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CCAAT/Enhancer Binding Protein α Regulates p21 Protein and Hepatocyte Proliferation in Newborn Mice

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CCAAT/enhancer binding protein α (C/EBPα) is expressed at high levels in quiescent hepatocytes and in differentiated adipocytes. In cultured cells, C/EBPα inhibits cell proliferation in part via stabilization of the p21 protein. The role of C/EBPα in regulating hepatocyte proliferation in vivo is presented herein. In C/EBPα knockout newborn mice, p21 protein levels are reduced in the liver, and the fraction of hepatocytes synthesizing DNA is increased. Greater than 30% of the hepatocytes in C/EBPα knockout animals continue to proliferate at day 17 of postnatal life when cell division in wild-type littermates is low (3%). p21 protein levels are relatively high in wild-type neonates but undetectable in C/EBPα knockout mice. The reduction of p21 protein in the highly proliferating livers that lack C/EBPα suggests that p21 is responsible for C/EBPα-mediated control of liver proliferation in newborn mice. During rat liver regeneration, the amounts of both C/EBPα and p21 proteins are decreased before DNA synthesis (6 to 12 h) and then return to presurgery levels at 48 h. Although C/EBPα controls p21 protein levels, p21 mRNA is not influenced by C/EBPα in liver. Using coimmunoprecipitation and a mammalian two-hybrid assay system, we have shown the interaction of C/EBPα and p21 proteins. Study of p21 stability in liver nuclear extracts showed that C/EBPα blocks proteolytic degradation of p21. Our data demonstrate that C/EBPα regulates hepatocyte proliferation in newborn mice and that in liver, the level of p21 protein is under posttranscriptional control, consistent with the hypothesis that protein-protein interaction with C/EBPα determines p21 levels.

C/EBPα belongs to the bZIP protein family of nuclear transcription factors, which contain a basic region and a leucine zipper domain in the C-terminal part of the molecule (18–20). These proteins are DNA binding molecules that are involved in the regulation of several biological processes such as the acute-phase response (16), control of cell proliferation and differentiation (11, 12, 32, 35), and control of energy metabolism (33). In addition, several publications have described a growth-inhibitory role for C/EBPα in vitro (3, 15, 28, 31, 32). Expression of C/EBPα is primarily restricted to highly differentiated nondividing cells such as adipocytes, hepatocytes, and type II cells of the lung (18). Human hepatoma cell lines express very low levels of C/EBPα protein compared to the liver (13). Expression of C/EBPα is reduced in tumor nodules relative to surrounding normal quiescent tissue (10). An increase in proliferating cell nuclear antigen (PCNA) staining and in expression of the growth-promoting factors c-Jun and c-Myc in the livers of C/EBPα-deficient mice has been described (9). All of these observations suggest that C/EBPα can modulate cell growth. Our studies provide genetic evidence that C/EBPα regulates hepatocyte growth in vivo and that this activity is mediated, in part, through the cell cycle-dependent kinase inhibitor p21.

The intronless C/EBPα gene encodes a single 2.7-kb mRNA that can be translated into two C/EBPα isoforms, 42 and 30 kDa, with different antimitotic properties (22, 27). Leaky ribosome scanning has been suggested to be the process by which C/EBPα isoforms are generated (2). C/EBPα growth arrest depends on several factors, including the level of C/EBPα (21), relative levels of the C/EBPα isoforms (22, 27), and the levels of other C/EBP family proteins (2, 35). Previously we studied C/EBPα-mediated growth inhibition in a stably transformed cell line, HT1, that contained an inducible C/EBPα gene under Lac repressor control. In this line, human C/EBPα inhibits proliferation via elevation of the level of the p21 (WAF-1/CIP-1/SDI-1) protein (31). The increase in p21 level was the result of stabilization of the p21 protein in C/EBPα-expressing cells. The p21 protein was discovered as an mRNA that is overexpressed in senescent fibroblasts (26), as a protein interacting with cyclin-dependent kinases (CDKs) (14), and as the product of a p53-regulated gene (5). p21 is an inhibitor of CDKs and arrests cell proliferation primarily at the G_1 phase of the cycle (29). The involvement of p21 in the control of cell proliferation in vivo is unclear. Although the p21 protein is a key element of growth regulation in cells in culture, p21 knockout mice are morphologically normal and do not exhibit increased tumor formation (1a).

A possible role for p21 in the regulation of liver proliferation was proposed by Wu et al. in experiments with p21 transgenic mice (34). Overexpression of p21 in liver resulted in a dramatic inhibition of liver proliferation during development as well as in response to the proliferative stimulus of partial hepatectomy (PH) (34). These results show that p21 is a growth inhibitor in vivo when it is overexpressed. Another CDK inhibitor, p27, is responsible for growth control in tissues where it is expressed at high levels such as thymus and testis (7, 17, 25). The level of p27 in liver is significantly lower than that in the latter two tissues (7, 17, 25).

To understand the mechanisms of tissue-specific growth regulation in vivo, we chose to study the regulation of hepatocyte growth in the newborn animals and, as a second model, the highly orchestrated process of liver regeneration. Hepatocyte growth control may or may not be identical in these two systems. In this report, we present evidence that C/EBPα controls hepatocyte proliferation in vivo in the developing liver, p21

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protein levels are reduced in the absence of C/EBPα, and changes in p21 protein levels do not correlate with p21 mRNA levels. In the regenerating rat liver, C/EBPα and p21 protein levels are coordinated. Interaction of C/EBPα with p21 occurs in liver and may be the mechanism regulating p21 protein levels. Both models, C/EBPα knockout mice and rat liver regeneration, suggest that C/EBPα is a critical element in the regulation of hepatocyte cell division.

MATERIALS AND METHODS

Animals and PH. Since C/EBPα knockout mice die within several hours after birth, C/EBPα knockout mice and genetically normal littermates were sacrificed immediately after birth. For RNA and protein analyses, livers were collected, frozen in liquid nitrogen, and kept at −80°C. For analysis of DNA synthesis, newborn animals were injected with bromodeoxyuridine (BrdU) for 1 h before sacrifice. Liver sections were fixed in 10% formalin and BrdU was detected by specific immunohistochemistry. Fischer 344 rats, 6 to 10 months old, were used for investigation of liver regeneration. Approximately 70% of the liver was surgically removed, and regeneration was allowed to proceed for 30 min and for 3, 6, 12, 24, and 48 h. Three animals at each time point were used. Livers from untreated animals were used as the point 0 controls. Sham operations were performed in parallel to those of the hepatectomized animals and served as a control for the stress response.

Protein isolation and Western analysis. The procedure for protein isolation is described in our previous publications (30, 31). Briefly, liver nuclear extracts (NEs) were isolated as follows. Livers were homogenized in buffer A containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 5 mM dithiothreitol (DTT). Nuclei were pelleted by centrifugation at 5,000 rpm for 10 min and washed with buffer A. The supernatant (cytoplasm) was frozen. High-salt extraction of nuclear proteins was performed by incubation of nuclei with buffer B (25 mM Tris-HCl [pH 7.5], 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 25% sucrose) for 30 min on ice. After centrifugation, the supernatant (NE) was divided into small fractions and kept at −80°C. Western analysis was carried out as described previously (31). Briefly, 50 to 100 μg of nuclear proteins was loaded on a 0.1% sodium dodecyl sulfate (SDS)–12% polyacrylamide gel. A 15% gel was used for low-molecular-weight proteins. After separation, proteins were transferred onto membranes (NitroBind; Micron Separation, Inc.) by using electroblotting. To equalize the protein loading, a preliminary filter was stained with Coomassie blue to verify the measured protein concentration. After detection of specific proteins, each filter was reprobed with antibodies to β-actin (Sigma) or with antibodies to cdk4. The β-actin control for Western blotting was used for quantitation of protein expression. The level of each protein was calculated as the ratio to β-actin. For calculation of C/EBPα levels, densitometric analysis of both the 42- and 30-kDa isoforms was used. Filters were blocked by 10% dry milk–2% bovine serum albumin (BSA) prepared in TTBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20). Incubations with primary and secondary antibodies were carried out as recommended for each antibody. Dry milk (0.5%) was added to TTBS, and this solution was used for incubation with antibodies. For measurement of p21 protein, two different antibodies were used: monoclonal antiserum cp36 (gift from W. Harper, S. Elledge, and E. Harlow) and polyclonal antiserum M-143 (Santa Cruz Biotechnology). These antibodies showed similar results. Antibodies to C/EBPα (14AA), PCNA, cdk4, cdk2, p53, and p27 were from Santa Cruz Biotechnology. Antibody to p16 was a gift of C. J. Sherr. Immunoreactive proteins were detected by using the enhanced chemiluminescence protocol (Amersham).

IP-bandshift assay. The interaction of C/EBPα and p21 in rat liver was studied by immunoprecipitation of p21 coupled with electrophoretic mobility shift assay for C/EBPα (IP-bandshift assay). NEs from rat liver (500 μg) were incubated with antibodies to p21 and protein A-agarose overnight. Three types of antibodies to p21 (polyclonal M-143 and H-164; monoclonal F5) from Santa Cruz Biotechnology were used in separate experiments. Immunoprecipitation with anti-p27, cdk2, PCNA, and Sp1 (Santa Cruz Biotechnology) was also carried out and served as controls for specific C/EBPα-p21 interaction. After four washes with phosphate-buffered saline, immunoprecipitates (IPs) were incubated in a binding buffer (10 μl) for 60 min on ice. Samples were centrifuged, and supernatant was added to the binding reaction containing an oligomer with a C/EBP consensus site. Conditions for the gel shift assay have been described elsewhere (30, 31).

Coimmunoprecipitation of bacterially expressed homogeneous C/EBPα and p21 with ^{35}S-labeled proteins. C/EBPα or p21 was translated in the Promega transcription-translation system in the presence of [^{35}S]methionine and purified by gel electrophoresis. Homogeneous histidine-tagged C/EBPα was isolated from bacteria by using the pET system (Clontech). Homogeneous glutathione *S*-transferase (GST)–p21 was kindly provided by J. Smith. Histidine-tagged p21 protein was a gift of J. Albrecht. The GST-p21 or His-p21 was attached to protein A-agarose through a specific polyclonal (H-164) or monoclonal (187) antibody to p21 (Santa Cruz Biotechnology) and incubated overnight with purified ^{35}S-labeled C/EBPα. Protein A-agarose with antibodies alone (no p21 added) was used as the control. We also incubated His-p21 and GST-p21 with ^{35}S-labeled NF-κB protein, which served as a control for nonspecific interaction. Samples were washed four times with phosphate-buffered saline and loaded on a 1% SDS–12% polyacrylamide gel.

Detection of C/EBPα-p21 interaction in mammalian cells by using the Matchmaker two-hybrid assay. The mammalian Matchmaker two-hybrid assay (Clontech) was used for detection of functional interaction between C/EBPα and p21. The coding region of C/EBPα (1.3 kb) was cloned in frame and downstream of the VP16 activation domain. The p21 coding region was inserted downstream of a Gal4 DNA binding domain (see Fig. 7A). The expression of the fusion proteins in mammalian cells was verified by Western blotting using transient-transfection experiments. We have observed that the VP16-C/EBPα fusion protein has a lower electrophoretic mobility than the predicted molecular weight of the protein. Restriction analysis, sequencing, and Western blotting with different antibodies to C/EBPα showed that the VP16-C/EBPα construct codes for the correct fusion protein, despite its reduced electrophoretic mobility. Three plasmids, pVP16-C/EBPα, pGal4-p21, and reporter pGCAT, were coelectroporated into HT1080 cells. Proteins were isolated 48 h after plasmid delivery and used for enzymatic chloramphenicol acetyltransferase (CAT) assay as described previously (31). VP16-large T antigen and Gal4-p53 were used as positive controls (Clontech). Cotransfection of VP16-C/EBPα with reporter alone or with Gal4-p53 served as a negative control. Radioactive spots were cut out and counted. Activation of the reporter gene by interaction of C/EBPα and p21 was measured as the ratio to CAT activity of pG5CAT alone and then as the percentage of the positive control, p53-T antigen, to control for transfection efficiency.

RNA isolation and Northern analysis. Total RNA was isolated as described previously (31). Samples (25 μg) of total RNA were loaded on a 1% agarose–2.2 M formaldehyde gel, transferred onto a membrane, and hybridized with specific probes. Each filter was hybridized sequentially with C/EBPα-, p21-, and 18S rRNA-specific probes as described previously (31). Quantitation of Northern blots was performed by phosphorimaging. The levels of C/EBPα and p21 mRNAs were normalized to those of the 18S rRNA control.

Stability of p21 in NEs from rat liver. To measure p21 stability in NEs, we used bacterially expressed, purified, full-length His-p21 protein and two truncated forms: the N-terminal portion of p21 (9 kDa; N-p21) and the C-terminal portion (12 kDa; C-p21). All three proteins were a gift of J. Albrecht. We have found that both full-length p21 and C-p21 are the subject of specific degradation in the liver. The N-terminal portion of p21 was resistant to degradation under the conditions of the experiment. The use of the C-p21 truncated form of p21 allowed us to distinguish this protein from endogenous p21 since the C-p21 protein is much smaller (12 kDa). Bacterially expressed p21 proteins (100 ng) were incubated with rat liver NE (5 to 10 μg) that was isolated without protease inhibitors in a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, 1 mM $MgCl_2$, and 5% sucrose for 30 min at 37°C and loaded on a 0.1% SDS–15% polyacrylamide gel. To examine the effect of C/EBPα on p21 stability in the presence of rat NEs, bacterially expressed homogenous His-C/EBPα (100 ng) was mixed with bacterially expressed p21 proteins and incubated for 15 min at room temperature prior to addition of nuclear extract. BSA was added to other samples to equilibrate the amount of protein in the reaction mixture. Proteins were electrotransferred onto a membrane and probed with a polyclonal antibody to p21 (H-164; Santa Cruz Biotechnology) as described above.

RESULTS

C/EBPα controls p21 protein in the liver. We have previously shown that C/EBPα inhibits proliferation of human fibrosarcoma cells through elevation of p21 protein, an inhibitor of CDKs (31). Overexpression of C/EBPα resulted in an increase of p21 half-life and growth arrest (31). To determine whether C/EBPα controls p21 protein in liver and arrests proliferation of hepatocytes in vivo via the p21 protein, as has been observed in cultured cells, we measured levels of p21 protein in NEs isolated from the livers of newborn C/EBPα −/− and +/+ mice (Fig. 1A). Although p21 protein is detectable in the livers of wild-type mice, little or no p21 protein was observed in livers from the four C/EBPα knockout animals. This remarkable reduction of p21 in C/EBPα null mice was specific for this CDK inhibitor, as no difference in p27 level was detected between C/EBPα null and wild-type mice. Expression of p21 has been shown to be regulated by p53; however, no differences in the amount of p53 were detected among the genotypes (Fig. 1A). The finding of equal levels of p53 in C/EBPα +/+ and −/− mice indicates that liver proliferation in C/EBPα null animals is unlikely to be due to a secondary response to DNA damage. The levels of p21 and p27 were also examined in rare, older, surviving C/EBPα null animals by using Western analysis. C/EBPα is present in genetically nor-

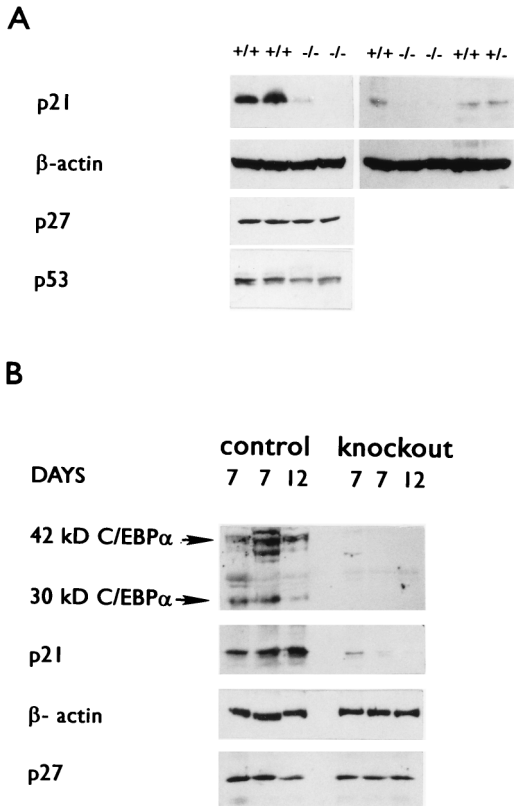

FIG. 1. (A) Expression of p21, p27, and p53 in livers of newborn mice. Nuclear proteins (100 μg) were analyzed by Western blotting. A monoclonal antibody to p21, cp36 (gift from W. Harper, S. Elledge, and E. Harlow), was used for detection of p21. The levels of p27 and p53 were examined with antibodies N20 (p27) and Pab-246 (p53) (Santa Cruz Biotechnology). (B) Western analysis of CDK inhibitors p21 and p27 in livers of older C/EBPα knockout mice (7 and 12 days old). The 12-day-old control mouse was heterozygous; 7-day-old mice were wild type. Antibodies to C/EBPα (A-144), to p21 (M-143), and to p27 (N20) were from Santa Cruz Biotechnology. Positions of 42- and 30-kDa C/EBPα isoforms are shown by arrows.

mal littermates at days 7 and 12 after birth (Fig. 1B). In these animals, p21 protein is expressed at high levels, but in C/EBPα knockout mice of the same age, p21 is not detectable in two animals and greatly reduced in the third. As in the newborn mice, p27 expression is not changed, indicating that the reduction in p21 is specific. Thus, these results show that C/EBPα affects the p21 protein level in vivo. We had previously determined that although C/EBPα can activate the p21 promoter in cultured cells, p21 mRNA induction was moderate and transient and did not account for 12- to 20-fold of p21 protein (31). In agreement with these observations, the levels of p21 mRNA in liver from C/EBPα knockout mice are identical to those in wild-type controls (data not shown). This observation suggests that the regulation of p21 protein by C/EBPα does not involve transcriptional control of p21 mRNA but rather involves a posttranscriptional event that is C/EBPα dependent.

The loss of C/EBPα results in increased DNA synthesis in hepatocytes in the livers of newborn mice. To test the growth-regulatory function of C/EBPα in vivo, we investigated the level of DNA synthesis as a measure of liver proliferation in C/EBPα knockout mice (33). Two approaches were used. The frequency of hepatocytes in S phase in the livers of C/EBPα knockout newborn mice was compared to that in wild-type littermates following BrdU uptake. Second, the amount of the S-phase-specific protein PCNA was determined by Western analysis. In livers of newborn mice from wild-type controls, 12 to 15% of the hepatocytes incorporate BrdU over a 1-h period of exposure to this thymidine analog. Twice as many BrdU-positive hepatocytes (27 to 30%) were found in C/EBPα knockout livers (Fig. 2A). Given the short labeling period, the percentage of BrdU-positive hepatocytes in C/EBPα knockout animals indicates a very high level of DNA synthesis. These data are consistent with the increased number of PCNA-positive hepatocytes reported by Flodby et al. (9). Although newborn livers of genetically normal mice continue to proliferate, the 2.0- to 2.5-fold elevation of BrdU-positive hepatocytes in the C/EBPα null livers over that of the control provides strong evidence that in the absence of C/EBPα, the rate of DNA synthesis, and thereby hepatocyte liver proliferation, in vivo is increased. To substantiate this conclusion, we measured the levels of the S-phase-specific protein PCNA in the livers of C/EBPα +/+ and −/− mice by Western blotting (Fig. 2B). Five animals of each genotype were analyzed. PCNA levels were 2.5- to 3-fold greater in the animals lacking C/EBPα than in genetically normal littermates. In contrast, the levels of β-actin did not change. Flodby et al. have described induction of β-actin mRNA in C/EBPα-deficient mice (9), but we did not detect any differences in the β-actin protein levels.

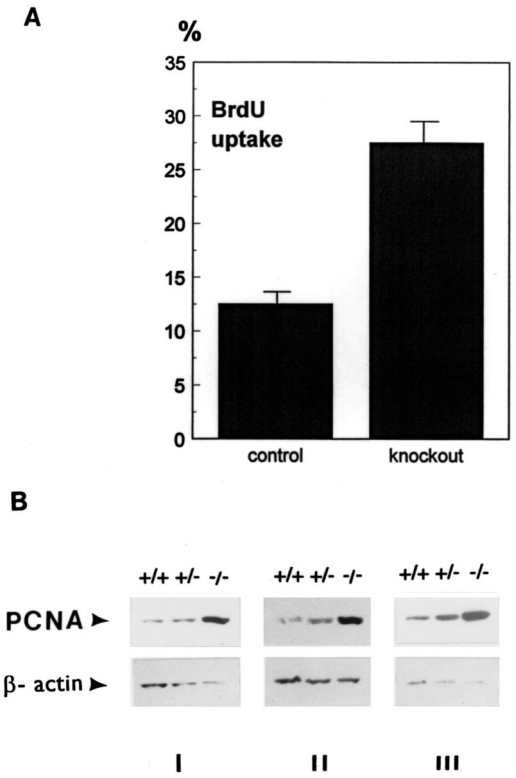

FIG. 2. DNA synthesis is increased in the absence of C/EBPα. (A) Measurement of DNA synthesis in livers by BrdU incorporation. Newborn mice were injected with BrdU 1 h before sacrifice. Liver sections were stained with antibodies to BrdU (Sigma), and the percentages of BrdU-positive hepatocytes in C/EBPα +/+ and +/− liver (control) and in C/EBPα −/− liver (knockout) were determined; 943 hepatocytes from one wild-type and one heterozygous animal (control) and 1,188 hepatocytes from two C/EBPα −/− mice were counted. (B) Protein levels of PCNA in the livers of C/EBPα knockout mice and in normal and heterozygous littermates. NEs were isolated as described previously (30, 31), and proteins (60 μg) were used for Western analysis with monoclonal antibodies to PCNA (Santa Cruz Biotechnology). Representative results with three littermate sets (I, II, and III) are shown.

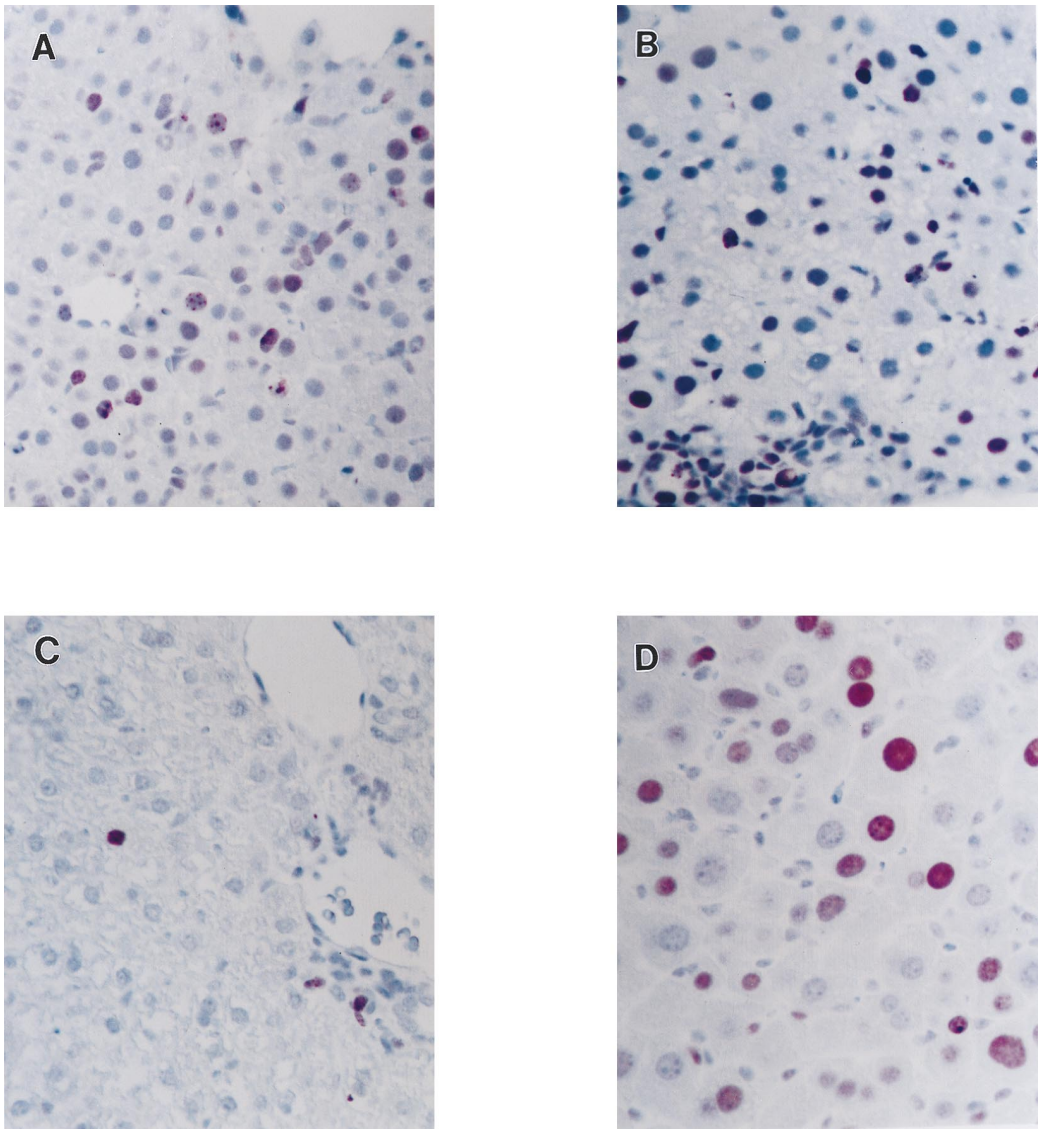

FIG. 3. DNA synthesis in hepatocytes is elevated in C/EBPα −/− mice. (A) Immunostaining of the livers from 7 (A and B)- and 17 (C and D)-day-old C/EBPα −/− and genetically normal mice with antibodies to PCNA. (A and C) Livers of genetically normal littermates; (B and D) C/EBPα knockout livers.

DNA synthesis is also increased in older C/EBPα knockout mice. The loss of C/EBPα also dramatically influences inhibition of DNA synthesis at later stages of development when cell division in the liver normally declines to a small percentage. We examined rare C/EBPα −/− mice that survived for several days (less than 1% of mutant animals live beyond the perinatal period). We obtained 7-, 12-, and 17-day-old C/EBPα −/− mice and control littermates of the same ages. Hepatocyte proliferation was measured by immunostaining for PCNA (Fig. 3). The numbers of PCNA-positive hepatocytes in 7-day-old C/EBPα +/+ and −/− mice show a twofold increase in the mutant animals. A more dramatic difference in PCNA staining is observed at 17 days of age. The livers of wild-type mice contain few (3%) PCNA-positive hepatocytes, while the frequency in animals that do not express C/EBPα is 30 to 40% (Fig. 3). Thus, in the absence of C/EBPα expression, inhibition of DNA synthesis in hepatocytes normally observed during the suckling period does not occur. In summary, an increase in the frequency of cells undergoing DNA synthesis and the elevation of PCNA suggest an increased rate of proliferation in C/EBPα knockout livers correlative with a reduction of p21 protein.

p21 protein but not RNA correlates with C/EBPα protein levels in liver. We have previously shown that the regulation of p21 by C/EBPα in cultured cells is complex and includes transient induction of the p21 mRNA and an increase of p21 protein that was posttranslationally stabilized by C/EBPα (31). In this study, we have detected a dramatic reduction of the p21 protein in C/EBPα knockout mice (Fig. 1); however, p21 mRNA levels are not altered (data not shown). This observation suggests that C/EBPα regulates p21 protein but not p21 mRNA. To test this hypothesis, we examined the expression of C/EBPα and p21 and their corresponding mRNAs in regenerating rat liver at different times after PH where C/EBPα mRNA and protein transiently decrease. p21 expression, as well as expression of other cell cycle-related proteins, was measured in regenerating livers of young (6- to 10-month-old) rats by Western analysis. Three animals were examined at each time point with the exception of 48 h (two animals). Proteins

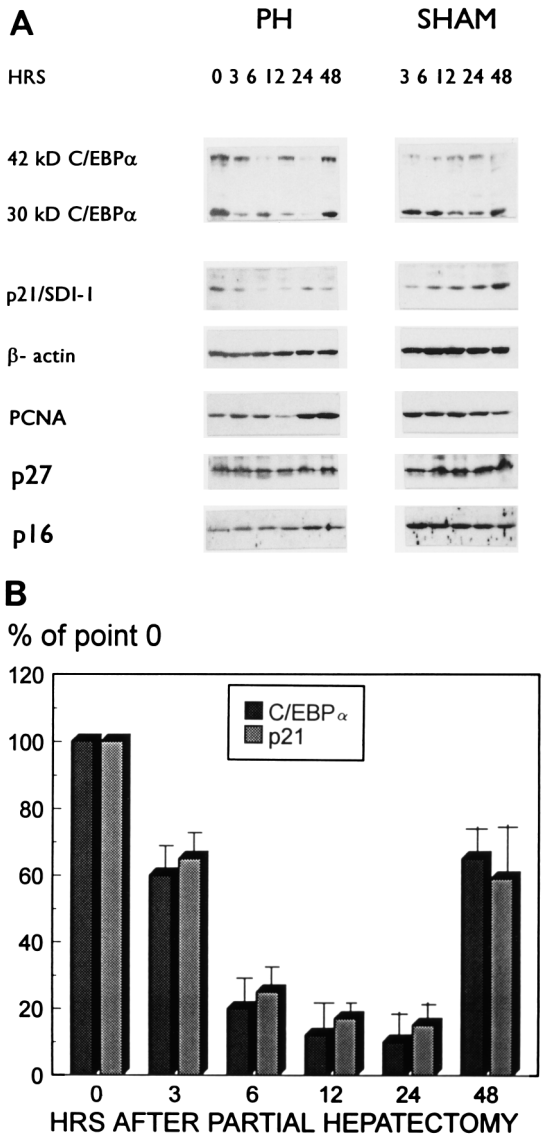

FIG. 4. C/EBPα and p21 protein are reduced during liver regeneration. (A) Western analysis of liver regeneration. Nuclear proteins were isolated at different times (indicated at the top) after surgery from hepatectomized (PH) and from sham-operated rats. Three animals for each time point were studied. Three to five Western analyses were done with each animal. (B) Summary of Western analyses with p21 and C/EBPα. The protein levels were calculated as the ratio to β-actin, using laser densitometry of five separate Western blots. Since two isoforms of C/EBPα are expressed, both were used in the calculations.

isolated from sham-operated rats served as the control. A representative Western blot and a summary of these analyses are shown in Fig. 4. The amounts of the 42- and 30-kDa isoforms of C/EBPα decline over time after PH, as has been previously described (4, 8, 24). In the same animals, the level of p21 protein was correlatively decreased. Levels of other proteins, including p16 and p27, did not change, suggesting that these CDK inhibitors are not involved in hepatocyte growth control following PH. Figure 4B shows a summary of five independent experiments. The maximum decrease of C/EBPα and p21 is observed at 12 and 24 h after PH, immediately before the peak DNA synthesis. We also tested the expression of p53 by Western analysis, and these data (not shown) confirmed an increased expression of p53 at 8 to 16 h after PH as previously described (6). These observations indicate that the reduction of p21 in dividing hepatocytes is p53 independent. To demonstrate that the proliferative response of animals to PH was normal, we measured the amount of the S-phase-specific protein PCNA, which was appropriately increased three- to fourfold at 24 and 48 h after surgery. The levels of both p27 and p16 were identical in hepatectomized and sham-operated animals. Taken together, our results show that the p21 protein levels are reduced in regenerating hepatocytes before DNA synthesis and that this reduction correlates with a reduction in the amount of C/EBPα. Although the amounts of C/EBPα and p21 protein are positively correlated in C/EBPα knockout mice and in regenerating liver, p21 mRNA expression in the liver is not influenced by C/EBPα and does not correlate with p21 protein levels. Figure 5 shows that p21 mRNA in liver is increased at times when C/EBPα mRNA and protein are decreasing (3 to 6 h). p21 mRNA was induced at 3 h in both sham-operated and hepatectomized animals, suggesting that this induction is not related to proliferation. Thus, the p21 protein is controlled by a mechanism that does not involve p21 mRNA regulation during liver regeneration. The reduction of p21 in C/EBPα knockout mice and the correlative decrease of both C/EBPα and p21 in response to PH suggest that C/EBPα controls p21 protein via protein-protein interaction rather than by transcriptional activation of the p21 gene (see below).

C/EBPα interacts with p21 in vitro. Because p21 mRNA expression is not influenced by C/EBPα, the mechanism of p21 regulation in the liver is likely to be at the posttranslational level. We have previously shown that C/EBPα increased the half-life of p21 protein in cultured HT1 cells (31). To further address the possibility that p21 regulation by C/EBPα is due to a protein-protein interaction, we used three approaches. IP-bandshift was carried out with nuclear proteins isolated from rat liver. A second approach, coimmunoprecipitation of bacterially expressed, gel-purified GST-p21 (or His-p21) with ^{35}S-C/EBPα labeled in reticulocyte lysates and gel purified, was used to test whether the interaction is direct. Functional interaction of C/EBPα and p21 was then examined in the mammalian two-hybrid assay (Matchmaker; Clontech). For the IP-bandshift, NEs from rat livers were incubated with antibodies to p21 (three different anti-p21 sera were used; see Materials and Methods), and the IPs were analyzed by bandshift assay. Figure 6A shows that C/EBPα binding activity is present in p21 IPs, but not in IPs with p27, indicating a specific interaction of C/EBPα with p21. We did not detect USF (a non-C/EBP transcription factor) binding activity in the p21 IPs, showing specific interaction with C/EBPα (data not shown). C/EBPα binding activity is also not detectable in IPs obtained in assays using antisera for cdk2 or PCNA (data not shown), showing

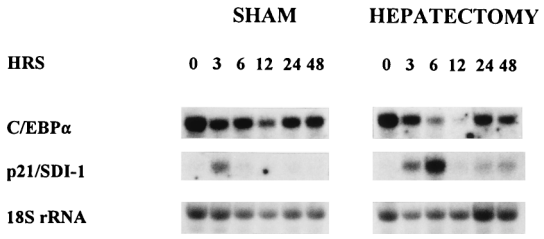

FIG. 5. Expression of p21 mRNA in regenerating liver. Total RNA (25 μg) was isolated from livers at different times after PH and analyzed by Northern blotting with specific probes. The filter was probed sequentially with p21, C/EBPα, and 18S probes as described previously (31).

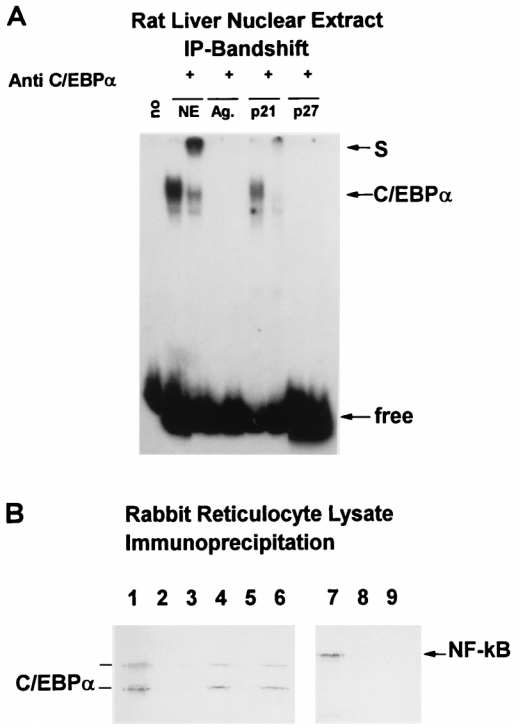

FIG. 6. (A) Interaction of p21 with C/EBPα in the liver. p21 was immunoprecipitated from liver NE by using antibodies to p21 and to p27 and protein A-agarose alone (Ag.). C/EBPα binding activity in IPs was analyzed by bandshift assay. IPs with three antibodies to p21 (see Materials and Methods) contained C/EBPα binding activity. IP-bandshift with each antibody was repeated three to four times with similar results. The result shown was obtained with p21 (H-164) and p27 (N20) antibodies (Santa Cruz Biotechnology). S, supershift with anti-C/EBPα. (B) Interaction of the purified C/EBPα and p21 proteins. C/EBPα was translated in the TNT transcription-translation reticulocyte lysate system in the presence of [^{35}S]methionine. Two C/EBPα isoforms are generated in this system (lane 1). Homogenous preparations of GST-p21 or His-p21 were attached to protein A-agarose by using monoclonal antibody 187 or polyclonal antibody H-164 (Santa Cruz Biotechnology) and incubated with ^{35}S-C/EBPα overnight. Lane: 1, C/EBPα translated in reticulocyte lysate; lane 2, protein A-agarose alone; lane 3, protein A-agarose and anti-p21 (187); lane 4, protein A-agarose, anti-p21 (187), and GST-p21; lane 5, protein A-agarose and anti-p21 (H-164); lane 6, protein A-agarose, anti-p21 (H-164), and GST-p21. Lanes 2 to 6 were incubated with ^{35}S-C/EBPα. p21 interacts with C/EBPα. Lanes 7 to 9, NF-κB negative control; lane 7, NF-κB translated in reticulocyte lysate; lane 8, protein A-agarose and anti-p21 (187); lane 9, protein A-agarose, anti-p21 (187), and GST-p21. Lanes 8 and 9 were incubated with ^{35}S-NF-κB. p21 protein does not interact with NF-κB.

that C/EBPα does not interact with either of these two proteins despite their ability to complex with p21.

To demonstrate direct interaction of C/EBPα with p21, gel-purified GST-p21 or His-p21 was attached to protein A columns and incubated with C/EBPα labeled with [^{35}S]methionine in reticulocyte lysates. Figure 6B shows a representative experiment in which labeled molecules of the appropriate mobility of two C/EBPα isoforms are observed in samples incubated with GST-p21 (lanes 4 and 6) but not with antibodies alone (lanes 3 and 5). Under the same conditions, GST-p21 protein does not bind to NF-κB (lanes 8 and 9), indicating a specific interaction with C/EBPα. A similar result was obtained with His-p21 protein (data not shown). We also performed experiments with bacterially expressed homogenous C/EBPα and ^{35}S-labeled p21 and again observed the specific interaction of C/EBPα and p21. Bacterially expressed, gel-purified C/EBPα was attached to protein A-agarose and incubated with ^{35}S-p21 protein that was translated in reticulocyte lysates and purified by gel electrophoresis. These results strongly indicate that C/EBPα interacts with p21 and suggest that the C/EBPα-p21 interaction is direct.

Functional interaction of C/EBPα and p21 in mammalian cells. C/EBPα and p21 interact in mammalian cells in the mammalian Matchmaker two-hybrid assay. Two constructs coding for VP16-C/EBPα and Gal-4-p21 (Fig. 7A) were cotransfected into SAOS2 cells together with the pG5CAT reporter construct, which contains Gal4 binding sites in its promoter. The result of these experiments is shown in Fig. 7B. The positive control (VP16-large T antigen cotransfected with Gal4-p53) activated the reporter construct to express the CAT gene.

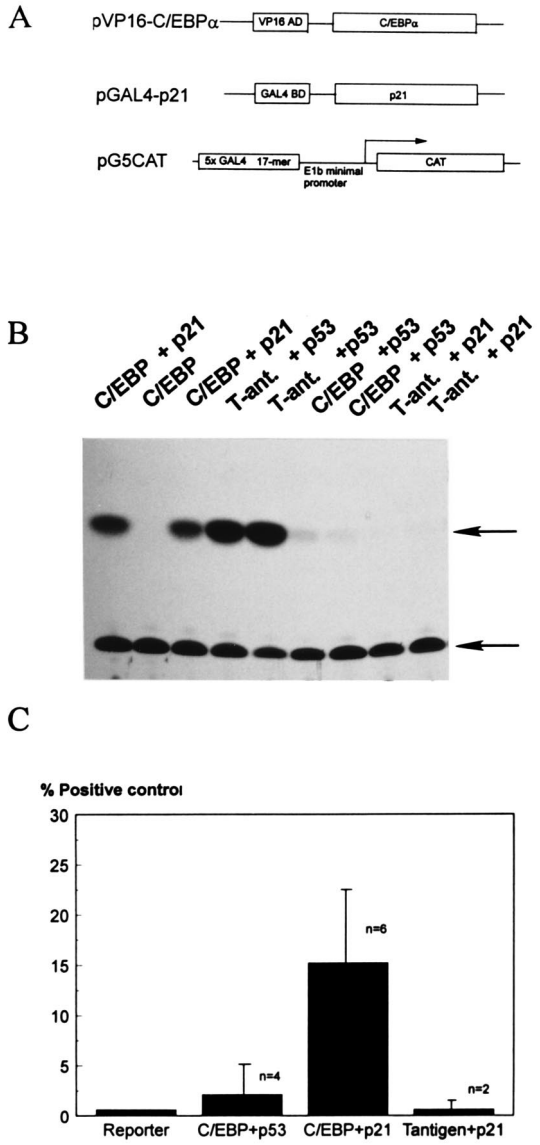

FIG. 7. Detection of C/EBPα-p21 interaction in the mammalian Matchmaker two-hybrid assay. (A) Schematic representation of the constructs used in these experiments. (B) Activation of pG5CAT reporter gene by interaction between C/EBPα and p21. Constructs (A) coding for the fusion proteins were cotransfected with pG5CAT into SAOS2 cells, and CAT activity was measured 48 h after transfection. (C) Summary of three independent experiments. The p53-T antigen interaction resulted in 50 to 70% conversion of chloramphenicol. Activation of the reporter gene by C/EBPα-p21 interaction was calculated as a percentage of the p53-T antigen positive control.

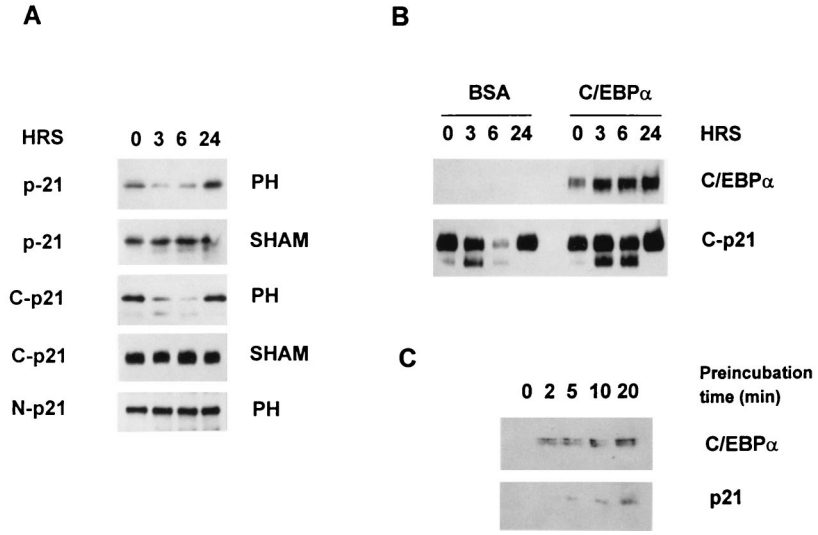

FIG. 8. C/EBPα blocks p21 degradation in liver nuclear extracts. (A) Bacterially expressed full-length His-p21, C-p21, and N-p21 (100 ng of each) were incubated with 10 μg of NEs isolated from rat livers at different times (indicated at the top) after PH or sham surgery and analyzed by Western assay with antibodies to p21 (H-164) as described in Materials and Methods. (B) C-p21 was preincubated with His-C/EBPα or with BSA for 15 min, and NEs were added. Samples were analyzed by Western blotting with anti-p21 as described above (bottom panel). The membrane was stripped and probed with antibodies to C/EBPα (upper panel). (C) Effect of time of C/EBPα preincubation on p21 resistance to proteolytic degradation. His-p21 and His-C/EBPα were incubated for 2, 5, 10, and 20 min before addition of NE (from the 6-h time point after PH). After 30 min of incubation with the NE the samples were analyzed by Western blotting with p21 antibody (bottom part) and with anti-C/EBPα (upper part).

When VP16-C/EBPα alone is expressed, no activation of the pG5CAT reporter is observed (lane C/EBP). However, the expression of cotransfected VP16-C/EBPα and Gal4-p21 results in the activation of the pG5CAT reporter construct. Cotransfection of VP16-C/EBPα with Gal4-p53 did not show the activation of pG5CAT, nor did the cotransfection of Gal4-p21 with VP16-T antigen. Figure 7C shows a summary of three independent experiments. Activation of the reporter gene by C/EBPα and p21 interaction was calculated as a percentage of the interaction of the positive control, p53-T antigen. The activation of the reporter construct by C/EBPα and p21 interaction was 15 to 20% of that of the p53-T antigen positive control (Fig. 7C). Activation of the reporter by VP16-C/EBPα or p21-Gal4 alone was negligible. Therefore, we conclude that C/EBPα interacts with p21 in mammalian cells. Taken together, our results show that C/EBPα forms complexes with the p21 protein, suggesting that this interaction may be the mechanism whereby p21 is stabilized.

C/EBPα blocks p21 degradation in NEs from regenerating livers. To examine the effect of C/EBPα-p21 interaction on p21 stability in rat liver, full-length His-p21, N-p21, and C-p21 were incubated with 10 μg of nuclear protein isolated from regenerating livers without protease inhibitors and analyzed by Western blotting. Figure 8A shows that the full-length p21 and C-p21 are degraded in NEs isolated at 3 and 6 h after PH but not in NEs isolated from quiescent livers. The N-terminal part of the p21 protein is resistant to proteolytic digestion during the test period, indicating specific cleavage of p21. Since C-p21 and full-length p21 showed identical results, we subsequently used the C-p21 protein because it allowed us to distinguish endogenous p21 (21 kDa) and His-C-p21 (12 kDa). The p21 degradation is specific to proliferating livers because no degradation is observed in sham-operated animals. Since p21 degradation correlates with reduction of C/EBPα levels in NEs (3- and 6-h NEs have low C/EBPα levels [Fig. 4]) and because C/EBPα interacts with p21 in rat livers, we suggest that C/EBPα can block p21 degradation via this interaction. To test this suggestion, bacterially expressed gel-purified His-C/EBPα was incubated with His-p21 for 15 min prior to addition of NEs. As shown in Fig. 8B, in the presence of C/EBPα, p21 degradation is significantly reduced, but addition of BSA did not alter p21 stability in NEs. The upper section of Fig. 8B shows the same membrane reprobed with anti-C/EBPα. C/EBPα molecules are stable in the extracts tested and serve as a control for nonspecific degradation. These data suggest that C/EBPα regulates p21 protein levels in the liver NEs by blocking its degradation. One alternative explanation of increased p21 stability in the presence of C/EBPα is that C/EBPα interacts with and inhibits a protease that specifically cleaves p21. To test this possibility, preincubations of p21 and C/EBPα were done. If C/EBPα blocks a specific protease, preincubation of the purified proteins, His-p21 and His-C/EBPα, prior to NE addition will not change the level of p21 stability. However, if C/EBPα protects p21 through direct interaction, one would expect an increase of p21 stability as a function of the preincubation with His-C/EBPα. His-C/EBPα and His-p21 were mixed and incubated for 2, 5, 10, and 20 min before addition of NEs from 6-h PH animals. Figure 8C shows that increasing the time of preincubation of C/EBPα and p21 leads to increasing resistance of p21 to digestion. This observation suggests that interaction of C/EBPα with p21, but not with components of the proteolytic pathway, is necessary for p21 protection and suggests that C/EBPα blocks p21 degradation in vitro through interaction with p21. Further studies are under way to demonstrate whether the C/EBPα-p21 interaction leads to increased stability of the p21 protein in mammalian cells.

DISCUSSION

Regulation of cell proliferation involves a cascade of events that are likely to be similar in many cell types. However, tissue-specific regulation of cell proliferation, such as hepatocyte regeneration, may involve unique factors that specifically respond to the loss of liver mass. C/EBPα is abundant in quies-

cent hepatocytes and has been shown to be reduced when hepatocytes proliferate in response to PH (4, 24) or in carcinoma nodules (10). These observations suggest a growth-inhibitory function for C/EBPα in vivo. Although PCNA induction in C/EBPα-deficient mice has been described (9), the proliferative capacity of hepatocytes has not been confirmed by direct measurement of DNA synthesis. In this report, we show that in the absence of C/EBPα, hepatocyte proliferation is increased, as evidenced by a 2- to 2.5-fold induction of DNA synthesis (BrdU uptake) and induction of PCNA levels. In addition, the presence of S-phase-specific E2F complexes was observed in C/EBPα knockout livers but not in livers of genetically normal littermates (29a).

We have previously shown that in cultured cells, C/EBPα-mediated stabilization of p21 protein is the major mechanism of C/EBPα growth arrest (31). The C/EBPα knockout mouse model has allowed us to investigate whether C/EBPα arrests hepatocytes in vivo through the p21 protein. We conclude from our finding that C/EBPα controls the protein levels of p21 but not the level of p21 mRNA. In two animal models in which C/EBPα levels are altered, PH in rats and C/EBPα knockout mice, reduced levels of p21 in NEs correlated with C/EBPα reduction and increased hepatocyte proliferation. Because overexpression of p21 in transgenic mice has been shown to block hepatocyte proliferation in newborn mice and during liver regeneration (34), we suggest that p21 is an important regulator of hepatocyte growth in newborn mice. It is notable that expression of p21 mRNA in the liver is not under C/EBPα control despite the presence of C/EBPα binding sites in the p21 promoter (data not shown). The induction of p21 mRNA in sham-operated animals indicates that this elevation is not connected with hepatocyte proliferation and does not result in protein induction. Most significantly, the p21 mRNA levels in C/EBPα knockout livers are not different from those in wild-type littermates. Our data show that p21 is controlled in the liver by a posttranslational mechanism(s). In agreement with this suggestion, we have observed that C/EBPα interacts with p21 in the liver (Fig. 7A). This interaction could well contribute to the regulation of p21 protein levels by C/EBPα. It is not clear whether this interaction is direct in the liver or involves other cellular proteins; however, coimmunoprecipitation of purified C/EBPα and p21 suggests a direct interaction (Fig. 7B). The mammalian Matchmaker two-hybrid assay showed that the interaction between p21 and C/EBPα also occurs in mammalian cells. In addition, C/EBPα protects p21 from degradation, presumably through interaction with p21 (Fig. 8). Recently, Maki and Howley showed that p21 protein is degraded by the ubiquitin-proteasome pathway (23), and we are exploring the possibility that this pathway exists in the liver and might be affected by C/EBPα in mammalian cells.

The C/EBPα pathway of growth arrest involves p21, but apparently not other CDK inhibitors, such as p16 and p27, that are abundant in liver, as their levels are not altered in dividing hepatocytes either during development or in the regenerative response to PH. Consistent with these in vivo findings that C/EBPα-mediated growth arrest does not involve p27 and p16 CDK inhibitors is the observation that protein levels of these two CDK inhibitors are not affected by C/EBPα expression in HT1080 cells (31). However, p27 and p16 can associate with cdk2 and cdk4. As the kinase activity of these complexes has not been examined, the involvement of p27 and p16 cannot be completely ruled out. Nevertheless, our data establish the interaction of p21 and C/EBPα and suggest that C/EBPα-mediated p21 regulation is one of perhaps multiple pathways of growth control in the liver and may be particularly important in the suckling neonate and during regeneration.

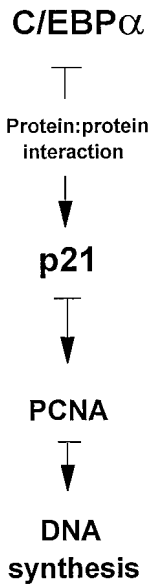

FIG. 9. C/EBPα-mediated growth arrest in hepatocytes.

C/EBPα-mediated pathway of hepatocyte growth arrest. Investigation of C/EBPα-mediated growth arrest in cultured cells and in vivo shows that p21 protein is a key element of the growth-regulatory pathway controlled by C/EBPα (31). Furthermore, C/EBPα regulates p21 levels by increasing the half-life of the p21 protein, rather than by transcription of the p21 gene (31). Although p21 knockout animals develop normally and do not have increased tumor formation in the liver, the involvement of p21 in the control of hepatocyte proliferation was not investigated in these animals. It is relevant that a growth-inhibitory role of p21 protein in the liver has been found by Wu et al. in p21 transgenic mice (34). In addition, the proliferative response to partial hepatectomy in p21 −/− animals shows an earlier entry into S phase and premature induction of a number of cell cycle-related genes (1). These observations and our results indicate that p21 plays a significant role in the control of liver proliferation during liver development and during liver regeneration (34). Based on our own observations and on those from the literature regarding the role of p21 in vitro and in vivo, we propose the following model for C/EBPα regulation of growth in hepatocytes (Fig. 9). In quiescent hepatocytes, high levels of C/EBPα maintain a p21 protein level that leads to the inhibition of PCNA activity and blocks DNA synthesis. When C/EBPα protein is reduced (liver regeneration) or absent (knockout mice), p21 protein is also decreased and permits an increase in the hepatocyte proliferation rate. Our data show that growth regulation by C/EBPα is not due to transcriptional regulation of the p21 gene. We have found that C/EBPα interacts with the p21 protein in liver NEs and in mammalian cells, suggesting that C/EBPα increases the p21 half-life through this interaction. Although the mechanism of p21 stabilization by C/EBPα in vivo is not known, our results showed that in vitro, bacterially expressed His-C/EBPα blocks specific p21 degradation presumably via direct interaction with p21. Our results, however, do not rule out the possibility that C/EBPα, in addition to interacting directly with p21, may activate other genes whose products stabilize the p21 protein. The dual activities of C/EBPα in regulation of differentiated gene expression and growth arrest may serve as a paradigm for

other tissue-specific transcription factors, whose expression is correlated with the quiescent differentiated state.

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