded in an automated tissue processor. 5-μm tissue sections were cut on a microtome and adhered to poly-λ-lysine–coated glass slides. The slides were dried overnight at 37°C.

Immunohistochemistry. Immunohistochemistry for detection of C/EBPα, β, c-Jun, and albumin was performed essentially as described (43). Tissue sections were rehydrated in xylene followed by graded alcohols. The Vectastain Elite ABC avidin biotin horseradish peroxidase detection system (Vector Labs, Inc., Burlingame, CA) was used according to the manufacturer's instructions with the following modifications. After the blocking step in 3% goat serum in PBS, avidin and biotin blocking steps were performed using the Vector Laboratories Avidin-Biotin blocking kit. Antibodies used include α-C/EBPα "C103" (raised against intact polypeptide corresponding to C/EBPα open reading frame) (gift from Steven McKnight); α-C/EBPβ; α-c-Jun (27); α-rat albumin (Cappel Laboratories); α-PCNA (Santa Cruz Biotechnology); and α-BrdU (Boehringer-Mannheim). C/EBPα and β primary antibodies were diluted 1:250 in 3% goat serum in PBS. c-Jun and albumin were diluted 1:500 and 1:750, respectively. Tissue sections were incubated for 45 min at room temperature in a humidified chamber. After the secondary antibody incubation step, the sections were incubated with 0.3% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity. Detection of PCNA was performed as above with the following modifications. After rehydration, tissue sections were incubated for 20 min in 2 N HCl at 37°C followed by a 5-min wash in dH_2O. The remainder of the procedure was according to the manufacturer's instructions. Double labeling of tissue sections was performed using the Vector Laboratories Vectastain Elite system to detect C/EBPα or β and rhodamine immunofluorescence to detect BrdU incorporation. After rehydration, tissue sections were incubated with pepsin (Sigma P-6887) 0.05 mg/ml in 0.1 N HCl at 37°C for 7 min, followed by three 5-min washes in PBS. A peroxidase quench step was then performed as described above. Tissue sections were then rinsed for 5 min in dH_20 followed by a 5-min incubation in PBS. Sections were then incubated for 10 min in 0.25% NH_4Cl in PBS to quench endogenous fluorescence. Blocking steps in 3% goat serum/PBS, avidin, or biotin were according to the Vectastain ABC Elite kit manufacturer's instructions. A mixture of both primary antibodies (either α-C/EBPα or α-C/EBPβ and α-BrdU) was diluted 1:250 in 3% goat serum/3% horse serum/PBS. Tissue sections were incubated for 60 min at 37°C in a humidified chamber and washed in PBS for 10 min. A mixture of biotinylated goat α-rabbit secondary antibody and affinity-purified rhodamine-conjugated goat α-mouse secondary antibodies was diluted 1:200 and 1:250, respectively, in 3% goat serum/3% horse serum/PBS, and tissue sections were incubated for 30 min at room temperature. Sections were then washed in PBS for 10 min followed by two 5-min washes in 0.25% Triton X-100/PBS. ABC reagent and substrate incubations were performed according to the manufacturer's instructions. Tissue sections were then briefly incubated in PBS and mounted with 25% glycerol/PBS.

Transient transfections. The plasmids used in the cotransfection assays include the expression vectors pCMV-p65/RelA (p65/RelA, under the control of the cytomegalovirus promoter) and pMSV-C/EBP-α and pMSV-C/EBP-β (C/EBP-α and -β genes with the murine sarcoma virus long terminal repeat promoter) (16, 32). Reporter vectors include SRE-luc, containing four copies of the c-*fos* promoter SRE sequence (−320 to −290 of the c-*fos* gene) located upstream of a luciferase gene; insulin-like growth factor binding protein-1 (IGFBP-1)pro-luc, containing the mouse *IGFBP-1* promoter region (−791 to −1 of the *IGFBP-1* gene) (43) inserted upstream of the luciferase gene in a pGL2-Basic luciferase reporter vector (Promega, Madison, WI).

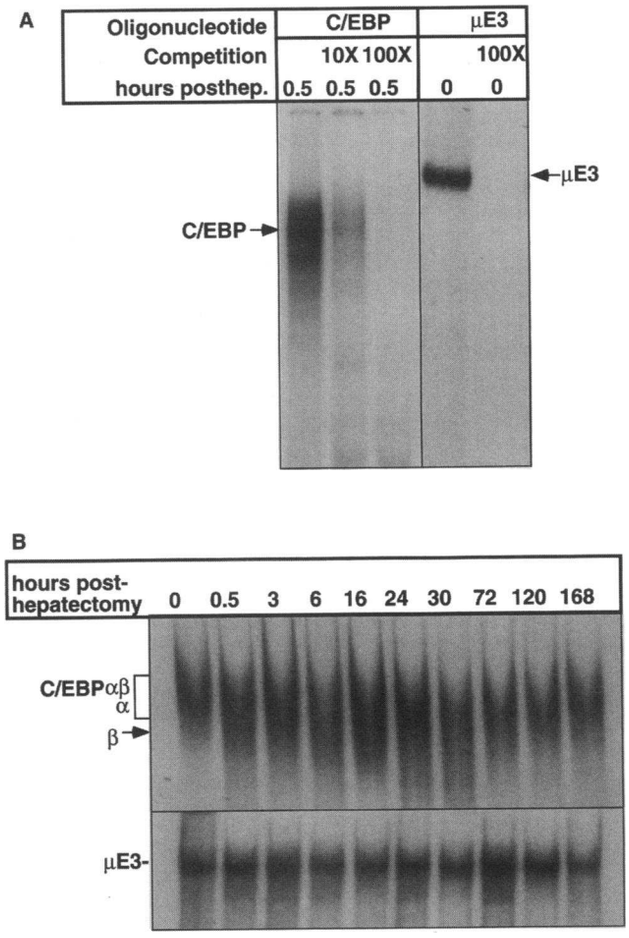

Figure 2. C/EBP binding activity is present in nuclear extracts throughout a 168-h period after hepatectomy. EMSAs were performed using ^{32}P-end labeled oligonucleotide containing a consensus C/EBPα/β site or μE3 enhancer motif in the presence of nuclear extracts. (*A*) Cold competition EMSA using regenerating liver nuclear extract from 0.5 h after hepatectomy (C/EBP) or 0 h (μE3) incubated with excess unlabeled oligonucleotide before the addition of labeled probe. (*B*) C/EBP binding in nuclear extracts prepared from regenerating liver at indicated times after hepatectomy. For clarity, the positions of the C/EBP isoforms as determined in Fig. 3 are indicated. μE3 binding activity was used to normalize the extracts. An excess of probe detected as free probe was used in all EMSAs; however, to conserve space, the free probe is not shown.

F9 embryonal carcinoma cells were grown in DME (supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 μg/ml penicillin/streptomycin 100 mm) on 100-mm dishes. At 50% confluency, the cells were transfected with the indicated amounts of expression plasmids, 8 μg of reporter plasmid, and 4 μg of CH110 β-galactosidase plasmid as a transfection control. In all cases, the amount of DNA transfected per dish was made constant with the addition of pCMV vector alone. At 12 h after calcium phosphate transfection, cells were washed twice in phosphate-buffered saline, harvested in 900 μl 1× luciferase reporter lysis buffer (Promega), freeze-thawed once, the cellular debris pelleted, and 20 μl of the supernatant removed and assayed for luciferase activity according to the instructions of the manufacturer. Luciferase activity was quantified using a luminometer (Moonlight 2001; Analytical Lumi-

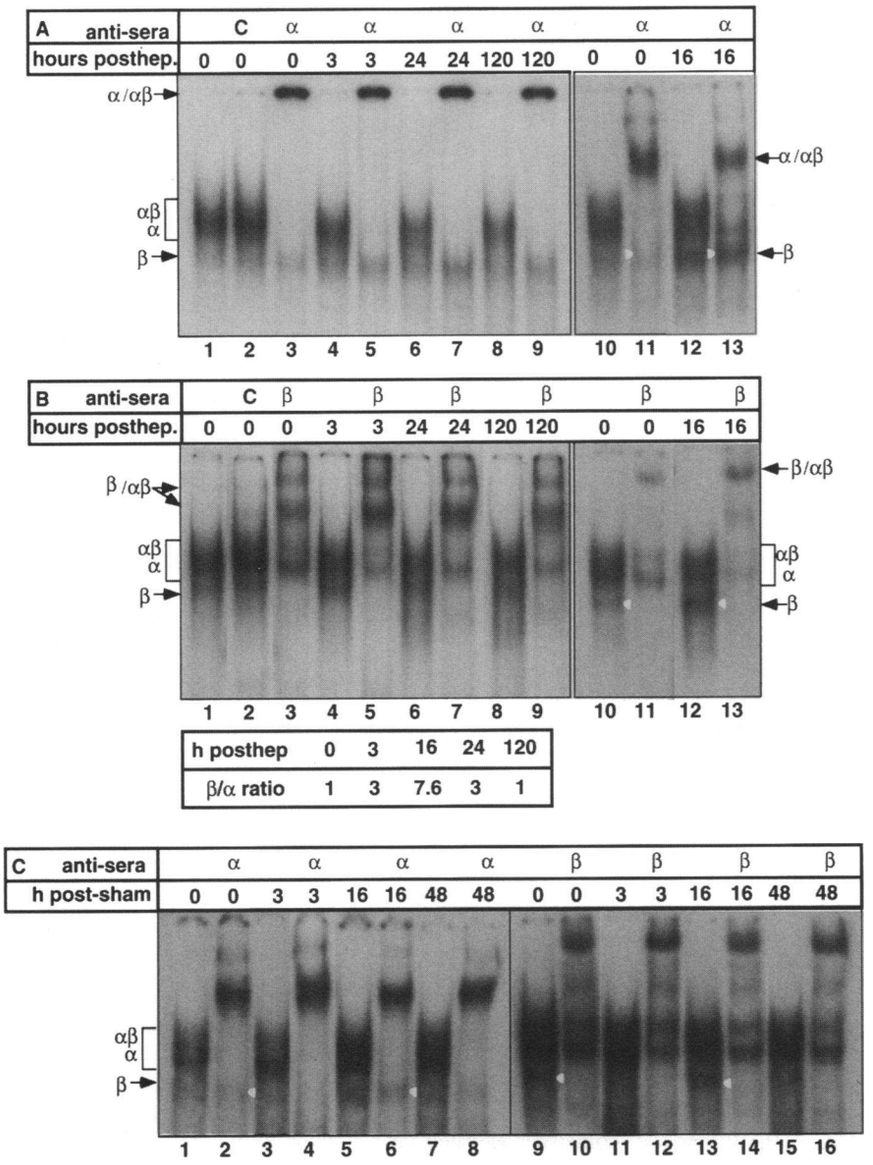

Figure 3. C/EBPα (*A*) and β (*B*) contribute to the DNA binding activity in nuclear extracts from regenerating liver. EMSAs were performed using a ^{32}P-end labeled C/EBPα/β consensus sequence oligonucleotide in the presence of nuclear extract prepared at the indicated times after hepatectomy. The labels *C*, α, or β over corresponding lanes indicate the addition of control, α-C/EBPα (a mixture of all C/EBPα antisera in the left panel, a single C/EBPα antibody α-14 in the right panel), or α-C/EBPβ antisera to the binding reaction after incubation of extract and oligonucleotide. The symbols on the left and right of the figure indicate the C/EBP isoform composition of the corresponding binding complexes and supershifted complexes. White arrows show high levels of binding at the β homodimer position in the 16-h extract. The relative β/α level was calculated by densitometry for each time point. (*C*) No change in C/EBP complex formation after sham surgery. EMSAs and supershifts as described in *A* and *B*. White arrows indicate β complex which is relatively unchanged.

nescence Laboratory, San Diego, CA). 40 μl of cell extract was also used to assay for β-galactosidase activity (44). Results from quantification of activity at OD 420 nm were used to normalize dishes for levels of transfection.

Results

Continued C/EBPα and β protein expression throughout a 168-h period after partial hepatectomy and increase in the relative level of C/EBPβ from 3 to 24 h after hepatectomy. Previously we showed that *C/EBPα* mRNA levels decline slightly during the first 24 h after partial hepatectomy (4, 34) and increase with a broad peak between 60 and 216 h after hepatectomy. Using additional hybridization controls, we reconfirmed these results finding a maximal decrease of twofold in *C/EBPα* mRNA at 16 h after hepatectomy (not shown). However, the major determinate of C/EBP activity during liver regeneration is the level of active C/EBP protein. We characterized the expression of C/EBPα and C/EBPβ proteins during the entire temporal course of regeneration after partial hepatectomy. Immunoblots of whole nuclei prepared at indicated times after hepatectomy were probed with either C/EBPα (Fig. 1 *A*) or C/EBPβ (Fig. 1 *B*) specific antibodies. The 42- and 30-kD bands detected represent alternative translation products of C/EBPα that have different transcription activation functions (21, 45). "LIP" represents an alternative translation product of C/EBPβ (25).

As surgery itself may induce a stress or acute phase response that could modify the level of nuclear proteins, we demonstrated that sham surgery has little effect on the levels of C/EBPα and C/EBPβ protein levels during the time period when posthepatectomy changes in C/EBPα and C/EBPβ levels are most pronounced (Fig. 1 *C*).

The level of protein expression was determined using densitometric scanning of several immunoblots probed with C/EBPα or C/EBPβ antibodies (Fig. 1 *D*). We did not observe any

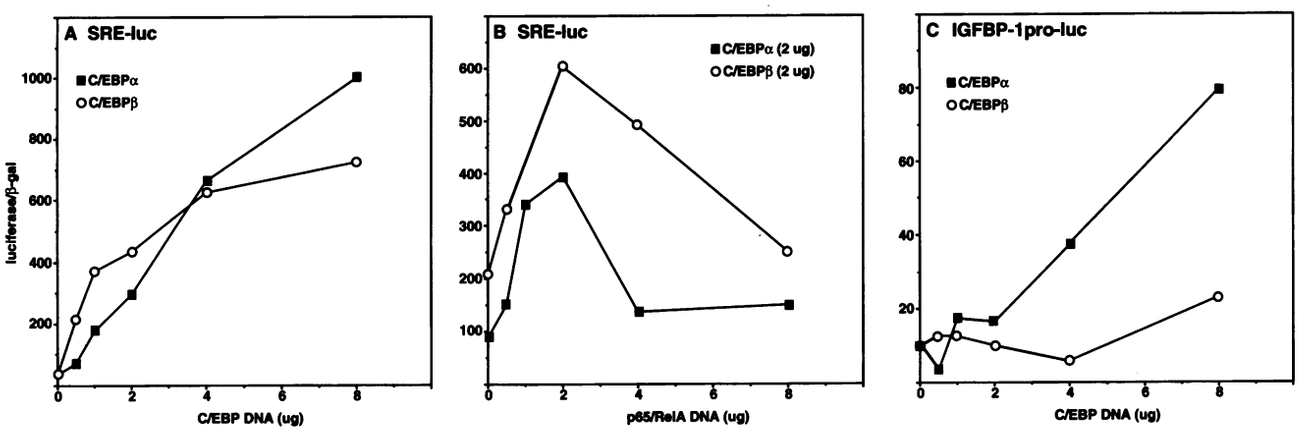

Figure 4. Differential transactivation of potential immediate-early gene target promoters by C/EBPα and β. Transfections were performed as described in Methods using (*A* and *B*) SRE-luc, (*C*) IGFBP-1pro-luc reporters in the presence of indicated concentrations of either C/EBPα or β (*A and C*); or indicated amounts of p65/RelA (*B*) DNA with constant amounts of C/EBPα or β DNA. Activity was calculated after setting the value of the PGL2-Basic luciferase vector to 1 after normalization to β-gal activity. In *C*, background activity of PGL2-Basic in the presence of each amount of C/EBP DNA was subtracted for each point. These data are derived from a single representative experiment. Each experiment was performed three times.

difference in the relative expression of the two isoforms of C/EBPα or β; thus, graphic representation of protein level is shown only for C/EBPα (42 kD) and C/EBPβ (36 kD). Although in Fig. 1 *A* there is an apparent increase in the level of C/EBPα protein 30 min after hepatectomy, densitometric quantitation of multiple immunoblots demonstrates that the protein level remains unchanged during this time period. The levels of C/EBPα 42- and 30-kD proteins progressively decrease to < 50% of prehepatectomy levels from 8–24 h after hepatectomy, which corresponds to the decrease in *C/EBPα* mRNA. C/EBPα protein renormalizes at 48 h after hepatectomy and increases to slightly above normal at 120 h after hepatectomy. Although Northern blot analysis shows a major mRNA peak 168 h after hepatectomy, we did not observe a corresponding peak in protein expression at this time point. Immunoblot analysis of C/EBPβ protein in whole nuclei demonstrates a reciprocal pattern of expression as compared with C/EBPα. C/EBPβ protein levels increase with a peak at 8 h after hepatectomy that is up to threefold above baseline and remain slightly elevated during the period 24–120 h after hepatectomy. Sham surgery does not result in significant changes in protein levels. This finding predicts that the relative level of β to α is increased during the 3–24 h time period after hepatectomy when C/EBPβ levels are maximal and α levels are depressed.

Total C/EBP DNA binding activity in nuclear extracts from regenerating liver during a 168-h period after partial hepatectomy. To detect any changes in the level of functional protein, we next measured C/EBP DNA binding activity in nuclear extracts. Nuclear extracts were incubated with a radiolabeled oligonucleotide encoding a consensus C/EBPα/β binding sequence, or μE3, a control probe, to demonstrate effective DNA binding (Fig. 2 *A*). The addition of excess unlabeled C/EBP or μE3 oligonucleotide to the reaction successfully competes for the binding activity, indicating that the binding is specific. Nuclear extracts prepared from regenerating livers at indicated times after hepatectomy were incubated with the C/EBP probe to quantitate the relative level of binding during 168 h after hepatectomy. Constitutive binding activity to the μE3 immunoglobulin enhancer (46, 47) was used to normalize the extracts (Fig. 2 *B*). We found that total DNA binding activity to the C/EBP consensus oligonucleotide remains relatively constant during the posthepatectomy period examined.

Relative C/EBPα and C/EBPβ binding activities in regenerating liver nuclear extracts. To determine the relative contributions of C/EBPα and β to the binding activity at various times after hepatectomy, DNA binding complexes from normal liver 3, 16, 24, and 120 h after hepatectomy were incubated with an equal mixture of C/EBPα antibodies (α-N; α-14, α-C103; α-C104, α-C106) or α-C/EBPβ (Fig. 3, *A* and *B*, indicated by the symbol α or β above the corresponding lanes). These times were chosen because they represent normal liver (0); early G1 (3 h), when the level of β is increased relative to α; late G1 (16 h) when the relative changes in C/EBP levels are maximal; S phase (24 h), when the level of β is still somewhat elevated; and after proliferation (120 h) when the level of α has renormalized. The addition of α-C/EBPα to the binding complex results in disruption of the upper portion of the binding complex at each time point examined. Addition of C/EBPβ antibody to the binding complex results in the formation of a band with decreased mobility (Fig. 3, *arrows*) and concomitant disappearance of the upper and lower portions of the binding complex. Taken together, the results of these supershift analyses suggest that the supershifted band that results from the addition of α-C/EBPβ likely contains C/EBPβ homodimer and C/EBPα/β heterodimer binding activity. The residual band that is not supershifted by α-C/EBPβ most likely contains C/EBPα binding activity. Similarly, the disruption of the upper portions of the DNA binding complex by the addition of α-C/EBPα suggests that these complexes contain C/EBPα/β heterodimer and C/EBPα homodimer binding activity. The remaining nondisrupted complex most likely contains C/EBPβ homodimer binding activity. We cannot exclude the presence of other proteins in these bands, such as C/EBPδ, because our C/EBPδ antibody was less effective in immunoblot and supershift studies. However, there appears to be relatively little contribution from homodimers other than C/EBPα and β be-

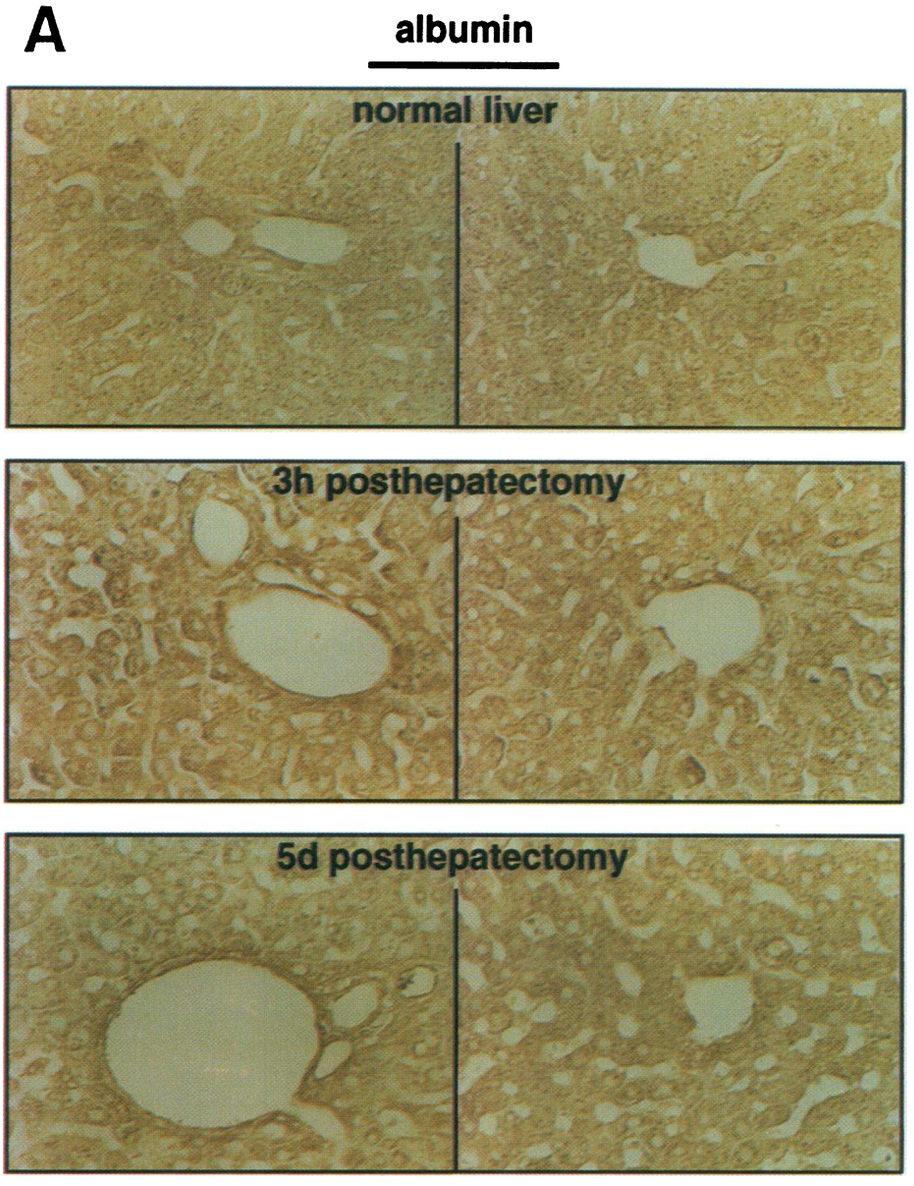

Figure 5. Expression of albumin, c-Jun, PCNA, and BrdU at appropriate times in regenerating liver. (*A*) Albumin, portal vein (*left*) and central vein (*right*) from normal liver and 3 and 120 h after hepatectomy incubated with an albumin-specific antibody are shown; (*B*) c-Jun, at 0 and 3 h after hepatectomy; (*C*) PCNA, at 0 and 24 h after hepatectomy; (*D*) BrdU in BrdU-pulsed animals, at 0 and 24 h after hepatectomy. ×62.5.

cause there is virtually no activity that is unshifted by either α or β specific antisera.

There is a notable increase in the contribution of C/EBPβ to the DNA binding complex, particularly when a comparison is made between 0 and 16 h after hepatectomy (see *white arrows* in lanes *11* and *12* in Fig. 3 *A*, and lanes *10* and *12* in Fig. 3 *B*). There is a smaller decrease in the C/EBPα complex at 3 and 16 h (Fig. 3 *B*, lane *3* vs. *5*, lane *11* vs. *13*). By calculating the amount of material in the supershift or residual bands, we were able to determine that the ratio of C/EBPβ to C/EBPα DNA binding increases up to 7.6-fold at 16 h after hepatectomy, which is consistent with the immunoblot analyses.

As shown, sham surgery does not result in any significant alteration in the ratio of C/EBPα/β DNA binding complex as both α and β complexes are unchanged after sham surgery (Fig. 3 *C*). White arrows in Fig. 3 *C* denote the consistent low level of the β complex.

Differential activation of immediate-early gene target promoters by C/EBPα and C/EBPβ. As we observe no change in the total C/EBP binding activity throughout the temporal course of liver regeneration but a significant change in the relative ratio of C/EBPβ to α, it was important to assess potential differences in the ability of the two C/EBP proteins to activate target promoters that are regulated during liver regeneration. Studies indicate that the relative ability of C/EBPα and β to activate various target promoters may be different (16, 48–50), but their relative impact on immediate-early gene promoters has not been assessed. We have observed previously that the peak of transcription of the *IGFBP-1* gene occurs at 30 min after hepatectomy (51). Similarly, we have shown that c-*fos* mRNA peaks 30 min after hepatectomy (4) with peak transcriptional initiation at 30 min after hepatectomy (52). As the C/EBPα to β ratio for this time period is reflective of normal liver, we reasoned that C/EBPα and/or β may participate in the activation of the c-*fos* and *IGFBP-1* promoters. As an initial study, we assessed the impact on the c-*fos* gene serum response element (SRE) which

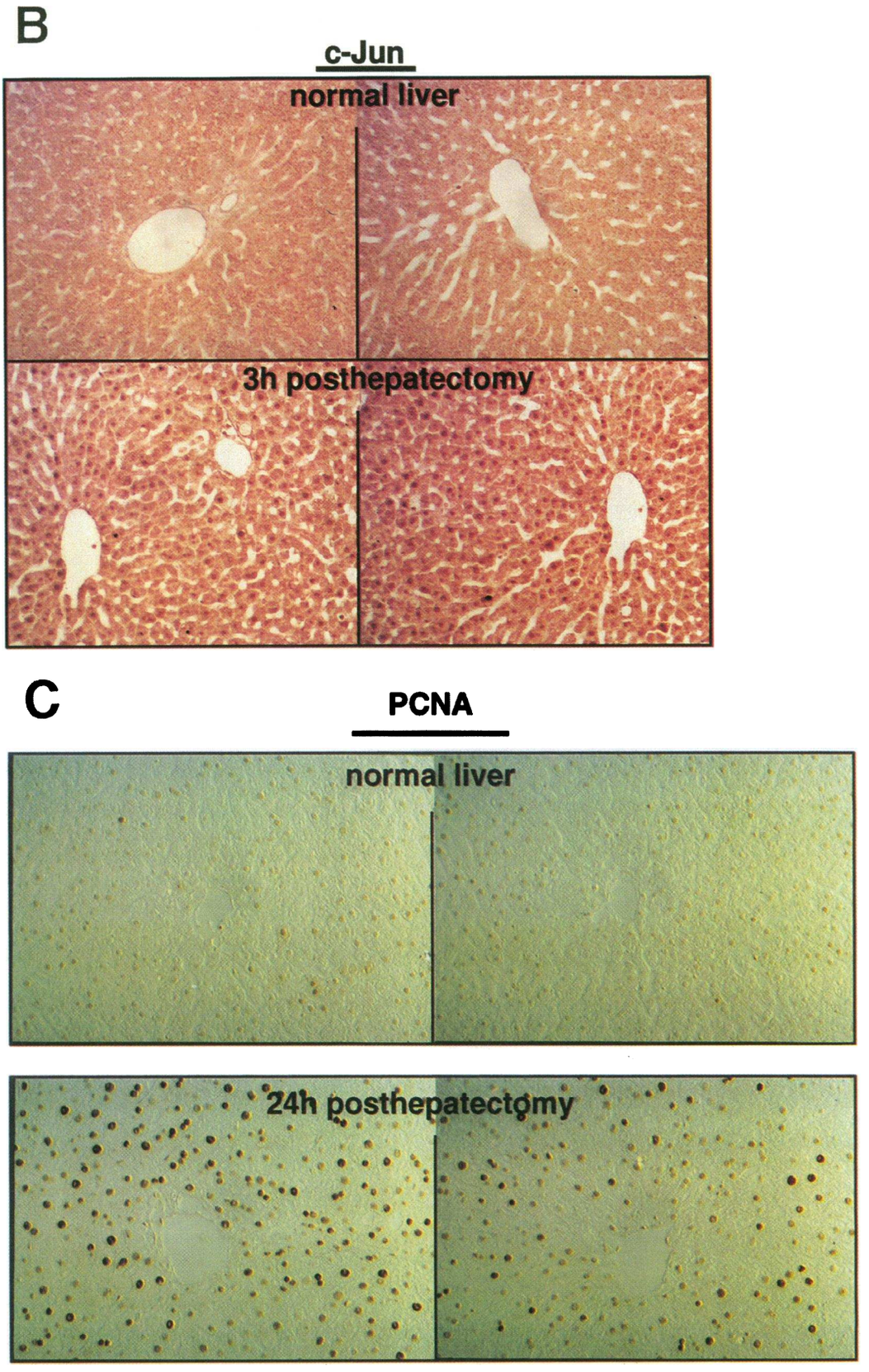

Figure 5 (Continued)

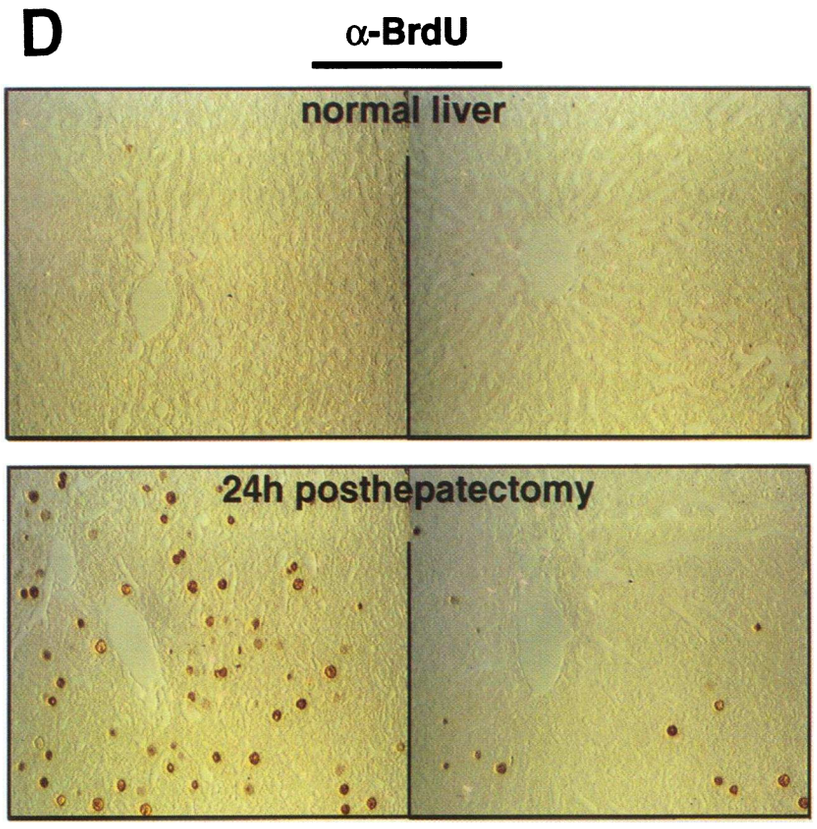

Figure 5 (Continued)

has previously been shown to be activated by C/EBPβ via an overlapping C/EBP site (53) (Fig. 4 *A*). In that study, no comparison was made between the activity of C/EBPα and β. We used F9 cells which have a low level of all of the transcription factors tested (16) and therefore provide a clean background in which to test the relative transactivation potential of individual factors. As shown, both C/EBPα and β strongly transactivate the promoter, although C/EBPβ is more effective at lower concentrations.

In regenerating liver, an NF-κB complex designated PHF/NF-κB which contains p65/RelA and p50/NF-κB1 is rapidly activated at 30 min after partial hepatectomy, a temporal course that is compatible with c-*fos* gene activation (13, 40). Therefore, c-*fos* is a potential target gene of PHF/NF-κB. Previously it has been shown that p65/RelA acts synergistically with C/EBPβ to transactivate the SRE element (16). As C/EBPs and NF-κB are simultaneously present in the early phases of liver regeneration, we assessed their combined impact on the SRE. Although both interact synergistically with p65/RelA, C/EBPβ interacts more effectively than C/EBPα (Fig. 4 *B*).

As C/EBPs are found in hepatocytes in regenerating liver, we reasoned that they might participate in transactivating hepatocyte-specific immediate-early genes. *IGFBP-1* is a liver-restricted immediate-early gene that is transcriptionally activated in regenerating liver at 0.5–2 h after hepatectomy when the levels of C/EBPα have not yet fallen (51). The *IGFBP-1* promoter is more effectively transactivated by C/EBPα (Fig. 4 *C*) than β, which has virtually no activity. These studies provide initial support for the hypothesis that changes in the relative ratios of C/EBPα and β may result in differential target gene activation during liver regeneration.

Markers for normal liver, G1 and S phase in liver regeneration. Virtually all hepatocytes undergo proliferation after partial hepatectomy, but the timing of DNA synthesis varies in different zones (1). We reasoned that one mechanism by which growth and differentiation could occur simultaneously in the regenerating liver would be if differentiated function were maintained at some times during the cell cycle and not others. If this were true, we would expect to see a mutually exclusive zonal distribution of proteins that are markers for growth and differentiation, respectively. Such distribution can be assessed by performing immunohistochemistry.

As a marker for hepatocytes, and for the integrity of the fixed sections, we demonstrated that albumin is detected throughout the liver before and after hepatectomy (Fig. 5 *A*). The immediate-early gene, c-*jun* is a marker for early G1 phase. We showed that c-Jun protein is present in the nuclei of hepatocytes throughout the liver at 3 h after hepatectomy (Fig. 5 *B*). There is no zonal distribution of the protein, no expression in normal liver, and the induction at 3 h indicates that the liver in this sample is appropriately regenerating.

We used two markers for S phase, PCNA, an S phase–specific protein, and anti-BrdU antibody, to detect cells specifically synthesizing DNA in animals that had been pulsed for 1 h with BrdU. We found that PCNA is expressed in a large percentage of cells at 24 h after hepatectomy (Fig. 5 *C*), and BrdU, a more specific indicator of DNA synthesis, is expressed at a high level in the periportal and lower level in the pericentral regions of the liver (Fig. 5 *D*). These findings confirm that many hepatocytes have entered S phase of the cell cycle at 24 h after hepatectomy, the peak of the first round of DNA synthesis. The expected periportal increase in DNA synthesis is observed.

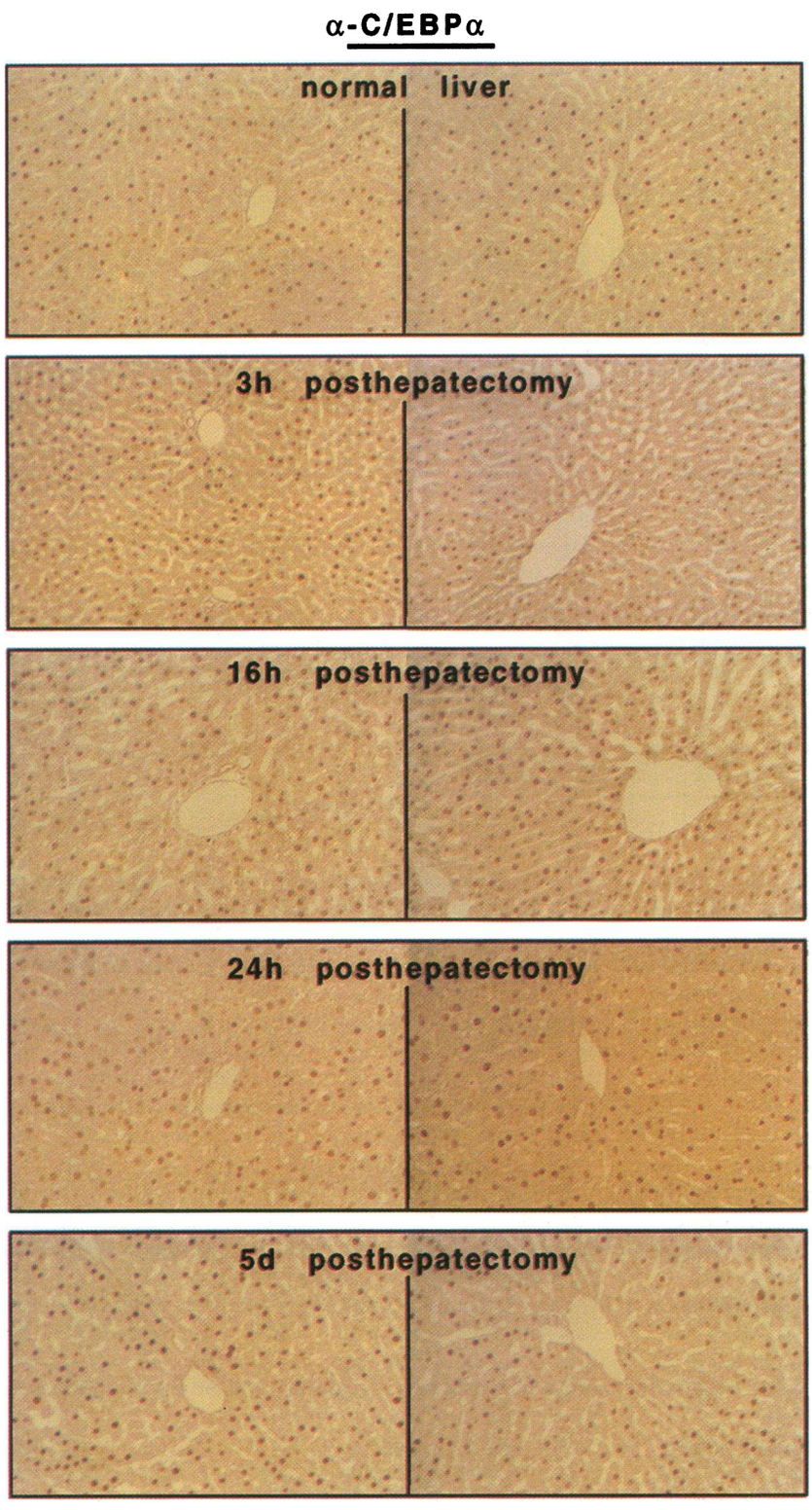

Figure 6. C/EBPα protein is found in virtually all hepatocyte nuclei in normal and regenerating liver. Livers were fixed as described in Methods. 5-μm tissue sections from the indicated times after hepatectomy were incubated with a C/EBPα specific antibody (α-C103) and antibody binding was detected using an avidin biotin horseradish peroxidase method. Portal vein and central vein regions are shown in the left and right panels, respectively. Identical exposure times, ×62.5.

C/EBPα and C/EBPβ expression in all hepatocytes throughout the temporal course of liver regeneration. Using a C/EBPα specific antibody, we were able to detect hepatocyte nuclear staining in normal and regenerating liver at 0, 3, 16, 24, and 120 h after hepatectomy (Fig. 6). We found that virtually all hepatocyte nuclei are positive for C/EBPα at all time points examined and this staining is distributed equally throughout the liver parenchyma without any zonal localization to periportal or pericentral areas. There is no obvious difference in the absolute level of C/EBPα at any time after hepatectomy, but immunohistochemistry may not be the best method for quantitation. Our ability to detect C/EBPα in all hepatocyte nuclei in 3-h

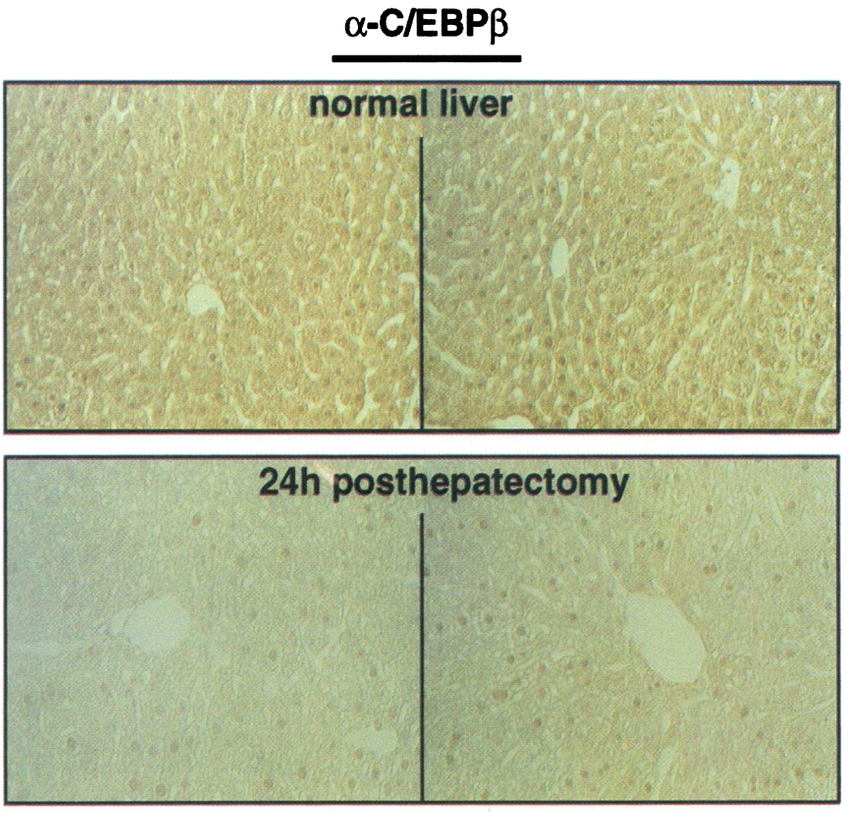

Figure 7. C/EBPβ protein is found in hepatocyte nuclei in normal liver and in regenerating liver 24 h after hepatectomy. Liver sections were prepared as described previously. Tissue sections from normal liver and 24 h after hepatectomy were incubated with a C/EBPβ specific antibody and antibody staining was detected using the avidin biotin horseradish peroxidase method. Portal vein and central vein regions are shown in the left and right panels, respectively, ×62.5.

posthepatectomy liver indicates that C/EBPα and c-Jun are coexpressed. Moreover, these findings suggest that C/EBPα expression coexists with DNA synthesis, but this fact was more firmly established by double labeling technology (see below).

Using an antibody to the C/EBPβ protein, we detected nuclear staining in hepatocyte nuclei in normal liver and 24 h after hepatectomy (Fig. 7). Nuclear staining appeared somewhat increased in the 24-h posthepatectomy liver as compared with normal liver. In addition, cytoplasmic staining is observed in the normal and regenerating liver, but as this antibody is not as effective for immunohistochemistry as the C/EBPα antibody, we do not know if the cytoplasmic staining is specific. As is the case for C/EBPα, there is no zonal pattern of nuclear staining. Because the C/EBPα and β antibodies used detect both isoforms of each protein, we cannot rule out region-specific differences in the levels of the different C/EBPα and β isoforms.

Continued expression of C/EBPα and β in hepatocytes that are synthesizing DNA. As a result of our observation that C/EBPα and β proteins are expressed in the majority of hepatocytes throughout the posthepatectomy period, it seemed probable that these proteins are expressed in proliferating hepatocytes. To confirm that C/EBPα and β are expressed in hepatocytes which are in S phase of the cell cycle, we next performed double labeling studies in which C/EBPα (Fig. 8 *A*) or C/EBPβ (Fig. 8 *B*) expression is colocalized with BrdU incorporation. We found that virtually all hepatocytes are positively stained for C/EBPα or β in normal and 24-h posthepatectomy liver, which is consistent with our results with single labeling studies (Fig. 8, *A* and *B, left panels*). Less than 1% of nuclei in the normal liver are positive for BrdU incorporation, with ~ 20% of hepatocyte nuclei positively stained for BrdU incorporation in the 24-h posthepatectomy liver. The majority of BrdU-positive hepatocytes are located in the periportal areas. Representative portal and central vein areas demonstrate that BrdU incorporation is simultaneously detected in hepatocytes that are positive for C/EBPα or C/EBPβ expression (Fig. 8, *arrows*). These results suggest that C/EBPα and β proteins continue to be expressed in hepatocytes that are in S phase of the cell cycle.

Discussion

This study is a complete and informative analysis of C/EBP expression in the context of liver regeneration that (*a*) uses immunoblot and DNA binding assays to provide accurate and consistent quantitation of C/EBPα and β levels throughout the temporal course of liver regeneration and sham surgery; (*b*) assesses the relative impact of the C/EBP isoforms on expression of two regeneration-induced immediate-early genes; and (*c*) demonstrates the regional localization of C/EBP isoforms and their coexpression with markers of proliferation. The relative increase in C/EBPβ to α in the prereplicative phase of liver regeneration may have a significant impact on target gene activation that may help promote liver growth. Our conclusion that substantial levels of C/EBPα and β isoforms are compatible with DNA synthesis in regenerating hepatocytes predicts that C/EBPs do not have profound growth inhibitory roles in this cell type.

We see no changes in the relative ratios of the C/EBPα (42 and 30 kD) or C/EBPβ (36 and 20 kD) isoforms, which may be significant because different transactivation properties have been attributed to the various translational products (21, 25, 45). In preadipocytes, the 42-kD translational product of the

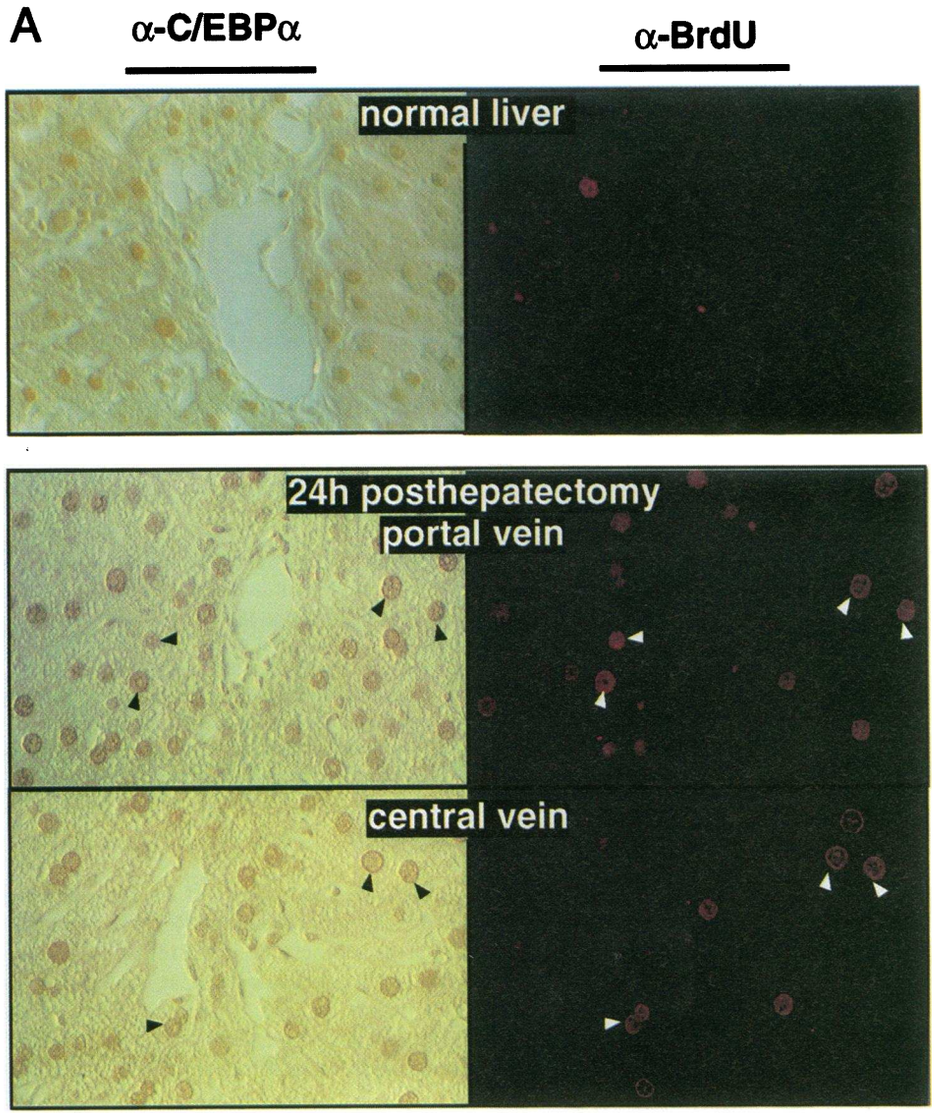

Figure 8. C/EBPα and β are expressed in hepatocytes that are in S phase of the cell cycle. Sections from normal and 24-h posthepatectomy liver were double-labeled with α- C/EBPα (*A*), or α-C/EBPβ and α-BrdU (*B*). Arrows indicate representative hepatocyte nuclei that are positively stained with both antibodies. Portal and central vein areas are shown in upper and lower panels, respectively, for the C/EBPα 24-h posthepatectomy sample, ×125.

C/EBPα message is antiproliferative, whereas the 30-kD alternative translation product is not antimitotic (21); it has been hypothesized that changes in the ratio of these two forms during rat liver development may regulate the balance between growth and differentiation. Either the liver is refractory to this antiproliferative effect of C/EBPα or the decline in C/EBPα expression seen in mid to late G1 and S phase is sufficient to permit entry into S phase.

The pattern of C/EBPβ expression after partial hepatectomy suggests that C/EBPβ is not likely to function as an antiproliferative factor in our model. Nuclear C/EBPβ including active DNA binding C/EBPβ is present throughout the early posthepatectomy period at levels that are consistently above prehepatectomy values. Like C/EBPα, C/EBPβ is detected in hepatocytes that are in S phase of the cell cycle. A potential caveat of this study is that the phosphorylation state of C/EBPβ may be important in determining its activity (54), but currently there is no reason to suspect that phosphorylation of nuclear C/EBPβ controls C/EBPβ activity during regeneration. Our findings contrast with a study (33) in which overexpression of LAP, the C/EBPβ rat homologue, prevents the progression of HepG2 cells from G_1 to S phase. However, studies in isolated hepatocytes suggest that C/EBPβ expression is compatible with DNA synthesis (55), and it is known that hepatoma cells can exhibit different growth characteristics than hepatocytes. For example, hepatocyte growth factor inhibits hepatoma cell proliferation (56) but is stimulatory to hepatocytes (57–59). It is not surprising that C/EBPβ expression blocks proliferation in a transformed cell line and is compatible with proliferation of hepatocytes in the regenerating liver.

Although C/EBPα levels drop abruptly in culture (55), in association with the disappearance of most hepatocyte-specific gene expression this observation differs from that seen in the intact liver. These contrasting observations may reflect a requirement for matrix communications to maintain the differentiated phenotype. When isolated primary hepatocytes are cultured on a reconstituted basement membrane gel matrix, C/EBPα expression is restored (55).

The absolute changes in the levels of C/EBPα and β are small but result in a more significant change of more than

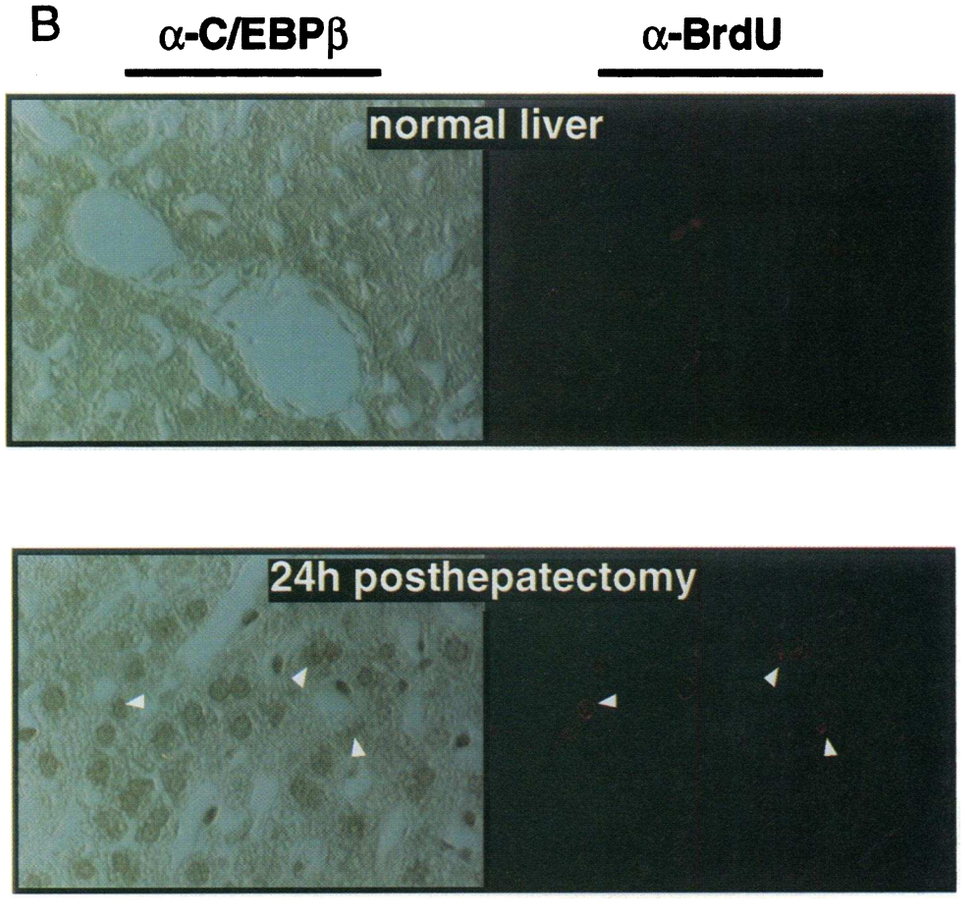

Figure 8 (Continued)

sevenfold in the relative DNA binding activity of C/EBPβ as compared with α. The relative increase of C/EBPβ to α activity during mid to late G1 and early S phase of hepatocyte proliferation may result in important changes in gene activation potential. For example, transfection analyses have demonstrated that the contribution of β and α to transcriptional activation of specific target promoters may differ. To name a few, *PEPCK, CYP2D5, ornithine transcarbamylase,* and the *α1-acid glycoproteins* genes have been shown to be differentially regulated by C/EBPα and β (16, 48–50). Our initial studies of immediate-early promoters extend these observations because we found differential activation by the two C/EBP isoforms of both the *fos* SRE and the liver-specific *IGFBP-1* gene promoter. In fact, an interesting outcome of our studies is the implication that c-*fos* gene expression may be controlled during liver regeneration by a cooperative interaction between a growth-induced transcription factor (PHF/NF-κB) and a liver-specific factor (C/EBP), thus not necessarily requiring serum response factor, a known upregulator of c-*fos* during cell growth (60).

It will be important to examine the interactions between the C/EBP proteins and other growth-induced transcription factors that are likely to participate in the regulation of a variety of genes associated with maintenance of the differentiated state of the liver. For example C/EBPα and HNF-1 via direct binding to the albumin promoter can synergistically activate the albumin gene in HeLa cells (18). C/EBP proteins may also participate in the regulation of liver-specific immediate-early genes through interactions with growth-induced transcription factors. As shown above, C/EBPs may interact with NF-κB. Cooperative interactions have also been identified between C/EBP and activator protein-1 transcription factors (61), which are induced during liver regeneration (40). Our ability to demonstrate C/EBP, NF-κB (13, 14), and c-Jun coexpression in regenerating hepatocytes provides a basis for examining potential interactions between these factors.

Possible targets for cooperative regulatory interactions are genes encoding gluconeogenic enzymes that are rapidly upregulated both at birth and in the remnant liver immediately after hepatectomy (4, 5, 61). These include *G6Pase, PEPCK,* and potentially others, although currently only *PEPCK* is known to be regulated by C/EBP isoforms. C/EBP levels are low in tyrosinemic animals that show blunted induction of gluconeogenic enzymes at birth (62). Animals in which both C/EBPα alleles have been deleted die within several hours of birth as a result of profound hypoglycemia and also show blunted induction of mRNAs encoding key gluconeogenic enzymes including *G6Pase* and *PEPCK* (63). Thus, the expression of these genes correlates with C/EBP levels, suggesting that C/EBPs coupled with hormonal changes (64) may contribute in helping the liver to maintain glucose homeostasis during the acute stress of birth and removal of two-thirds of the liver. The persistence of C/EBP expression during the major proliferative states of the liver during postnatal growth and hepatic regeneration suggests that differentiation-specific processes can cooperate with proliferation to support survival of the animal in these physiologic conditions.

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References

1. Michalopoulos, G. K. 1990. Liver regeneration: molecular mechanisms of growth control. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:176–187.

2. Fausto, N. 1994. Liver regeneration. *In* The Liver: Biology and Pathobiology. I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, editors. Raven Press, New York. 1059–1086.

3. Fausto, N., and M. E. Mead. 1989. Regulation of liver growth: protooncogenes and transforming growth factors. *Lab. Invest.* 60:4–13.

4. Mohn, K. L., T. M. Laz, A. E. Melby, and R. Taub. 1990. Immediate-early gene expression differs between regeneration liver, insulin-stimulated H-35 cells, and mitogen-stimulated 3T3 cells: liver specific induction patterns of gene 33, PEPCK, and the jun, fos and egr families. *J. Biol. Chem.* 265:21914–21921.

5. Haber, B. A., S. Chin, E. Chuang, W. Buikhuisen, A. Naji, and R. Taub. 1995. High levels of glucose-6-phosphatase gene and protein expression in proliferating liver and diabetes. *J. Clin. Invest.* 95:832–841.

6. Mohn, K. L., T. M. Laz, J.-C. Hsu, A. E. Melby, R. Bravo, and R. Taub. 1991. The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison to serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. *Mol. Cell. Biol.* 11:381–390.

7. Lau, L. F., and D. Nathans. 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3145–3151.

8. Lau, L. F., and D. Nathans. 1987. Expression of a set of growth-related immediate early genes in BALB/c3T3 cells: coordinate regulation with *c-fos* or *c-myc*. *Proc. Natl. Acad. Sci. USA.* 84:1182–1186.

9. Almendral, J. M., D. Sommer, H. MacDonald-Bravo, J. Burckhardt, J. Perera, and R. Bravo. 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol. Cell. Biol.* 8:2140–2148.

10. Zipfel, P. F., S. G. Irving, K. Kelly, and U. Siebenlist. 1989. Complexity of the primary genetic response to mitogenic activation of human T cells. *Mol. Cell. Biol.* 9:1041–1048.

11. Bravo, R. 1990. Growth Factors, Differentiation Factors, and Cytokines. Springer Verlag, Berlin. 1–476.

12. Herschman, H. R. 1991. Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* 60:281–319.

13. Cressman, D. E., L. E. Greenbaum, B. A. Haber, and R. Taub. 1994. Rapid activation of PHF/NFκB in hepatocytes, a primary response in the regenerating liver. *J. Biol. Chem.* 269:30429–30435.

14. Cressman, D. E., and R. Taub. 1994. Physiologic turnover of NF-κB by nuclear proteolysis. *J. Biol. Chem.* 269:26594–26597.

15. Cressman, D. E., R. H. Diamond, and R. Taub. 1995. Rapid activation of Stat3 DNA binding in liver regeneration. *Hepatology.* 21:1443–1449.

16. Stein, B., and A. S. Baldwin, Jr. 1993. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. *Mol. Cell. Biol.* 13:7191–7198.

17. Stein, B., P. C. Cogswell, and A. S. Baldwin, Jr. 1993. Functional and physical associations between NF-κB and C/EBP family members: a Rel domain-bZIP interaction. *Mol. Cell. Biol.* 13:3964–3974.

18. Wu, K.-J., D. R. Wilson, C. Shih, and G. Darlington. 1994. The transcription factor HNF1 acts with C/EBPα to synergistically activate the human albumin promoter through a novel domain. *J. Biol. Chem.* 269:1177–1182.

19. Flodby, P., P. Antonson, C. Barlow, A. Blanck, I. Porsch-Hällström, and K. G. Xanthopoulos. 1993. Differential patterns of expression of three C/EBP isoforms, HNF-1, and HNF-4 after partial hepatectomy in rats. *Exp. Cell Res.* 208:248–256.

20. Qian, X., U. Samadani, A. Porcella, and R. H. Costa. 1995. Decreased expression of hepatocyte nuclear factor 3α during the acute-phase response influences transthyretin gene transcription. *Mol. Cell. Biol.* 15:1364–1376.

21. Lin, F.-T., O. A. MacDougald, A. M. Diehl, and M. D. Lane. 1993. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein α message: transcriptional activator lacking antimitotic activity. *Proc. Natl. Acad. Sci. USA.* 90:9606–9610.

22. Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. Graves, and S. L. McKnight. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes & Dev.* 2:786–800.

23. Friedman, A. D., and S. L. McKnight. 1990. Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene. *Genes & Dev.* 4:1416–1426.

24. Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight. 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes & Dev.* 3:1146–1156.

25. Descombes, P., and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell.* 67:569–579.

26. Friedman, A. D., W. H. Landschulz, and S. L. McKnight. 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes & Dev.* 3:1314–1322.

27. Costa, R. H., D. R. Grayson, K. G. Yanthopoulos, and J. E. J. Darnell. 1988. A liver-specific DNA binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, alpha-1-antitrypsin, albumin, and simian virus 40 genes. *Proc. Natl. Acad. Sci. USA.* 85:3840–3844.

28. Park, E. A., A. L. Gurney, S. E. Nizielski, P. Hakimi, Z. Cao, A. Moorman, and R. W. Hanson. 1993. Relative roles of CCAAT/enhancer-binding protein β and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphenolpyruvate carboxykinase (GTP). *J. Biol. Chem.* 268:613–619.

29. Christy, R. J., K. H. Kaestner, D. E. Geiman, and M. D. Lane. 1991. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA.* 188:2593–2597.

30. Cao, Z., R. M. Umek, and S. L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes & Dev.* 5:1538–1552.

31. Lin, F.-T., and M. D. Lane. 1992. Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes & Dev.* 6:533–544.

32. Umek, R. M., A. D. Friedman, and S. L. McKnight. 1991. CCAAT/Enhancer binding protein: a component of a differentiation switch. *Science (Wash. DC).* 251:288–291.

33. Buck, M., H. Turler, and M. Chojkier. 1994. LAP (NF-IL-6), a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:851–860.

34. Haber, B. A., K. L. Mohn, R. H. Diamond, and R. Taub. 1993. Induction patterns of 70 genes during nine days after hepatectomy define the temporal course of liver regeneration. *J. Clin. Invest.* 91:1319–1326.

35. Mischoulon, D., B. Rana, N. Bucher, and S. R. Farmer. 1992. Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBPα) gene expression during hepatocyte proliferation in the regenerating liver and in culture. *Mol. Cell. Biol.* 12:2553–2560.

36. Diehl, A. M., and S. Q. Yang. 1994. Regenerative changes in C/EBPα and C/EBPβ expression modulate binding to the C/EBP site in the c-*fos* promoter. *Hepatology.* 19:447–456.

37. Higgins, G. M., and R. M. Anderson. 1931. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol.* 12:186–202.

38. Hattori, M., A. Turgores, L. Veloz, M. Karin, and D. A. Brenner. 1990. A simplified method for the preparation of transcriptionally active liver nuclear extracts. *DNA Cell Biol.* 9:777–781.

39. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 18.62–18.63.

40. Hsu, J.-C., R. Bravo, and R. Taub. 1992. Interactions between LRF-1, JunB, c-Jun and c-Fos define a regulatory program in the G_1 phase of liver regeneration. *Mol. Cell. Biol.* 12:4654–4665.

41. Tewari, M., P. Dobrzanski, K. L. Mohn, D. E. Cressman, J.-C. Hsu, R. Bravo, and R. Taub. 1992. Rapid induction in regenerating liver of RL/IF-1, an IκB that inhibits NH-κB, Rel-p50; and PHF, a novel κB site binding complex. *Mol. Cell. Biol.* 12:2898–2908.

42. Schutte, B., M. M. J. Reynders, F. T. Bosman, and G. H. Blijham. 1987. Effect of tissue fixation on anti-bromodeoxyuridine immunohistochemistry. *J. Histochem. Cytochem.* 35:1343–1345.

43. Lee, J., L. Greenbaum, B. A. Haber, D. Nagle, V. Lee, V. Miles, K. L. Mohn, M. Bucan, and R. Taub. 1994. Structure and localization of the *IGFBP-1* gene and its expression during liver regeneration. *Hepatology.* 19:656–665.

44. Herbomel, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell.* 39:653–662.

45. Ossipow, V., P. Descombes, and U. Schibler. 1993. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc. Natl. Acad. Sci. USA.* 90:8219–8223.

46. Church, G. M., A. Euphrussi, W. Gilbert, and S. Tonegawa. 1985. Cell

type specific contacts to immunoglobulin enhancers in nuclei. *Nature (Lond.)*. 313:798–801.

47. Ephrussi, A., G. Church, S. Tonegawa, and W. Gilbert. 1985. B lineage specific interactions of an immunoglobulin enhancer factor with cellular factors in vivo. *Science (Wash. DC)*. 227:134–140.

48. Alam, T., R. M. An, R. C. Mifflin, C.-C. Hsieh, X. Ge, and J. Papaconstantinou. 1993. *trans*-activation of the a_1-acid glycoprotein gene acute phase responsive element by multiple isoforms of C/EBP and glucocorticoid receptor. *J. Biol. Chem.* 268:15681–15688.

49. Nishiyori, A., H. Tashiro, A. Kimura, K. Akagi, I. Yamamura, M. Mori, and M. Takiguchi. 1994. Determination of tissue specificity of the enhancer by combinatorial operation of tissue-enriched transcription factors. *J. Biol. Chem.* 169:1323–1331.

50. Lee, Y.-H., M. Yano, S.-Y. Liu, E. Matsunaga, P. F. Johnson, and F. J. Gonzalez. 1994. A novel *cis*-acting element controlling the rat *CYP2D5* gene and requiring cooperativity between C/EBPβ and an Sp1 factor. *Mol. Cell. Biol.* 14:1383–1394.

51. Mohn, K. L., A. E. Melby, D. S. Tewari, T. M. Laz, and R. Taub. 1991. The gene encoding rat insulin like growth factor-binding protein 1 is rapidly and highly induced in regenerating liver. *Mol. Cell. Biol.* 11:1393–1401.

52. Morello, D., M. J. Fitzgerald, C. Babinet, and N. Fausto. 1990. c-*myc*, c-*fos*, and c-*jun* regulation in the regenerating livers of normal and H-2K/c-*myc* transgenic mice. *Mol. Cell. Biol.* 10:3185–3193.

53. Metz, R., and E. Ziff. 1991. cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to *trans*-locate to the nucleus and induce c-*fos* transcription. *Genes & Dev.* 5:1754–1766.

54. Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier. 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature (Lond.)*. 364:544–547.

55. Rana, B., D. Mischoulon, Y. Xie, N. L. R. Bucher, and S. R. Farmer. 1994. Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: reciprocal expression of C/EBPα and immediate-early growth response transcription factors. *Mol. Cell. Biol.* 14:5858–5869.

56. Shiota, G., D. B. Rhoads, T. C. Wang, T. Nakamura, and E. V. Schmidt. 1992. Hepatocyte growth factor inhibits growth of hepatocellular carcinoma cells. *Proc. Natl. Acad. Sci. USA*. 89:373–377.

57. Nakamura, T., K. Nawa, and A. Ichihara. 1984. Partial purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* 122:1450–1459.

58. Nakamura, T., H. Teramoto, and A. Ichihara. 1986. Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary culture. *Proc. Natl. Acad. Sci. USA*. 83:6489–6493.

59. Zarnegar, R., and G. Michalopoulos. 1989. Purification and biological characterization of human hepatopoeitin A, a polypeptide growth factor for hepatocytes. *Cancer Res.* 49:3314–3320.

60. Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman. 1993. Functional analysis of a growth factor-responsive transcription factor complex. *Cell.* 73:395–406.

61. Hsu, W., T. K. Kerppola, P. L. Chen, T. Curran, and S. Chen-Kiang. 1994. Fos and Jun transcription activation by NF-IL6 through association at the basic zipper region. *Mol. Cell. Biol.* 14:268–276.

62. Ruppert, S., M. Boshart, F. X. Bosch, W. Schmid, R. E. K. Fourier, and G. Shütz. 1990. Two genetically defined *trans*-acting loci coordinately regulated overlapping sets of liver-specific genes. *Cell.* 61:895–904.

63. Wang, N. D., M. J. Finegold, A. Bradley, C. N. Ou, S. V. Abdelsayed, M. D. Wilde, L. R. Taylor, D. R. Wilson, and G. J. Darlington. 1995. Impaired energy homeostasis in C/EBPα knock-out mice. *Science (Wash. DC)*. In press.

64. O'Brien, R. M., P. C. Lucas, T. Yamasaki, E. L. Noisin, and D. K. Granner. 1994. Potential convergence of insulin and cAMP signal transduction systems at the phosphoenolpyruvate carboxyinase (PEPCK) gene promoter through CAAT/enhancer binding protein (C/EBP). *J. Biol. Chem.* 269:30419–30428.