CCAAT Enhancer–binding Protein β Is Required for Normal Hepatocyte Proliferation in Mice after Partial Hepatectomy

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Abstract

After two-thirds hepatectomy, normally quiescent liver cells are stimulated to reenter the cell cycle and proliferate to restore the original liver mass. The level of bZIP transcription factor CCAAT enhancer-binding protein β (C/EBPβ) increases in the liver during the period of cell proliferation. The significance of this change in C/EBP expression is not understood. To determine the role of *C/EBPβ* in the regenerating liver, we examined the regenerative response after partial hepatectomy in mice that contain a targeted disruption of the *C/EBPβ* gene. Posthepatectomy, hepatocyte DNA synthesis was decreased to 25% of normal in *C/EBPβ* −/− mice. The reduced regenerative response was associated with a prolonged period of hypoglycemia that was independent of expression of C/EBPα protein and gluconeogenic genes. *C/EBPβ* −/− livers showed reduced expression of immediate-early growth-control genes including the *Egr-1* transcription factor, mitogen-activated protein kinase protein tyrosine phosphatase (*MKP-1*), and *HRS*, a delayed–early gene that encodes an mRNA splicing protein. *Cyclin B* and *E* gene expression were dramatically reduced in *C/EBPβ* −/− livers whereas *cyclin D1* expression was normal. The abnormalities in immediate-early gene expression in *C/EBPβ* −/− livers were distinct from those seen in *IL-6* −/− livers. These data link *C/EBPβ* to the activation of metabolic and growth response pathways in the regenerating liver and demonstrate that C/EBPβ is required for a normal proliferative response. (*J. Clin. Invest.* 1998. 102: 996–1007.) Key words: hepatic gluconeogenesis • liver regeneration • CCAAT enhancer-binding protein α • cytokine • cyclin genes • transcription factor

Introduction

The adult liver retains the capacity to restore its mass in response to partial hepatectomy or liver transplantation, or after toxin or inflammatory-mediated injury (1–4). After a 70% partial hepatectomy, in which the two larger lobes are removed without injury to the remaining lobes, ~ 95% of the remnant normally quiescent liver cells reenter the cell cycle and proliferate with the restoration of the original liver mass within 7–10 d. Studies with blocking antibodies and tumor necrosis factor α receptor (*TNFR*)[1] (5) and *IL-6* knockout mice (6) demonstrated that the TNF-α and IL-6 cytokines are necessary for normal liver regeneration after partial hepatectomy, and defined a pathway of cytokine activation in the partial hepatectomy model that involves TNF-α activation of nuclear factor κB (NF-κB) followed by IL-6 activation of serum transducer and activator of transcription-3 (STAT3) and selected growth–response genes (5, 6). The reduced expression in *IL-6* −/− and *TNFR* −/− mice of a subset of immediate–early genes important for cell cycle progression provides an additional link between these cytokines and the regenerative response.

During the proliferative response posthepatectomy, the liver must also maintain organ-specific functions that are necessary for synthetic and metabolic homeostasis (1). In response to the abrupt loss of glycogen stores, the regenerating liver increases glucose production through the induction of immediate–early genes encoding for gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6pase*) (7, 8). The mechanisms that regulate this homeostatic response during liver regeneration are not well understood, but studies have supported the importance of the CCAAT enhancer-binding proteins (*C/EBP*) *α* and *β* in this process (9). The C/EBPs, leucine–zipper transcription factors that are highly expressed in quiescent liver (9), are able to heterodimerize with other C/EBP proteins (10–15), possess similar DNA binding affinities, and differ primarily in the transactivation properties conferred by their amino-terminal domains. *C/EBPα* expression is important for normal glucose homeostasis at birth; mice that do not express C/EBPα protein die of profound hypoglycemia shortly after birth and fail to induce normal levels of the genes for *PEPCK* and *G6pase* that encode for enzymes involved in gluconeogenesis (16). The role of *C/EBPβ* in glucose homeostasis is more complex. Studies with *C/EBPβ* −/− mice have noted a failure to achieve the expected Mendelian ratio of homozygous *C/EBPβ* −/− animals at weaning (17, 18). Croniger et al. (18) noted a subset of mice that die at birth of profound hypoglycemia and fail to express *PEPCK* mRNA or mobilize hepatic glycogen. Animals that survive to 4–6 mo of age develop an age-related lymphoproliferative disorder associated with progressive elevations in serum IL-6 levels (19). The relative importance of *C/EBPα* and *β* to glucose homeostasis in the adult animal after partial hepatectomy has not been studied.

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Received for publication 17 February 1998 and accepted in revised form 8 July 1998.

J. Clin. Invest.

0021-9738/98/09/996/12 $2.00
Volume 102, Number 5, September 1998, 996–1007
http://www.jci.org

1. *Abbreviations used in this paper:* C/EBP, CCAAT enhancer-binding protein; *Egr*-1, early-growth response gene-1; G6pase, glucose-6-phosphatase; MKP-1, mitogen-activated kinase phosphatase-1; NF-κB, nuclear factor κB; PEPCK, phosphoenolpyruvate carboxykinase; TNF, tumor necrosis factor.

C/EBPα and *C/EBPβ* are antiproliferative in some experimental models (20–25) and two recent studies have shown that forced expression of *C/EBPα* and *C/EBPβ* is associated with cell arrest via induction of the cyclin dependent kinase inhibitor *p21* (23, 26). In the regenerating liver, we and others have demonstrated that C/EBPβ protein and DNA binding activity increase relative to C/EBPα during the G1 phase of the hepatocyte cell cycle (27, 28). C/EBPα and β are coexpressed with markers of DNA synthesis (27), suggesting that the expression of these proteins is compatible with liver cell proliferation. *C/EBPβ* can be regulated by transcriptional and posttranslational mechanisms (29–35) and has been linked to the IL-6 signaling pathway based on its ability to activate the *IL-6* promoter (36) and its increased transactivation potential in response to IL-6 signaling (37). However, the normal activation of *C/EBPβ* mRNA in *IL-6* −/− mice (6) and normal C/EBPβ DNA binding in *TNFR* −/− mice (5) posthepatectomy argues against its position as a downstream effector in the TNF-α/IL-6 cytokine activation pathway after partial hepatectomy.

With the availability of *C/EBPβ* −/− mice (19), we were able to directly examine the contribution of *C/EBPβ* to the expression of growth–control and liver function genes induced in response to partial hepatectomy. In this report, we show that *C/EBPβ* −/− hepatocytes did not proliferate normally after partial hepatectomy and this abnormal regenerative response was associated with prolonged hypoglycemia and dysregulation of several genes important for hepatocyte gluconeogenesis and proliferation.

Methods

Animals. *C/EBPβ* −/− mice were generated on a C57/BL6/SV129 background. Homozygous progeny were identified by characteristic ruffled fur coat. Southern blot analysis of tail DNA was performed as described (19) for additional confirmation with selected mice. All experiments were performed with male and female mice between 12 and 16 wk of age. Animals were maintained on an ad libitum diet of rodent laboratory chow (Ralston-Purina Co., St. Louis, MO) 5008 (6.5% fat). For regenerating liver, animals were anesthetized with metafane and subjected to midventral laparotomy with ∼ 70% liver resection (left lateral and median lobes) (38). Animals exhibiting exhibited signs of lethargy and/or hypothermia after > 2 h posthepatectomy were euthanized by cervical dislocation and were used to calculate morbidity rates. Animals that exhibited lethargy, bleeding, hypothermia, or respiratory distress or those who died within the first 2 h posthepatectomy were considered to demonstrate perioperative morbidity and were not included in the determination of morbidity or mortality rates. Postoperative morbidity and mortality were similar in *C/EBPβ* −/− and *C/EBPβ* +/+ mice posthepatectomy. 32% of *C/EBPβ* −/− and 27% of *C/EBPβ* +/+ mice were euthanized 24 h posthepatectomy because of lethargy, and 5% of *C/EBPβ* −/− and 2% of *C/EBPβ* +/+ mice died after the perioperative period. *C/EBPβ* +/− female heterozygotes were bred with *C/EBPβ* −/− male homozygotes to produce *C/EBPβ* −/− males and females used for hepatectomies. Animals were sacrificed by cervical dislocation. For BrdU-treated mice, animals were injected intraperitoneally with 50 mg/kg bromodeoxyuridine (0.2% solution in PBS) 1 h before fixation (39). Statistical analysis of animal liver weights was determined using StatWorks (Apple Software, Cupertino, CA) and Student's *t* test.

Serological analysis. Blood was obtained at the time of killing via cannulation of the inferior vena cava; serum was collected and analyzed by Ani Lytics, Inc. (Gaithersburg, MD).

Preparation of nuclear extracts, whole nuclei, and whole-cell extracts. Nuclear extracts from livers were prepared from the remaining liver lobes as described previously (40, 41) with some modifications. The remnant liver was washed in ice-cold PBS and then machine-homogenized with 3 ml/g liver homogenization buffer (Hepes [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]), pH 7.6, 25 mM KCl, 1 mM EDTA, 2 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol, 1.0 mM DTT, 0.1 mM PMSF, 1 mM NaF, 1 mM Na_2MoO_4, and protease inhibitors (below) (41). After centrifugation at 90,000 *g* for 50 min, pelleted nuclei were resuspended and lysed by Dounce homogenization in 150 μl/g liver buffer C (20 mM Hepes, pH 7.9, 0.2 mM EDTA, 420 mM NaCl, 1.5 mM $MgCl_2$, 1.0 mM DTT, 0.1 mM PMSF with protease inhibitors, 1 mM NaF, 1 mM Na_2MoO_4). Lysed nuclei were incubated with vigorous shaking at 4°C for 30 min and centrifuged at 29,000 *g* for 30 min. The supernatant was dialyzed vs. buffer D (20mM Hepes, pH 7.6, 0.2 mM EDTA, 100 mM KCl, 20% glycerol, 1.0 mM DTT, 0.1 mM PMSF with protease inhibitors, 1 mM NaF, 1 mM Na_2MoO_4) overnight; protein concentration was determined using a protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots were stored in liquid nitrogen. All extracts were prepared in the presence of 2 μg/ml antipain, aprotinin, bestatin, and leupeptin (Boehringer-Mannheim Corp., Indianapolis, IN). Protease inhibitors were added immediately at the time of remnant liver removal. Whole nuclei were prepared by resuspending pelleted nuclei in a nonlysing suspension buffer (42) (100 mM NaCl, 10 mM Tris-Cl [pH 7.6] 1 mM EDTA). After protein concentration determination, an equal volume of 2× SDS loading buffer (100 mM Tris-HCl, 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. An equal volume of 2× SDS buffer was added to denaturing samples followed by boiling for 10 min. Whole-cell extracts were prepared by extensive homogenization of liver tissue in buffer C to which protease inhibitors were freshly added, pelleted by centrifugation and stored at −70°C.

Electrophoretic gel mobility shift assays. Binding reactions were performed essentially as described (43) using nuclear extracts from posthepatectomy mouse liver cells. All experiments were performed using an excess of probe. 4 μg of extract were incubated with radiolabeled oligonucleotide (1 ng) for 20 min at room temperature in binding buffer and electrophoresed on nondenaturing polyacrylamide gels in 0.5× TBE buffer (44 mM Tris, 44 mM EDTA, 1.0 mM DTT, 10% glycerol) (20). The gels were dried and exposed to x-ray film. 2 μg of poly dI-dC (Boehringer-Mannheim Corp.) were used as a nonspecific DNA competitor in each reaction. For C/EBP EMSAs, nuclear extract dialysis buffer D containing freshly added protease inhibitors (see nuclear extract methods) was added to each reaction to achieve a final reaction volume of 10 μl. For STAT3 EMSA, ddH20 was added to each reaction to reach the final reaction volume. STAT3 supershift experiments were performed by incubating nuclear extract with STAT3 antibody for 2 h at 4°C followed by addition of oligonucleotide. The probes used were HPLC-purified double-stranded oligonucleotides; C/EBPα/β consensus-binding sequence (TACACCATTACACAATTCA) and STAT3-binding sequence from the sis-inducible factor-binding element in the c-*fos* promoter, (GATCCTCCAGCATTTCCCGTAAATCCTCCAG) (44) and end-labeled with [γ-^{32}P]ATP. C/EBPα and β supershift experiments were performed by incubating 1 μl of primary antibody with the nuclear extracts in binding buffer for 1 h at 4°C after the addition of the labeled oligonucleotide. Primary antibodies used in supershift experiments include α-STAT3, α-p50/NF-κB, α-C/EBPα "14AA", and α-C/EBPβ "C-19" (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoblots. For C/EBP immunoblots, 20 μg of whole nuclei was electrophoresed on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected by chemiluminescence (Amersham ECL, Buckinghamshire, UK) according to the manufacturer's instructions. Membranes were blocked overnight at 4°C in 5% dry milk. 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.

All incubations occurred in PBS, 0.05% Tween-20. p21 immunoblot detection was essentially as described above with some modifications. Primary antibody was diluted in TBS-0.5% Tween-20/0.5% milk. For cyclin D1, 25 μg of whole cell extract was electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred as described above. For p21, 75 μg of whole nuclei was electrophoresed on a 15% SDS-polyacrylamide gel. The membrane was blocked overnight in 10% dry milk/TBS-0.1% Tween-20 at 4°C. Primary antibody was diluted 1:1,000 in 5% dry milk/TBS-0.1% Tween-20 and incubated for 2 h at room temperature. Secondary antibody was diluted 1:5,000 in 5% milk/TBS-0.1% Tween-20 and incubated for 1 h at room temperature.

Immunoblots were scanned densitometrically to quantitate protein levels (Image-Quant Software; Molecular Dynamics, Sunnyvale, CA) and densitometry of the Coomassie-stained gel after transfer was used to normalize protein loading. In one experiment, the same nitrocellulose membrane was sequentially probed with C/EBPβ followed by C/EBPα antibody. Protein loading for the C/EBPα immunoblot was normalized based on densitometric scanning of a noninduced cross-reactive band detected by the C/EBPβ antibody.

Primary antibodies used in immunoblots, electrophoretic gel mobility supershift, and immunohistochemistry studies were α-C/EBPα "14AA", α-C/EBPβ "C-19", α-cyclin D1, (Santa Cruz Biotechnology), and α-p21 (OS100) (45; a gift from Wafik el-Deiry, University of Pennsylvania, Philadelphia, PA).

Tissue fixation and immunohistochemistry. Hepatectomized mice were reanesthetized and ventral laparotomy was performed. 1 h before harvest and fixation of the remnant liver, animals received an intraperitoneal injection of bromodeoxyuridine (BrdU), a thymidine analogue capable of incorporation into actively replicating DNA (39). The liver was removed and cut into 5-mm slices with a razor blade followed by a 16–24-h fixation in 10% neutral buffered formalin (Formalde-Fresh; Fisher Scientific, Fairlawn, NJ). Livers were then placed at 4°C in PBS until paraffin embedded in an automated tissue processor. 5-μm tissue sections were cut on a microtome and adhered to poly-L-lysine-coated glass slides. The slides were dried overnight at 37°C.

Immunohistochemical detection was performed essentially as has been described (27). An avidin–biotin horseradish peroxidase detection system (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions with the following modifications. After the blocking step in 1.5% goat serum in PBS, avidin- and biotin-blocking steps were performed using the avidin–biotin blocking kit (Vector Laboratories). α-BrdU (Boehringer-Mannheim) primary antibody was diluted 1:250 in 1.5% goat serum in PBS. Tissue sections were incubated for 45 min at 37°C and sections were washed in PBS for 10 min. ABC reagent and substrate incubations were performed according to the manufacturer's instructions. The percent BrdU labeled hepatocytes was determined by counting positively stained hepatocyte nuclei in at least three low-power (10×) microscope fields and calculating the mean. This value was expressed as a fraction of the total number of hepatocytes per 10× field (estimated to be equal to 800 hepatocytes/low-power field).

RNA preparation and Northern blots. For RNA preparation, animals were killed at indicated time points and total liver RNA was prepared as described previously (46). For Northern blots, 10 μg of heat-denatured, total RNA per lane was separated by electrophoresis on a 1% agarose, 0.6% formaldehyde/MOPS denaturing gel and transferred to Optibind (Schleicher and Schuell, Keene, NH) supported nitrocellulose. Recombinant plasmids or isolated cDNA inserts were labeled through the incorporation of [α-^{32}P]dCTP by nick-translation (nick-translation reagent kit; Gibco-BRL, Gaithersburg, MD). Blots were hybridized overnight at 42°C in hybridization buffer (10% dextran sulfate, 25% formamide, 0.6 M NaCl, 60 mM sodium citrate, 7 mM Tris pH 7.5, 0.8× Denhardt's solution, 0.0002% heat-denatured, sonicated salmon sperm DNA), washed 3 times for 15 min each at room temperature (0.3 M NaCl, 30 mM sodium citrate, 0.5% SDS) and twice for 15 min each at 60°C (15 mM NaCl, 1.5 mM sodium citrate) and exposed to autoradiograph film.

Results

DNA synthesis was decreased in hepatocytes of C/EBPβ −/− mice posthepatectomy. We predicted that if *C/EBPβ* is important for normal liver regeneration, the DNA synthetic response should be delayed or decreased in the *C/EBPβ −/−* livers posthepatectomy. We measured hepatocyte BrdU incorporation using immunohistochemistry to quantitate the number of S phase hepatocytes at various times after partial hepatectomy. There were no BrdU-positive hepatocytes in quiescent liver in either the *C/EBPβ +/+* or *C/EBPβ −/−* livers (not shown), consistent with the fact that the majority of cells in the adult liver are in the G0 stage. Hepatocyte DNA synthesis was markedly reduced in *C/EBPβ −/−* animals (Fig. 1, *A* and *B*) and this difference reached statistical significance at 40 ($P < 0.0001$) and 48 h posthepatectomy ($P < 0.03$) (Fig. 1 *A*). At 40 h posthepatectomy, which represented the peak of DNA synthesis, we observed a reduction in hepatocyte DNA synthesis in *C/EBPβ −/−* livers to between 25 and 30% of *C/EBPβ +/+* levels. Although the number of BrdU labeled hepatocytes was significantly higher in *C/EBPβ −/−* livers at 72 h ($P < 0.05$), the absolute percentage of labeled hepatocytes was quite low in both groups of animals at this time point. We did not detect any zonal differences in hepatocyte DNA synthesis in the two groups (Fig. 1 *B*). Morbidity and mortality were similar as was elevation of serum ALT. There was no statistically significant difference in the rate of mass reconstitution in the *C/EBPβ −/−* when compared to *C/EBPβ +/+* livers (not shown) suggesting that cell size increases may be independent of S phase.

Metabolic dysregulation was associated with decreased C/EBPα protein levels in both C/EBPβ +/+ and C/EBPβ −/− livers posthepatectomy. A subset of *C/EBPβ −/−* animals die at birth of profound hypoglycemia and fail to mobilize hepatic glycogen (18). We reasoned that a failure to adequately compensate for the rapid loss of liver glycogen could contribute to the diminished regenerative response observed in *C/EBPβ −/−* livers posthepatectomy. Serum glucose levels were assessed during the first 60 h posthepatectomy to determine if impaired glucose homeostasis was present in *C/EBPβ −/−* animals. Serum glucose levels were normal prior to surgery in both groups of animals, but between 4 and 16 h posthepatectomy, both groups of mice became moderately hypoglycemic, with mean serum glucose values of 102±6 in *C/EBPβ +/+* and 97±24 in *C/EBPβ −/−* mice (normal values: 124–262 mg/dl). Glucose values returned to the normal range by 36–40 h in the *C/EBPβ +/+* animals (mean 158±23) but remained low in *C/EBPβ −/−* animals (mean 98±25) during this time period ($P = 0.012$) and did not normalize until 48 h posthepatectomy. Other parameters not significantly different were changes in serum cholesterol and triglycerides posthepatectomy.

C/EBPα expression is important for normal glucose homeostasis at birth (16). We therefore sought to determine if the sustained hypoglycemic response in *C/EBPβ −/−* animals posthepatectomy was associated with reduced levels of C/EBPα protein. The level of C/EBPα and C/EBPβ protein in *C/EBPβ +/+* and *C/EBPβ −/−* livers was quantitated by immunoblot analysis of whole nuclei isolated at indicated intervals during 48 h posthepatectomy (Fig. 2). Protein levels were determined using densitometric scanning of several immunoblots. We were unable to reliably detect the 30-kD alternative translation C/EBPα protein product, but previous studies have shown that its expression parallels the level of full-length C/EBPα

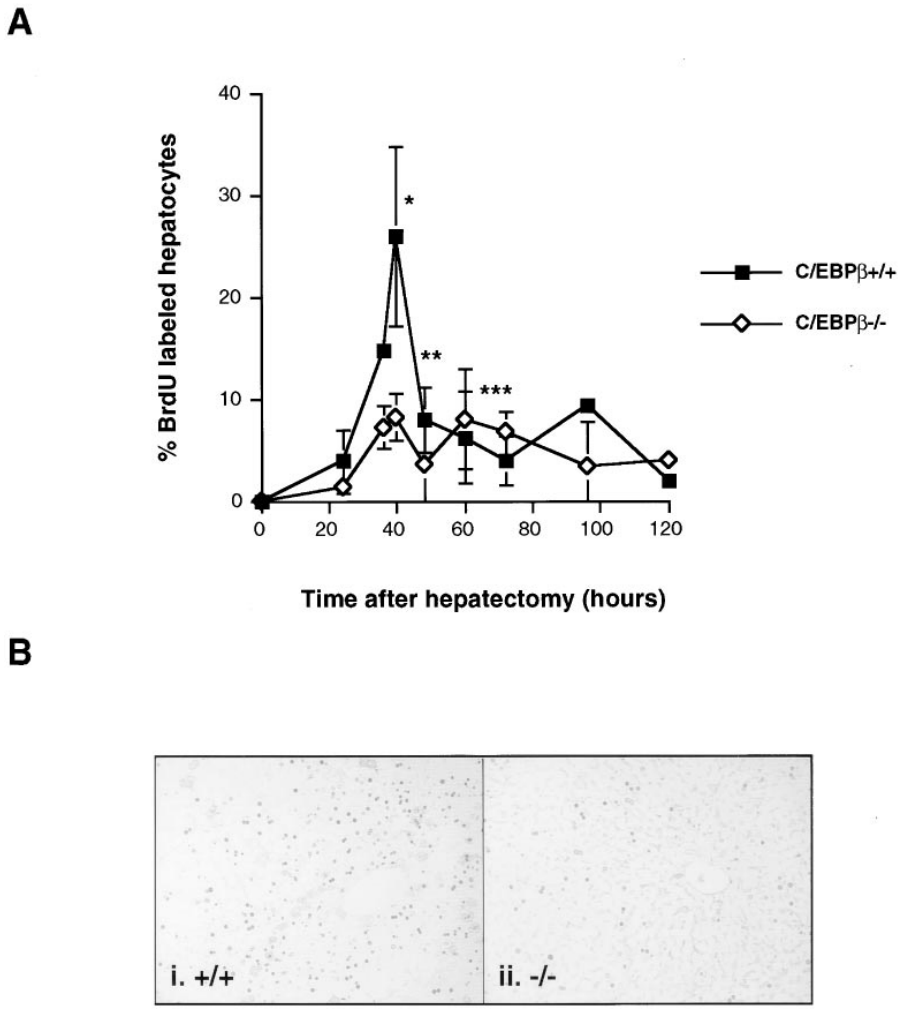


Figure 1. S phase hepatocytes are decreased in *C/EBPβ* −/− livers posthepatectomy *C/EBPβ* +/+ and −/− mice were subjected to partial hepatectomy. 1 h before harvest, animals received an intraperitoneal injection of BrdU. The remnant liver was harvested at indicated times, formalin-fixed, and stained with an α-BrdU antibody. BrdU-positive hepatocytes for each sample were quantitated by counting positively stained cells in three low power fields. The mean for each time point was expressed as a percentage total hepatocytes per low-power field (*A*) (800 hepatocytes/low-power field). Standard deviations are shown and statistical significance was calculated using at least three animals. (*B*) Low-power (*1*) *C/EBPβ* +/+ and (*2*) *C/EBPβ* −/− livers at 40 h posthepatectomy after α-BrdU immunohistochemical detection. Round, uniformly stained nuclei represent BrdU positive hepatocytes. $*P < 0.0001$; $**P < 0.03$; $***P < 0.05$.

protein (27). Prehepatectomy C/EBPα protein levels were ~3.0-fold higher in *C/EBPβ* −/− livers and this difference reached statistical significance ($P < 0.04$). Posthepatectomy, the level of C/EBPα protein decreased fivefold in *C/EBPβ* +/+ livers 8 h posthepatectomy, which was a twofold greater decrease than what we had detected in the rat (27). The level of C/EBPα protein in *C/EBPβ* −/− livers was similarly reduced at this time point, but this reduction represented a ninefold decrease from prehepatectomy levels. C/EBPα protein levels renormalized in both groups at 24 h posthepatectomy. The return to baseline C/EBPα protein expression correlated with normalization of serum glucose levels in the *C/EBPβ* +/+ livers but *C/EBPβ* −/− animals remained hypoglycemic, despite normalization of C/EBPα protein.

As expected, there was no detectable C/EBPβ protein in *C/EBPβ* −/− livers (Fig. 2 *B*). The timing of the induction of the 35-kD full-length C/EBPβ protein in *C/EBPβ* +/+ mice was similar to what we had observed in the rat liver but the protein level was induced 25-fold, as compared with 3-fold induction in the rat (27). An unidentified 42-kD protein was induced with similar kinetics as the 35-kD protein.

C/EBP dimer DNA binding activity posthepatectomy in C/EBPβ +/+ and C/EBPβ −/− liver nuclear extracts. Altered C/EBP protein levels and/or changes in relative composition of C/EBP isoforms bound to target promoters could contribute to changes in expression of C/EBP target genes posthepatectomy in *C/EBPβ* −/− livers. We therefore measured binding to a consensus C/EBP oligonucleotide sequence in nuclear extracts during 24 h posthepatectomy and used supershift analysis to determine the composition of the bound complexes (Fig. 3). Equal loading was not convincingly achieved so changes in total C/EBP-binding could not be reliably assessed. However, relative changes in contributions of C/EBPα and β isoforms to total C/EBP-binding activity could be determined in individual samples. In *C/EBPβ* +/+ extracts before hepatectomy, α/β heterodimers constituted the major C/EBP DNA-binding complex with the remaining activity represented by α/α, similar to our finding in rat liver (27). We detected a progressive rise in the percentage of β/β homodimers and a decrease in α/α homodimers during the first 8 h posthepatectomy in *C/EBPβ* +/+ livers which corresponded to the time when C/EBPβ protein levels were maximal. By 24 h the α/α homodimer fraction of the total binding activity had renormalized. In *C/EBPβ* −/− liver nuclear extracts, C/EBP DNA binding activity was almost completely supershifted by a C/EBPα antibody at every time point indicating that the majority of C/EBP-binding activity contained C/EBPα protein either as an α/α homodimer or complexed with an unidentified protein. At 3 and 8 h posthepatectomy, two novel complexes not supershifted by C/EBPα antibody were detected in *C/EBPβ* −/−

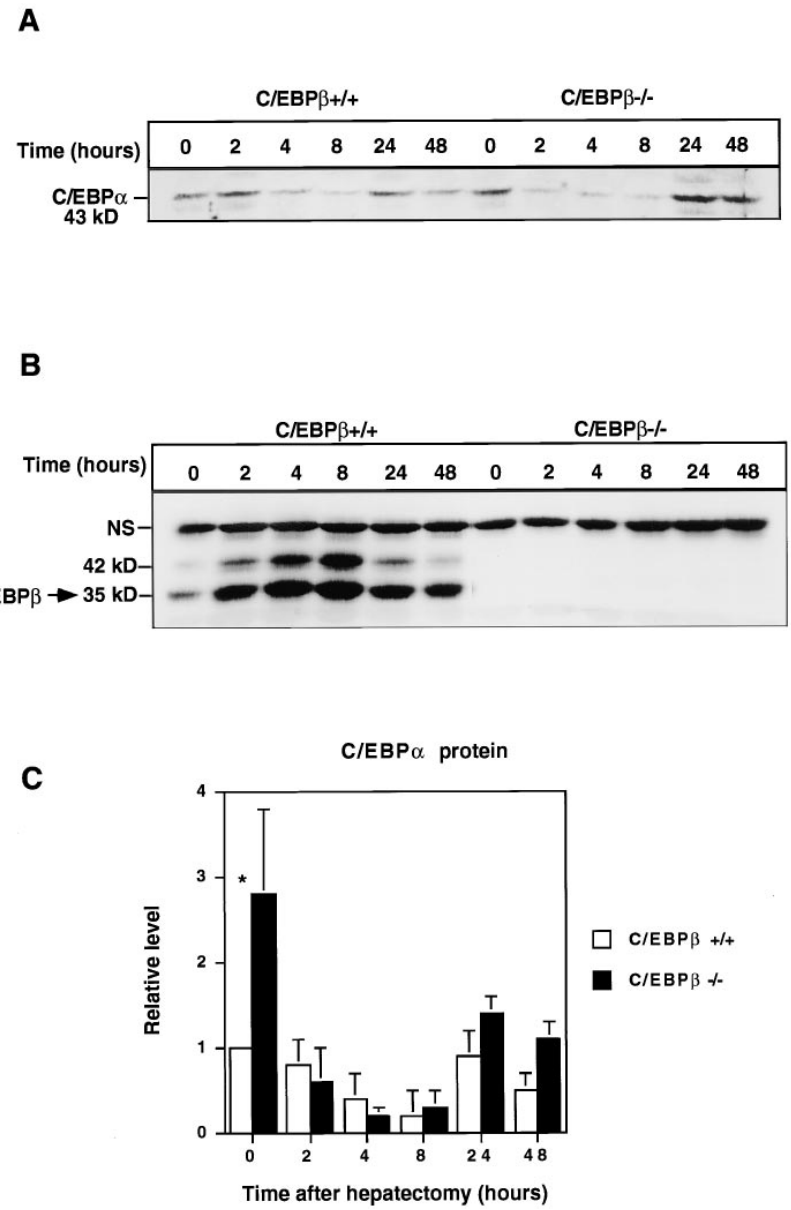


Figure 2. C/EBPα and β protein expression in *C/EBPβ* +/+ and *C/EBPβ* −/− livers posthepatectomy. Representative immunoblots of C/EBPα (*A*) and C/EBPβ (*B*) expression at indicated times after hepatectomy. 20 μg of whole nuclei were separated on a 12.5% SDS-polyacrylamide gel, transferred and probed with α-C/EBPα antibody (*A*) or α-C/EBPβ antibody (*B*). (*C*) Relative level of C/EBPα protein (43 kD) in *C/EBPβ* +/+ and *C/EBPβ* −/− liver nuclei at indicated times after hepatectomy. Scanning densitometry of three immunoblots was used to calculate protein levels. Each time point represents the mean of three animals except 2 h, which represents the mean of two animals. C/EBPα protein level in *C/EBPβ* +/+ quiescent liver (0 h) was arbitrarily set to 1.0. $*P <$ 0.04.

extracts, the lower of which was supershifted by a C/EBPδ antibody (not shown). These results indicate that C/EBPβ and α/β are the predominant C/EBP forms bound to DNA during the first 16 h posthepatectomy in normal liver and the composition of C/EBP binding complexes is markedly altered in *C/EBPβ* −/− livers.

C/EBPα target gene activation was inappropriate in response to hypoglycemia in both C/EBPβ +/+ and C/EBPβ −/− livers posthepatectomy. It was not clear whether the absolute level of C/EBPα protein or the percentage C/EBPα of the total C/EBP binding is the dominant effect that determines activation of C/EBPα target genes. We therefore determined if the sustained hypoglycemia in *C/EBPβ* −/− animals was associated with reduced activation of C/EBPα target genes important for gluconeogenesis such as *PEPCK* and *G6pase* (Fig. 4) (16). Basal expression of *PEPCK* and *G6pase* mRNAs was two- to threefold higher in *C/EBPβ* −/− livers and there was no difference in the early induction of these genes posthepatectomy. Expression of *PEPCK* and *G6pase* mRNA was inappropriately low in both groups 4–16 h posthepatectomy based on the degree of hypoglycemia observed. By 24 h posthepatectomy, when C/EBPα protein expression had returned to time zero level, *PEPCK* and *G6pase* mRNA were appropriately elevated in *C/EBPβ* −/− livers, although the animals remained hypoglycemic.

STAT3 DNA binding was present in C/EBPβ −/− livers and was expressed throughout 24 h following partial hepatectomy. C/EBPβ has been linked to the IL-6 signaling pathway (36, 37), and we reasoned that reduced IL-6 signaling could contribute to the decreased DNA synthetic response in the *C/EBPβ* −/− livers. We previously identified IL-6 as the cytokine responsible for STAT3 activation in the regenerating liver (41). We assessed STAT3 DNA-binding activity in *C/EBPβ* +/+ and −/− nuclear extracts prepared at various times after partial hepatectomy. In *C/EBPβ* +/+ remnant liver nuclear extract (Fig. 5 *A*, *left*) the temporal kinetics of STAT3 activation were similar to our previous observations (6, 41) characterized by undetectable activity in quiescent liver, maximal induction

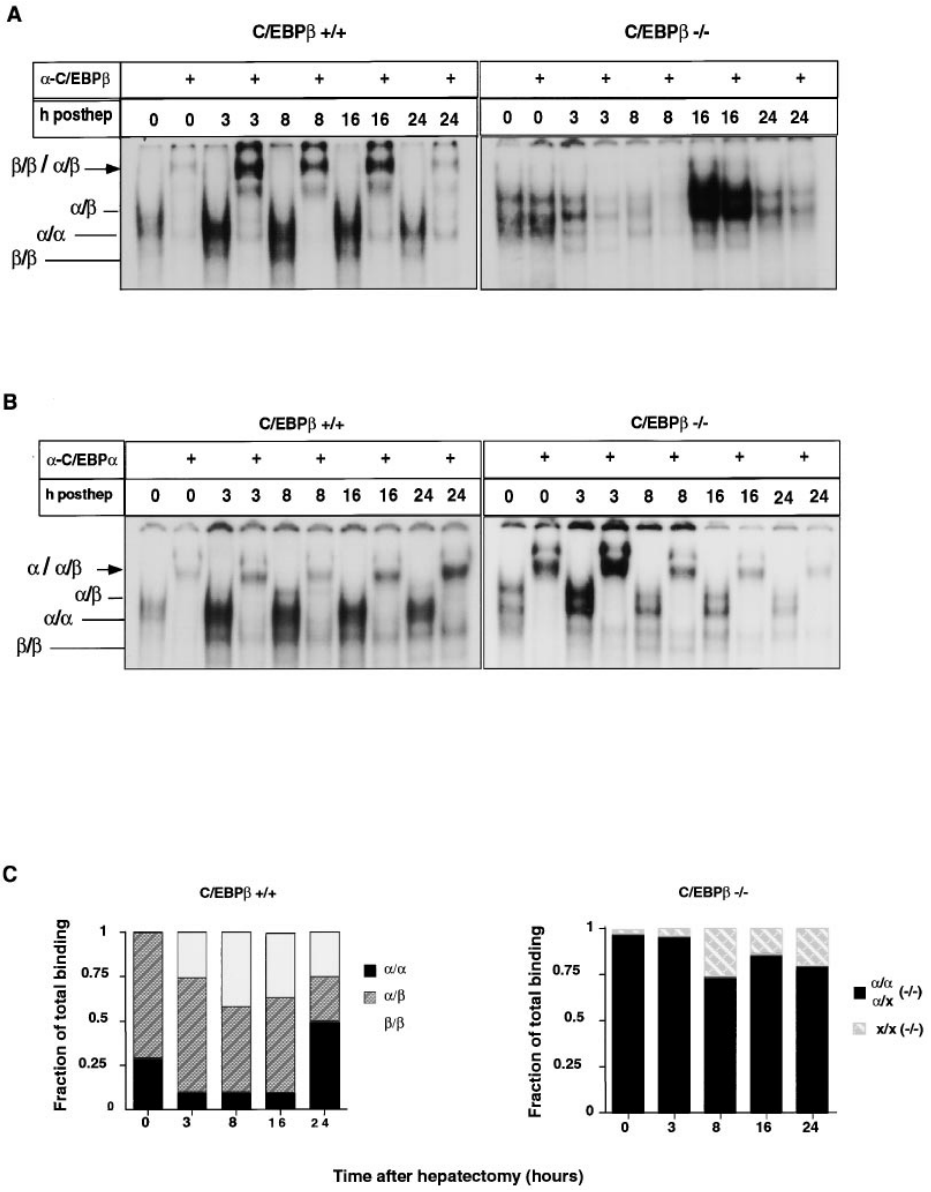


Figure 3. C/EBP isoform DNA binding in *C/EBPβ* +/+ and *C/EBPβ* −/− nuclear extracts posthepatectomy. Gel mobility shift and C/EBPα (*A*) or C/EBPβ (*B*) supershift assay of nuclear extracts from *C/EBPβ* +/+ (*left*: *A* and *B*) and *C/EBPβ* −/− (*right*: *A* and *B*) nuclear extracts at indicated times (hours) posthepatectomy. The oligonucleotide probe contains a C/EBPα/β consensus sequence oligonucleotide. + over corresponding lanes indicates the addition of α-C/EBPβ (*A*) or α-C/EBPα (*B*). The symbols on the left and right of the figure indicate the C/EBP isoform composition of the corresponding binding complexes and supershifted complexes. (*C*) Fraction of total DNA binding represented by C/EBPα and β homo- and heterodimers in *C/EBPβ* +/+ and *C/EBPβ* −/− nuclear extracts posthepatectomy. Scanning densitometry of blots shown in Fig. 4, *A* and *B* was used to calculate the total DNA-binding activity, supershifted, and nonshifted bands for each time point and was expressed as the percentage of the total DNA binding activity represented by α/α, α/β, β/β, and α/x dimers where "x" represents unidentified protein or proteins. Total C/EBP binding activity was arbitrarily set to 1.0.

by 3 h posthepatectomy and a return to undetectable levels by 16 h. STAT3 was appropriately induced in *C/EBPβ* −/− livers (Fig. 5 *A*, *right*). However, STAT3 DNA-binding activity was elevated in some *C/EBPβ* −/− quiescent livers and was persistently elevated at 24 h posthepatectomy consistent with known IL-6 elevation in these animals (Fig. 5 *A*, *right*). Densitometric quantitation of total STAT3 DNA-binding activity, and shifted and residual unshifted activity in a STAT3 supershift analysis (Fig. 5 *B*) demonstrated that > 90% of STAT-binding activity was either disrupted or supershifted by α-STAT3 antibody in *C/EBPβ* +/+ and *C/EBPβ* −/− 6 h posthepatectomy nuclear extracts. Less than 10% of total STAT-binding activity in *C/EBPβ* +/+ and *C/EBPβ* −/− extracts was not supershifted and may represent other STAT proteins.

Induction of a subset of growth-associated immediate-early and delayed-early genes was reduced in C/EBPβ −/− livers posthepatectomy. We were interested in determining if genes important for the growth response after partial hepatectomy were abnormally induced in the absence of C/EBPβ protein. Although the timing of C/EBPβ protein induction 2–8 h posthepatectomy would suggest that C/EBPβ would be more likely to transactivate delayed–early gene promoters, the potential for preexisting C/EBPβ protein to be activated by posttranslational modifications such as phosphorylation (29) suggested that C/EBPβ already present in the remnant liver could be rapidly activated in response to mitogenic signals resulting in the activation of immediate–early target genes. We performed Northern blot analyses with total RNA from *C/EBPβ* +/+ and *C/EBPβ* −/− livers (Fig. 6, *A* and *B*) to detect differences in the expression of immediate–early and delayed–early genes implicated in the regulation of the proliferative response in the regenerating liver. Because STAT3 DNA-binding levels were constitutively elevated in some *C/EBPβ* −/− livers, we examined the expression of two putative STAT3 target genes, c-*myc* and *junB*, in *C/EBPβ* +/+ and *C/EBPβ* −/− livers posthepatectomy (Fig. 6 *A*). We did not detect any differences in expression of these and other STAT3-responsive genes, including *LRF-1* and c-*fos* (not shown).

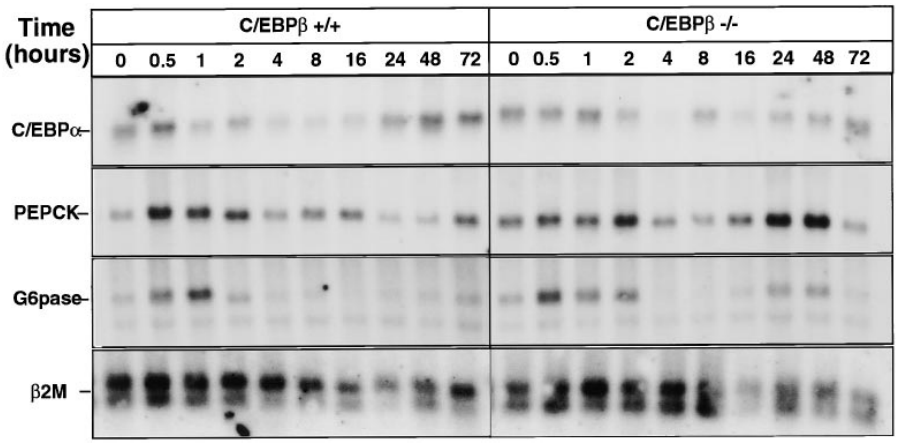
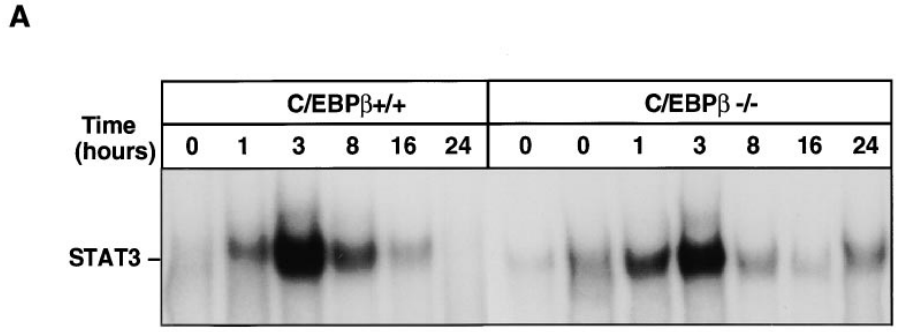


Figure 4. Reduced expression of C/EBPα target genes in *C/EBPβ* −/− posthepatectomy livers. RNA was prepared from *C/EBPβ* +/+ and *C/EBPβ* −/− animals at indicated times (hours) posthepatectomy. 10 μg of RNA was separated on a formaldehyde gel and probed with nick-translated cDNA probes. β2M was used as a normalization control.

Two immediate–early growth–response genes, *MKP-1* (47) and *Egr-1* (7, 48–52) showed the greatest difference in expression at 1 h posthepatectomy in *C/EBPβ* −/− livers when we detected a fourfold reduction in *MKP-1* and sixfold reduction in *Egr-1* mRNA (Fig. 6 *A*). *Egr-1* induction in IL-6 −/− livers was normal (data not shown). The early activation of *HRS* (53, 54), a delayed–early gene that encodes an mRNA splicing protein, was greatly reduced with a four- to sixfold reduction observed during the first 2 h posthepatectomy.

Although the expression of several growth genes was reduced in *C/EBPβ* −/− livers, the normal expression of *junB* and c-*myc* suggested that the observed decrease in DNA synthesis could reflect impaired progression of hepatocytes across the G1/S transition. We therefore measured the steady-state levels of several cell cycle–associated genes, including *p21*, *cyclin D1*, *A*, *B*, and *E*, and *Histone 3*. *p21* mRNA is induced during mid-to-late G1 after partial hepatectomy (55, 56) as a normal component of the growth response and is felt to be a target of C/EBPα regulation. *p21* mRNA induction occurred earlier and was more sustained in *C/EBPβ* −/− livers at several time points posthepatectomy (Fig. 7 *A*). The level of p21 protein was slightly higher in *C/EBPβ* −/− livers and correlated with the level of C/EBPα protein at 24 and 48 h posthepatectomy. Steady-state levels of *cyclin D1* mRNA and protein (Fig. 7 *B*) were similar in *C/EBPβ* +/+ and *C/EBPβ* −/− livers. Mean *cyclin A* expression was not different in



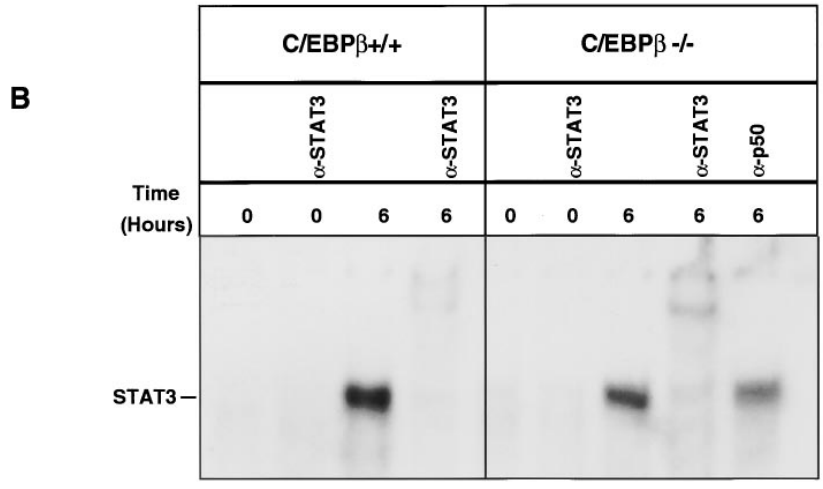


Figure 5. STAT3 DNA binding activity in *C/EBPβ* −/− livers. Gel mobility shift assay (*A*) and supershift assay (*B*) of nuclear extracts from *C/EBPβ* +/+ (*left*) and *C/EBPβ* −/− livers (*right*) at indicated times (hours) posthepatectomy. (*B*) Nuclear extracts were incubated with probe alone or with α-STAT3 or p50NF-κB (control) antibodies. The oligonucleotide probe contains the STAT-binding site of the sis-inducible element (*SIE*) from the c-*fos* promoter.

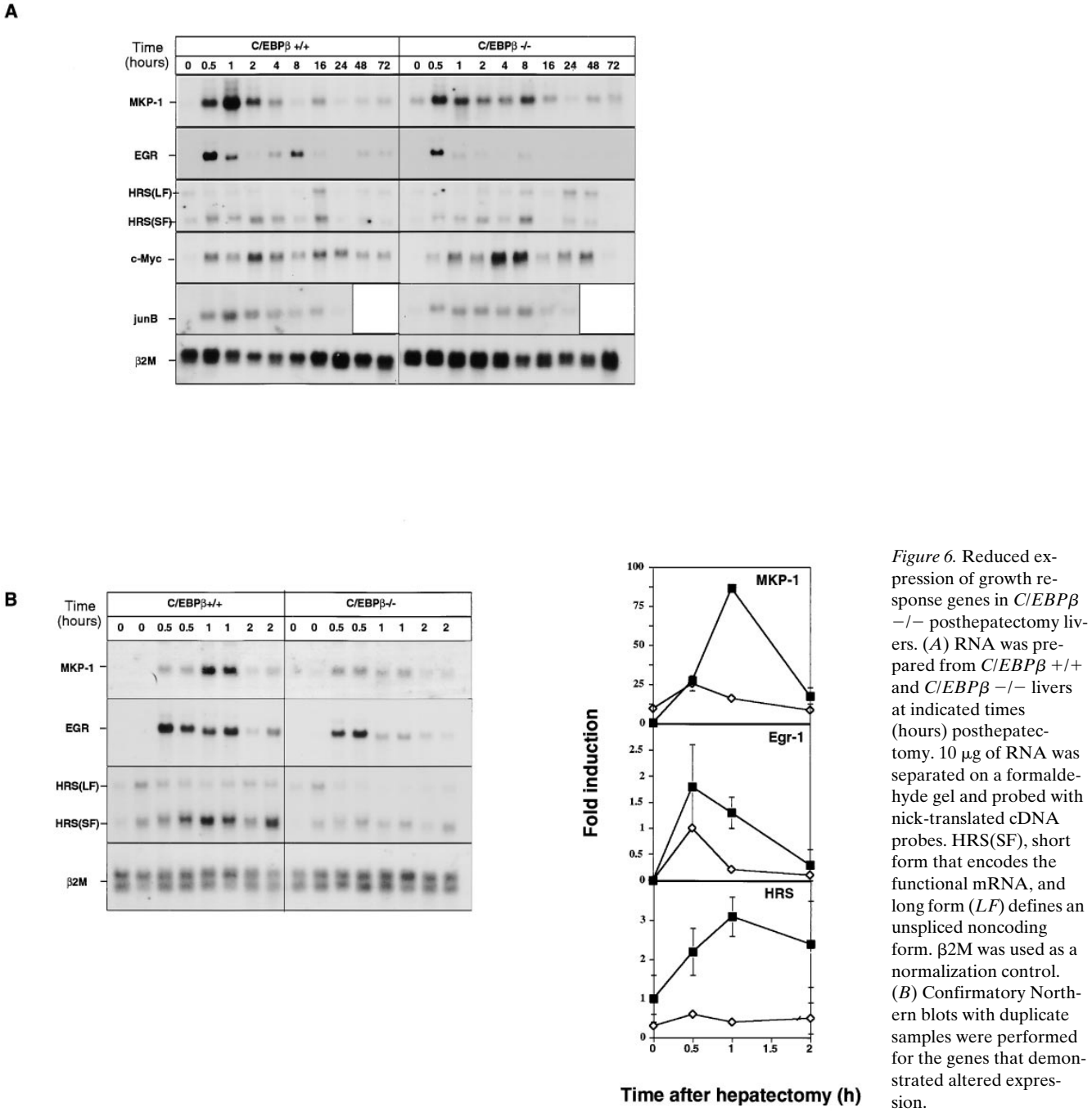


Figure 6. Reduced expression of growth response genes in *C/EBPβ −/−* posthepatectomy livers. (*A*) RNA was prepared from *C/EBPβ +/+* and *C/EBPβ −/−* livers at indicated times (hours) posthepatectomy. 10 μg of RNA was separated on a formaldehyde gel and probed with nick-translated cDNA probes. HRS(SF), short form that encodes the functional mRNA, and long form (*LF*) defines an unspliced noncoding form. β2M was used as a normalization control. (*B*) Confirmatory Northern blots with duplicate samples were performed for the genes that demonstrated altered expression.

C/EBPβ −/− livers, but there was significant variability between animals. *Cyclin A* mRNA was undetectable in two 36-h posthepatectomy *C/EBPβ −/−* livers and was relatively normal in a third animal. *Cyclin B* and *E* mRNA expression were 10- ($P < 0.02$) and 12-fold ($P < 0.001$) reduced, respectively 36 h posthepatectomy in *C/EBPβ −/−* livers (Fig. 7 *C*) and *Histone 3* expression was 10-fold lower 16 h posthepatectomy ($P < 0.24$).

Discussion

In this study, we demonstrated that hepatocyte DNA synthesis posthepatectomy was markedly reduced in animals that lack *C/EPBβ* and this finding was associated with alterations in metabolic homeostasis and abnormal expression of a subset of genes important for gluconeogenesis and growth regulation. The less dramatic differences in restitution of liver mass in *C/EBPβ −/−* animals may reflect the fact that increased cellular size that results in doubling of the liver mass occurs during G1 phase and is independent of DNA synthesis. Bennett et al. observed that mass restitution was similar in two different mouse strains posthepatectomy, despite a twofold difference in DNA synthesis (57).

We estimate that only ~ 50–60% of total hepatocytes were positive for DNA synthesis during the first 60 h posthepatectomy in our study. This DNA-synthetic response was less robust than expected as measurements of DNA synthesis in *IL-6*

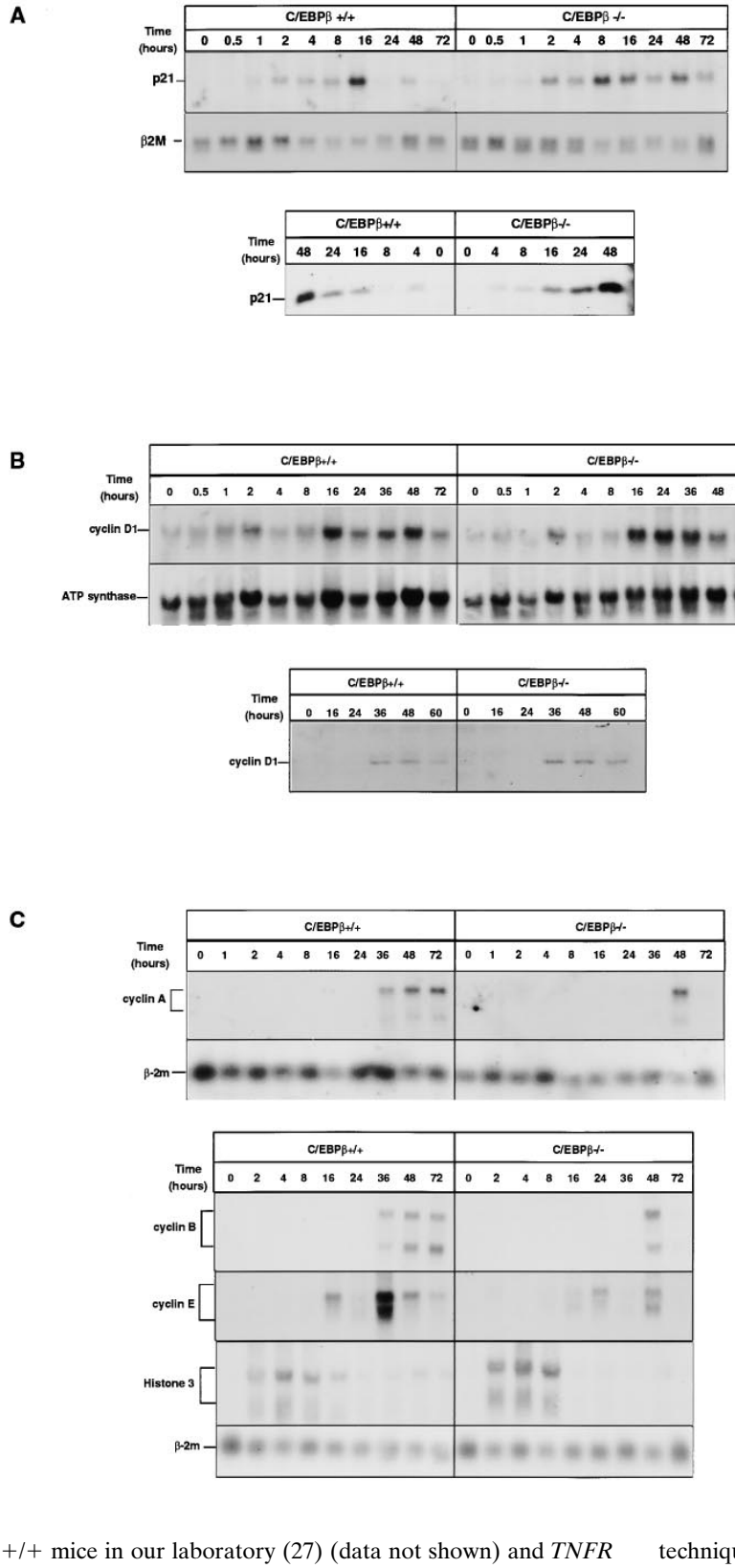


Figure 7. Altered expression of cell cycle regulatory genes in *C/EBPβ* −/− posthepatectomy livers. (*A–C*) RNA was prepared from *C/EBPβ* +/+ and *C/EBPβ* −/− livers. 10 μg (p21, cyclin D1, cyclin A) or 20 μg (cyclin B and E, Histone 3) of total RNA was separated on a formaldehyde gel and probed with nick-translated cDNA probes. For p21 Northern blot, one animal was used for each time point. For cyclin D1 and Histone 3, two animals were used for 16–72-h time points with three animals used for 16 h C/EBPβ +/+ time point. For cyclin A, B, and E blots, two animals were used for 24-, 36-, and 72-h *C/EBPβ* +/+ and 48- and 72-h *C/EBPβ* −/− time points. Three animals were used for 48-h *C/EBPβ* +/+; 24- and 36-h *C/EBPβ* −/− time points. Northern blots were densitometrically scanned and values normalized to the level of β2M or ATP synthase in each lane. Fold induction was calculated relative to the quiescent liver value (p21, cyclin D1, and Histone 3) or relative to the 36 h *C/EBPβ* +/+ value (cyclin A, B, and E). Mean, SD, and statistical significance were calculated to determine differences in cyclin A, B, and E, and Histone 3 gene expression. For p21 immunoblot, 75 μg of whole nuclei was separated on a 15% SDS-polyacrylamide gel, transferred and probed with α-p21 antibody. For cyclin D1 immunoblot (*B*), 25 μg of whole cell extract were separated on 12.5% SDS-PAGE and probed with α-cyclin D1. Protein expression was determined by densitometric scanning of the immunoblot and induction was normalized to Coomassie-stained gel after protein transfer.

+/+ mice in our laboratory (27) (data not shown) and *TNFR* +/+ mice reported by Yamada et al. (5) have found BrdU labeling in ∼100% of hepatocytes. The reduced regenerative response detected in *C/EBPβ* +/+ mice is most likely related to slight differences in mouse genetic background, as the mice used in this study were otherwise similar to those reported previously by our laboratory with respect to diet, age, and surgical technique. Bennett et al. observed similar strain-related differences in hepatocyte proliferation with a twofold reduction in DNA synthesis in C57BL/6 as compared with C3H mice (57).

During the first few hours posthepatectomy before C/EBPα protein levels decrease, gluconeogenic genes were induced in a compensatory response to the loss of glycogen stores. There was a subsequent loss of compensation in both *C/EBPβ* +/+

and *C/EBPβ −/−* livers resulting in hypoglycemia in both groups that correlated with the reduction in C/EBPα protein expression and inappropriate activation of C/EBPα target genes involved in gluconeogenesis during the period of hypoglycemia. In *C/EBPβ −/−* animals, this period of hypoglycemia was more sustained, suggesting that these animals were less able to respond to the metabolic changes that occur after partial hepatectomy. Despite renormalization of C/EBPα protein levels and induction of *G6pase* and *PEPCK* mRNA 24 h posthepatectomy, *C/EBPβ −/−* animals remained hypoglycemic until 48 h posthepatectomy. These findings suggest that the defect in glucose homeostasis in *C/EBPβ −/−* mice is only partially corrected by induction of C/EBPα target genes for gluconeogenesis. Croniger et al. have observed that 50% of *C/EBPβ −/−* mice die at birth due to hypoglycemia associated with a failure to express *PEPCK* and inability to mobilize hepatic glycogen stores (18). Administration of dibutyryl cAMP to these animals at birth induces hepatic *PEPCK* mRNA, glycogen mobilization, and normal glucose homeostasis for several hours, indicating that cAMP can normalize these parameters in the absence of C/EBPβ protein. The surviving animals exhibit apparently normal glucose homeostasis, suggesting that in this subset, C/EBPβ is not required for induction of *PEPCK* mRNA at birth. While these studies suggest that C/EBPα is the principal C/EBP isoform responsible for *PEPCK* mRNA induction at birth, *C/EBPβ −/−* animals that survive to adulthood may have a non–life-threatening defect in glucose metabolism that is not fully compensated in the setting of profound metabolic stress after partial hepatectomy. We predict that this inability to maintain normal metabolic homeostasis contributed to the blunted regenerative response that we observed in the livers of *C/EBPβ −/−* mice.

A few genes linked to the growth response showed reduced expression in *C/EBPβ −/−* livers after partial hepatectomy and the combined effect of this reduction in expression may have contributed to the reduced regenerative response in *C/EBPβ −/−* livers. Although IL-6 levels and STAT3 DNA binding were constitutively elevated in some *C/EBPβ −/−* livers, the induction of immediate–early genes linked to the IL-6/STAT3 activation pathway was normal suggesting *C/EBPβ −/−* animals have a defect in an IL-6 independent pathway(s) posthepatectomy. The greatest differences in gene expression were detected at the first hour posthepatectomy, after maximal induction of C/EBPβ protein, consistent with a posttranslational mechanism of C/EBPβ activation (29, 32). The transactivation potential of C/EBPβ for target gene promoters in hepatoma cells is increased in response to phosphorylation of specific serine residues by protein kinase C (29) and mitogen-activated protein kinase (MAP kinase) (32). MAP kinase activity rapidly increases in many cell types including regenerating rat hepatocytes in response to growth factor stimulation (58) and C/EBPβ could represent a potential downstream target of this activation pathway in the regenerating liver. In this way, C/EBPβ may resemble STAT3 and NF-κB as a rapid transducer of the earliest signals after partial hepatectomy. Alternatively, C/EBPβ could be important for the synthesis of a growth factor or cytokine required before the onset of liver cell proliferation.

Specific cell cycle regulatory genes showed reduced expression in *C/EBPβ −/−* livers. Steady-state levels of *cyclin B* and *E* were markedly reduced in *C/EBPβ −/−* livers at times near the peak of DNA synthesis and the level of *cyclin A* was similarly reduced in some animals. In contrast to *IL-6 −/−* livers, which demonstrated reduced *cyclin D1* mRNA and protein expression posthepatectomy (6), cyclin D1 mRNA and protein expression were normal in *C/EBPβ −/−* livers. *Cyclin D1* is transcriptionally regulated by AP-1 and c-*myc* (60, 61), both of which demonstrated normal activation in *C/EBPβ −/−* livers. We also detected small elevations at several time points in the expression of *p21* mRNA in *C/EBPβ −/−* livers. These results taken together suggest that in *C/EBPβ −/−* hepatocytes, the block to cell cycle progression may occur close to or at the G1/S phase transition. The possibility that decreased expression of these genes may have contributed to the reduced number of S phase hepatocytes in *C/EBPβ −/−* livers is supported by several studies demonstrating a correlation between cyclin expression and hepatocyte cell cycle progression (61–64). Cyclins have been shown to be regulated via transcriptional and posttranscriptional mechanisms in the liver (65, 66) although little is known about the specific factors that regulate their transcription. It is therefore not known if C/EBPβ regulates cyclin gene expression at the transcriptional level or via an indirect mechanism.

The regulation of liver cell proliferation and maintenance of metabolic homeostasis during liver regeneration must be coordinated to insure the health and survival of the organism. We have shown that glucose homeostasis and activation of specific genes associated with growth regulation are impaired in the livers of mice that do not contain C/EBPβ. Our findings are consistent with a model in which C/EBPβ regulates these two processes via different mechanisms. It is likely that C/EBPβ has a direct effect on metabolism as the prolonged hypoglycemia in some *C/EBPβ −/−* animals could not be completely explained on the basis of differences in the activation of C/EBPα dependent gluconeogenic genes. The reduced expression of several growth-associated and cell cycle regulatory genes is consistent with a model in which C/EBPβ participates in the liver's response to mitogenic signals after partial hepatectomy.

Acknowledgments

We thank Wafik el-Deiry for the 0S100 α-p21 antibody, Jeffrey Albrecht for cyclin A, B, E cDNA probes, and Clifford Steer for the cyclin D1 cDNA probe.

This work was in part supported by National Institutes of Health grants DK-44237, DK-49210, and DK-49629 (to R. Taub) and K08 DK-02366-01 (to L. Greenbaum).

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