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TO: Philip J. Migliore, M.D.
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SUBJECT: Progress Report for The Moran Foundation Project
"Regulation of hepatocyte proliferation by C/EBPα" (96-0086)

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The major goal of this project was to test the hypothesis that CCAAT/Enhancer binding protein α (C/EBPα) and p21 regulate proliferation of hepatocytes in vivo. The original application proposed to study several physiological conditions when hepatocytes are stimulated to proliferate (liver regeneration after partial hepatectomy) or when proliferation of hepatocytes declines (liver development before and after birth). The application contained 2 Specific Aims. At the present time, Specific Aims 2 is completed and Specific Aim 1 (part B) will be completed in approximately two to three months.

Specific Aim 1. Test the hypothesis that C/EBPα and p21 are correlatively expressed in liver regeneration and development.

A. Regenerating rat liver model.
We have investigated the expression of C/EBPα and p21 in rat livers after partial hepatectomy using Western blotting (for protein detection) and Northern blotting (for mRNA detection) assays. Expression of both proteins is reduced in dividing hepatocytes with maximum reduction observed before DNA synthesis. This result shows that normal hepatocyte proliferation requires a decrease of these proteins and suggests that both C/EBPα and p21 inhibit hepatocyte proliferation. We have also found that C/EBPα regulates p21 protein, but not p21 mRNA expression in regenerating liver. These data are included in the manuscript that has been submitted to Molecular Cellular Biology (see attached).

B. Developmental expression of p21 in C/EBPα knockout and wild type mice.
We examined the expression of p21 and C/EEBPα during liver development at 16, 18 days of gestation and in newborn mice. In wild type animals, both proteins are induced before birth suggesting that C/EBPα regulates p21 protein during this period. Investigation of C/EBPα knockout animals indicated that p21 protein is regulated by C/EBPα, but p21 mRNA levels are under C/EBPα independent control. We continue to investigate this issue.

Specific Aim II. Test the hypothesis that the C/EBPα pathway of growth inhibition is deranged in the livers of old rats.

Expression of C/EBPα and p21 after stimulation of hepatocyte proliferation by partial hepatectomy has been investigated. Our results clearly show that, in young animals, both C/EBPα and p21

proteins are declined before DNA synthesis, however in older animals (24 mo), no reduction for these proteins was observed in response to PH. These data demonstrate that older animals fail to slow the levels of two strong inhibitors of cell proliferation in response to PH. We have demonstrated that high levels of p21 and C/EBPα lead to significant alterations of cell cycle progression in older animals. These alterations include delay in DNA synthesis, reduced and delayed induction of PCNA and cdc2 p34, proteins that play an important role in cell cycle progression. We have also detected changes in the activities of a transcription factor E2F, putative target of p21 protein. This Specific Aim has been completed and the manuscript has been submitted to Journal of Biological Chemistry (manuscript is attached).

The data of this project have been presented at the Cold Spring Harbor Meeting, April, 1997 and included in two manuscripts that have been submitted to Mol. Cel. Biol. and to J. Biol. Chem.

I would like to thank you for supporting my research.

Sincerely,

Timchenko

Nikolai Timchenko, Ph.D.
Assistant Professor of Pathology

NAT/kkf

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CCAAT/ENHANCER BINDING PROTEIN α REGULATES p21 PROTEIN AND HEPATOCYTE PROLIFERATION IN NEWBORN MICE.

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Running title: C/EBPα controls liver proliferation via p21 protein.

Key Words: C/EBPα, p21, protein:protein interaction, liver proliferation.

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Abstract

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 litter mates 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 (6-12 hrs) and then return to pre-surgery levels at 48 hrs. Although C/EBPα controls p21 protein levels, p21 mRNA is not influenced by C/EBPα in liver. Using co-immunoprecipitation and a mammalian two hybrid assay system, we have shown the interaction of C/EBPα and p21 proteins. Our data demonstrate that C/EBPα regulates hepatocyte proliferation in newborn mice and that, in liver, the level of p21 protein is under post-transcriptional control consistent with the hypothesis that protein:protein interaction with C/EBPα determines p21 levels.

INTRODUCTION

C/EBPα belongs to the bZIP protein family of nuclear transcription factors that 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 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, 42kD and 30kD, 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 a p53 regulated gene (5). p21 is an inhibitor of cyclin dependent kinases and arrests cell proliferation primarily at the G1 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 (1).

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 (34). These results show that p21 is 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).

In order to understand the mechanisms of tissue specific growth regulation in vivo, we chose to study the regulation of hepatocyte growth during the newborn phase 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. However, C/EBPα is a likely candidate factor to be involved in hepatocyte growth control because of its growth inhibitory role in cultured cells and the reduction of its level in response to proliferative stimulus of PH (4,8,24), in proliferating hepatocellular carcinomas (10) and in established hepatoma cell lines (13). In this paper we present evidence that

C/EBPα controls hepatocyte proliferation in vivo at different stages of development. p21 protein levels are reduced in the absence of C/EBPα and changes in p21 protein levels do not correlate with p21 mRNA levels. 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 during perinatal growth and during liver regeneration.

MATERIALS AND METHODS

Animals and partial hepatectomy. 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 BrdU for 1 hr before sacrifice. Liver sections were fixed in 10% formalin and BrdU was detected by specific immunohistochemistry. Fischer 344, 6-10 mo old rats were used for investigation of liver regeneration. Approximately 70% of the liver was surgically removed and regeneration was allowed to proceed for 30 min, 3, 6, 12, 24 and 48 hrs. 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 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 (NE) were isolated as follows. Livers were homogenized in buffer A containing 25 mM Tris-HCL, pH 7.5, 50 mM KCL, 2mM MgCL2, 1mM EDTA and 5mM DTT. Nuclei were pelleted by centrifugation at 5000 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 MgCL2, 1mM DTT, 0.5 mM EDTA and 25% sucrose] for 30 min on ice. After centrifugation, the supernatant (nuclear extract) was divided onto small fractions and kept at -80°C. Western analysis was carried out as described (31). Briefly, 50-100 ug of nuclear proteins were loaded on 12% polyacrylamide -0.1%SDS gel. A fifteen percent gel was used for low molecular weight proteins. After separation, proteins were transferred onto membranes (NitroBind, Micron Separation, Inc.) using electro blotting. 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, shown for Westerns presented in the paper, was used for quantitation of protein expression. The level of the various proteins was calculated as the ratio to β-actin. Filters were blocked by 10% dry milk, 2% BSA prepared on TTBS (20mM Tris-HCL, pH 7.5, 150 mM NaCL and 0.05% Tween-20) buffer saline. Incubations with primary and secondary antibodies were carried out according to recommendations for each antibody. 0.5% dry milk was added to TTBS and this solution was used for incubation with antibodies. For measurement of p21 protein, two different antibodies were used: a monoclonal antiserum cp36 (gift from W. Harper, S. Elledge and E. Harlow) and a 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 Dr. C. J. Sherr. Immunoreactive proteins were detected using the enhanced chemiluminescent (ECL) protocol (Amersham).

Immunoprecipitation-Bandshift assay (IP-Bandshift)

Interaction of C/EBPα and p21 in rat liver was studied by IP-Bandshift assay. Nuclear extracts from rat liver (500 ug) 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. 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 washing with PBS (4x), immunoprecipitates were incubated in a binding buffer (10 ul) for 60 min on ice. Samples were centrifugated and supernatant was added to binding reaction containing bZIP probe. Conditions for gel shift assay have been described (30,31).

Co-immunoprecipitation of bacterially expressed homogenous C/EBPα and p21 with S-35 labeled proteins. C/EBPα or p21 were translated in the Transcription/Translation Coupled System (Promega) in the presence of S-35-methionine and purified by gel electrophoresis. Homogenous histidine-tagged C/EBPα was isolated from bacteria using the pET system (Clonotech). Homogenous GST-p21 was kindly provided by J. Smith. His-p21 protein is a gift of J. Albrecht. The GST-p21 or His-p21 were attached to Protein-A agarose through specific polyclonal (H-163) or monoclonal (187) antibodies to p21 (Santa Cruz Biotechnology) and incubated overnight with purified ^{35}S labelled C/EBPα. Protein-A agarose with antibodies alone was used as the control. We have also incubated His-p21 and GST-p21 with ^{35}S-labelled NF-kB protein that served as a control for nonspecific interaction. Samples were washed 4 times with PBS and loaded on 12%-PAG-1%SDS gel.

Detection of C/EBPα:p21 interaction in mammalian cells using the Matchmaker two hybrid assay.

The mammalian matchmaker two hybrid assay (Clonotech) 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. 8A). The expression of the fusion proteins in mammalian cells was verified by Western blotting using transient transfection experiments. We have observed that VP16-C/EBPα fusion protein has slower electrophoretical 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, although it displays an abnormal electrophoretic mobility. Three plasmids, pVP16-C/EBPα, pGal-4-p21 and reporter pGCAT, were co-electroporated into HT1080 cells. Proteins were isolated in 48 hours after plasmid delivery and used for enzymatic CAT assay as described (31). VP16-T-large antigen and Gal4-p53 were used as positive controls (Clonotech). Co-transfection of VP16-C/EBPα with reporter alone or with Gal4- p53 served as negative controls. 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 percent of the positive control: p53:T-large antigen to control for transfection efficiency.

RNA Isolation and Northern analysis. Total RNA was isolated as described (31). 25 ug of total RNA were loaded on 1%-agarose:2.2M 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 (31). Quantitation of Northerns was performed using phosphor imaging. The levels of C/EBPα and p21 mRNAs were normalized to the 18S rRNA control.

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 nuclear extracts 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 CKI 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 older surviving C/EBPα null animals using Western analysis. C/EBPα is present in genetically normal 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 p21 protein level in vivo. Although C/EBPα can activate the p21 promoter in cultured cells, p21 mRNA induction is moderate and transient and does not account for 12-20 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 a posttranscriptional event that is C/EBPα dependent.

The loss of C/EBPα results in increased hepatocyte proliferation in the livers of newborn mice. C/EBPα growth inhibitory function in cultured cells has been shown by several studies (3,15,28,31,32). To test the growth regulatory function of C/EBPα in vivo, we investigated 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 were compared to wild type littermates following BrdU uptake. Second, the amount of the S-phase specific protein, PCNA was determined by Western analysis. Livers of newborn mice from wild type controls have 12-15% of the hepatocytes that incorporate BrdU, over a one hour period of exposure to this thymidine analog. Twice as many BrdU positive hepatocytes (27-30%) 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 proliferation. Although newborn livers of genetically normal mice continue to proliferate, the 2.0 -2.5 fold elevation of BrdU positive hepatocytes in the C/EBPα null livers over that of control provides strong evidence that, in the absence of C/EBPα, the rate of 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 are 2.5-3 fold greater in the animals lacking C/EBPα than those 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.

Liver proliferation is increased in older C/EBPα knockout mice.

The loss of C/EBPα also dramatically influences inhibition of hepatocyte proliferation 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. Liver 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 two fold 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 30-40% (Fig. 3). Thus, in the absence of C/EBPα expression, inhibition of hepatocyte proliferation, 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 show an increased rate of proliferation in C/EBPα knockout livers correlative with a reduction of p21 protein.

21 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 post-translationally stabilized by C/EBPα (31). In this paper, 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, the expression of C/EBPα and p21 and their corresponding mRNAs were examined in regenerating rat liver at different times after partial hepatectomy (PH). p21 expression, as well as expression of other cell cycle related proteins, was measured in regenerating livers of young (6-10 months) rats by Western analysis. Three animals were examined at each time point with the exception of 48 hours (two animals). Proteins 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 42kD and 30 kD isoforms of C/EBPα decline over time after partial hepatectomy as has been previously described (4,8,24). In the same animals, the level of p21 protein was correlatively decreased. Other proteins, including p16 and p27, did not change suggesting that these cyclin dependent kinase inhibitors are not involved in hepatocyte growth control following PH. Figure 4 (bottom part) shows a summary of five independent experiments. (5 Westerns of 3 animals at each point). The maximum decrease of C/EBPα and p21 is observed at 12 and 24 hrs 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 8-16 hrs after partial hepatectomy 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 3-4 fold at 24 and 48 hrs 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 liver is not influenced by C/EBPα and does not correlate with p21 protein levels. Fig. 5. shows that p21 mRNA in liver is increased at times when C/EBPα mRNA and protein are decreasing (3-6 hrs). p21 mRNA was induced at 3 hrs in both sham operated and hepatectomized animals (data not shown) 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.

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 post-translational 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. Immunoprecipitation of p21 followed by Bandshift of C/EBPα (IP-Bandshift) was carried out with nuclear proteins isolated from rat liver. A second approach, co-immunoprecipitation of homogenous

bacterially expressed GST-p21 (or His-p21) with ^{35}S-C/EBPα labeled in reticulocyte lysates 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, Clonetech). For the IP-Bandshift, nuclear extract from rat livers were incubated with antibodies to p21 (three different anti-p21 sera were used, see Materials and Methods), and the immunoprecipitates (IPs) were analyzed by Bandshift assay. Fig. 6 A 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 binding activity in the p21 IPs showing specific interaction with C/EBPα (data not shown). C/EBPα binding activity is also not detectable in immunoprecipitates using antisera for cdk2 or PCNA, (data not shown) showing 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, homogenous GST-p21 or His-p21 were attached Protein A columns and incubated with C/EBPα labelled with ^{35}S-methionine in reticulocyte lysates. Fig. 6B shows a representative experiment in which labelled 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-kB (lanes 8 and 9) indicating a specific interaction with C/EBPα. A similar result was obtained with His-p21 protein (data not shown). We have also performed experiments with bacterially expressed homogeneous C/EBPα and ^{35}S-labelled p21 and also observe the specific interaction of C/EBPα and p21. Bacterially expressed homogeneous 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. In these experiments, C/EBPβ (another member of

C/EBP family) did not interact with p21 and served as the negative control (data not shown). 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 co-transfected into SAOS2 cells together with the pG5CAT reporter construct that contains Gal4 binding sites in its promoter. The result of these experiments is shown in Fig. 7B. The positive control (VP16-T-large antigen co-transfected with Gal4-p53) activated the reporter construct to express the CAT gene. When VP16-C/EBPα alone is expressed, no activation of the pG5CAT reporter is observed (lane C/EBP). However, the expression of co-transfected VP16-C/EBPα and Gal4-p21 results in the activation of the pG5CAT reporter construct. Co-transfection of VP16-C/EBPα with Gal4-p53 did not show the activation of pG5CAT, nor did the co-transfection of the Gal-4-p21 with VP16-T antigen. Fig. 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 15-20% 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. Further studies are underway to

demonstrate whether or not the C/EBPα:p21 interaction leads to increased stability of the p21 protein.

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 quiescent 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 the present paper we show that, in the absence of C/EBPα, hepatocyte proliferation is increased as evidenced by a 2-2.5 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 genetically normal littermates (Timchenko, unpublished observations).

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, partial hepatectomy in rats and C/EBPα knockout mice, reduced levels of p21 in nuclear extracts 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 liver is not under C/EBPα control despite the presence of C/EBP 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 liver by post-translational 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, co-immunoprecipitation 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. Recently Maki et al 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.

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 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 cdk 4. As the

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 but one of multiple pathways of growth control in the liver, one that may be particularly important in the suckling neonate and during regeneration.

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 of the half life of the p21 protein, rather then by transcription of 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 to note that a growth inhibitory role of the p21 protein in the liver has been described 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 (J. Albrecht, personal communication). These observations and our results indicate that p21 plays 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. 8). 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 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 nuclear extracts and in mammalian cells suggesting that C/EBPα increases the p21 half-life through this interaction. At least two modes of regulation of p21 by C/EBPα can be suggested; interaction of C/EBPα with p21 may result in an increase of the p21 half life and/or C/EBPα 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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LEGENDS TO FIGURES

Figure 1. A. Expression of p21, p27 and p53 in the livers of newborn mice. Nuclear proteins (100 ug) were analyzed by Western blotting. Monoclonal antibodies to p21, cp36, (gift from W. Harper, S. Elledge and E. Harlow) were used for detection of p21. The levels of p27 and p53 were examined with antibodies N20 (p27) and Pab-246 (p53) from Santa Cruz Biotechnology. **B.** Western analysis of CDK2 inhibitors p21 and p27 in the livers of older C/EBPα knockout mice (7 and 12 day old). The 12 day 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.

Figure 2. Liver proliferation is increased in the absence of C/EBPα. **A.** Measurement of DNA synthesis in the livers by BrdU incorporation. Newborn mice were injected with BrdU 1 hr before sacrifice. Liver sections were stained with antibodies to BrdU (Sigma) and the percentage of BrdU-positive hepatocyte was counted. Percent of BrdU-positive hepatocytes in C/EBPα +/+ and +/- liver (control), and in C/EBPα -/- hepatocyte (knockout). 943 hepatocyte from one wild type and one heterozygous animal (control) and 1188 hepatocyte from two C/EBPα -/- mice were counted. **B.** The protein levels of PCNA in the livers of C/EBPα knockout mice and in normal and heterozygous littermates. Nuclear extracts were isolated as described (30,31) and proteins (60 ug) used for Western analysis with monoclonal antibodies to PCNA (Santa Cruz Biotechnology). Representative results with three littermate sets (I, II and III) are shown.

Figure 3. Hepatocyte proliferation is elevated in C/EBPα-/- mice. A. Immunostaining of the livers from 7 (A, B) and 17 (C, D) day old C/EBPα -/- and genetically normal mice with antibodies to PCNA. A and C - genetically normal littermates, B and D - C/EBPα knockout livers.

Figure 4. C/EBPα and p21 protein are reduced during liver regeneration. **Upper part.** Western analysis of the liver regeneration. Nuclear proteins were isolated at different times (indicated on the top) after surgery from hepatectomized (PH) and from sham operated rats. Three animals for each time point were studied. 3-5 Western analyses were done with each animal. **Bottom part.** Summary of Western analyses with p21 and C/EBPα. The protein levels were calculated as the ratio to β-actin using laser densitometry of 5 separate Western blots.

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

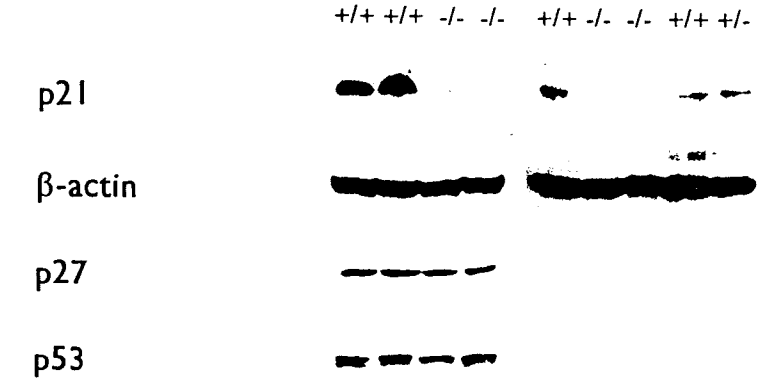
Figure 6. A. Interaction of p21 with C/EBPα in the liver. p21 was immunoprecipitated from liver nuclear extract using antibodies to p21, to p27 and Protein A-Agarose alone (Ag.). C/EBPα binding activity in immunoprecipitates was analyzed by Bandshift assay. IPs with three antibodies to p21 (see Material and Methods) contained C/EBPα binding activity. IP-Bandshift with each antibody was repeated 3-4 times with similar results. The result shown above 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 Transcription/Translation TNT Reticulocyte Lysate system in the presence of ^{35} S-methionine. Two C/EBPα isoforms are generated in this system (lane 1). Homogeneous preparations of GST-p21 or His-p21 were attached to Protein-A agarose through monoclonal 187 or polyclonal H-164 antibodies (Santa Cruz Biotechnology) and incubated with the ^{35}S-C/EBPα for overnight. 1 -C/EBPα translated in Reticulocyte Lysate, 2- Protein-A (Pr.-A) agarose alone, 3- Pr.-A agarose and anti p21 (187), 4- Pr.-A-agarose, anti p21 (187) and GST-p21, 5 - Pr.A-agarose and anti p21 (H164), 6 - Pr.A-agarose, anti p21 (H-164) and GST-p21. Samples 2-6 were incubated with ^{35}S-C/EBPα. p21 interacts with C/EBPα. 7-9 - NF-kB negative control. 7- NF-kB translated in reticulocyte lysate, 8- Pr.A-agarose and anti-p21 (187) and 9 - Pr.A-agarose, anti p21(187) and GST-p21. 8 and 9 were incubated with ^{35}S-NF-kB. p21 protein does not interact with NF-kB.

Figure 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 (indicated on the top) coding for the fusion proteins were co-transfected with pG5CAT into SAOS2 cells and CAT activity was measured 48 hrs after transfection. C. Summary of three independent experiments. The p53:T-antigen interaction resulted in 50-70% conversion of chloramphenicol. Activation of the reporter gene by C/EBPα:p21 interaction was calculated as percent of the p53:T-antigen positive control.

Figure 8. C/EBPα mediated growth arrest in hepatocytes.

A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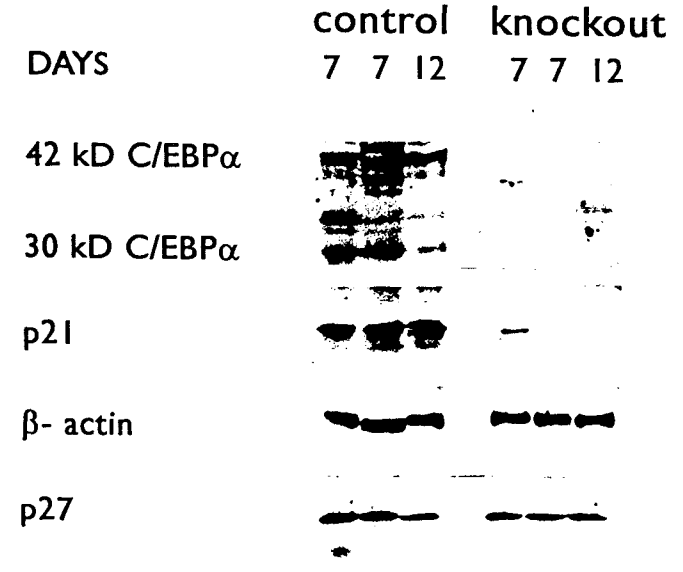
β-actin
p27
p53

B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knockout
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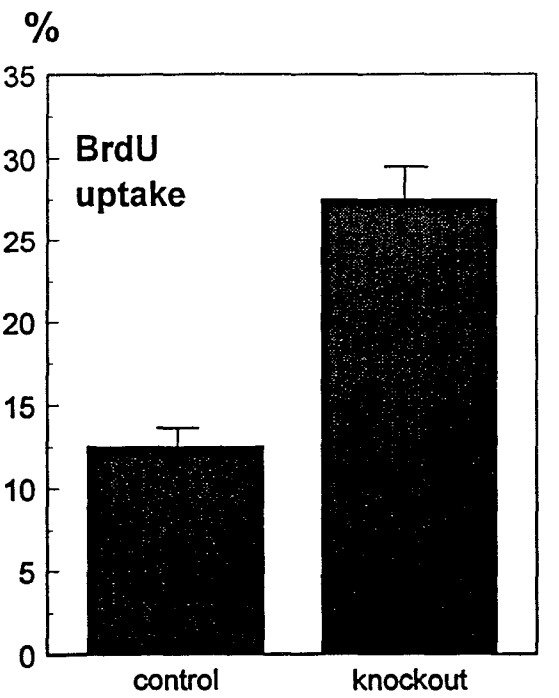
30 kD C/EBPα
p21
β- actin
p27

Fig. 1

A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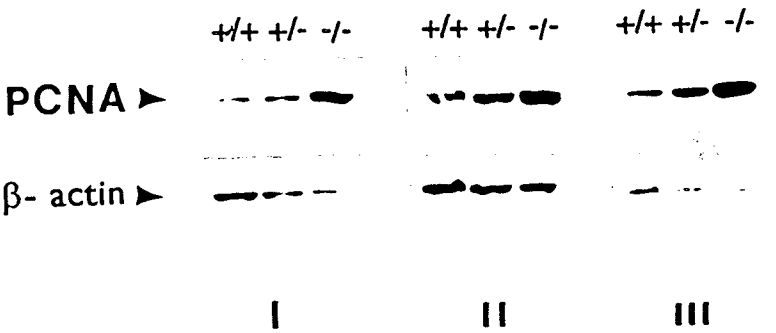
B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PCNA ►
β- actin ►
I
II
III

Fig. 2.

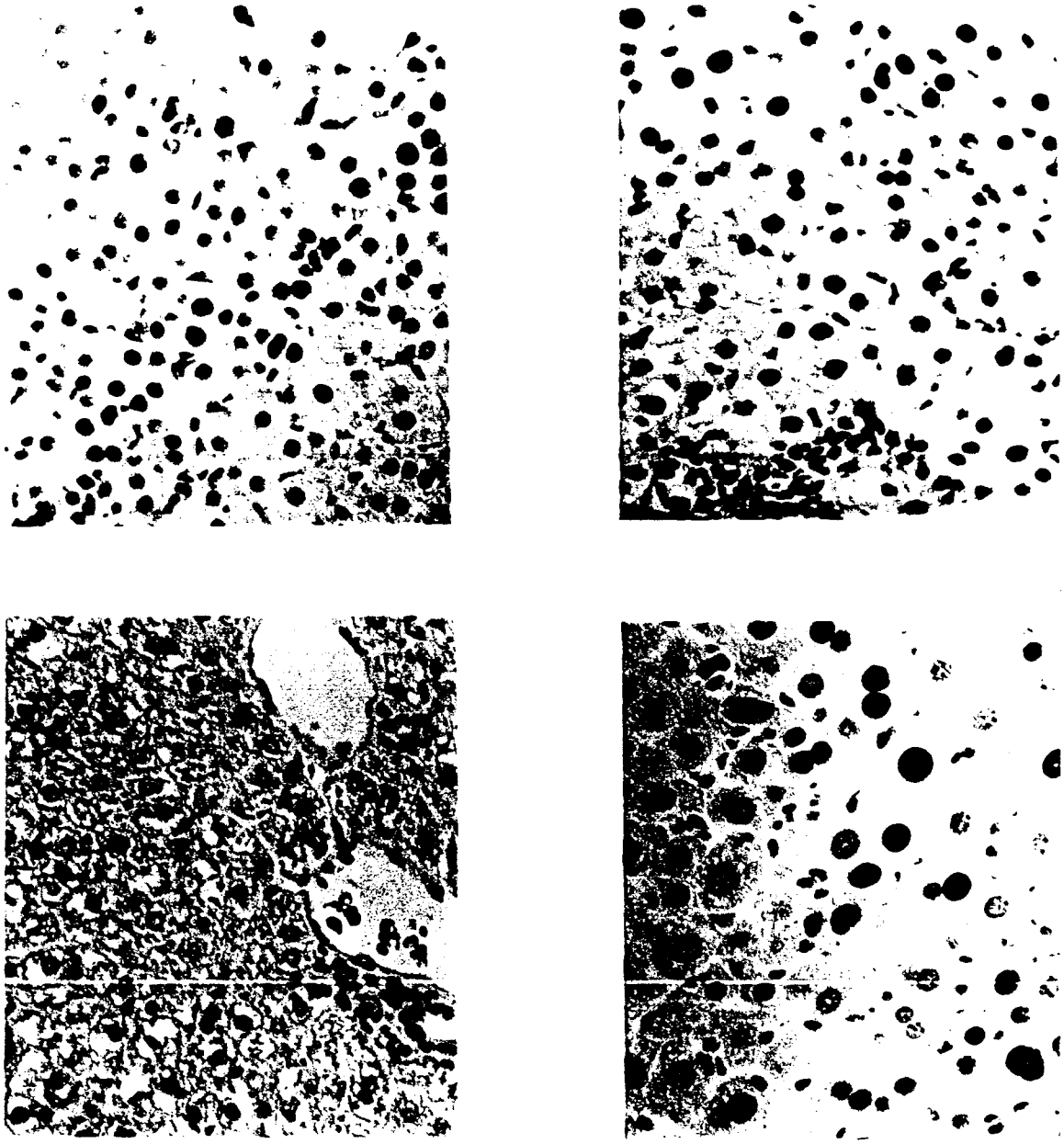
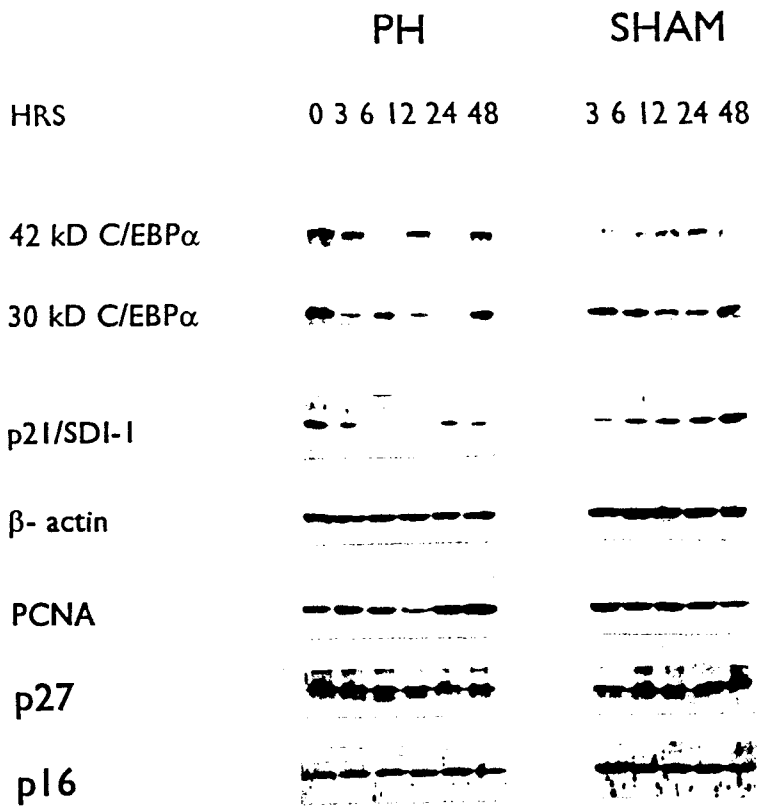


Fig. 3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SHAM
HRS
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p21/SDI-1
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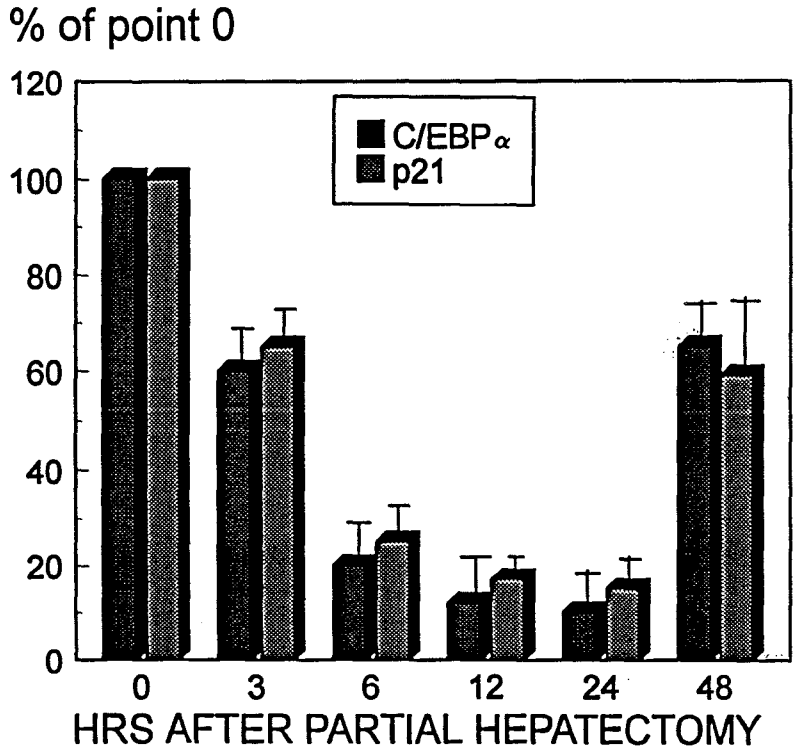
PCNA
p27
p16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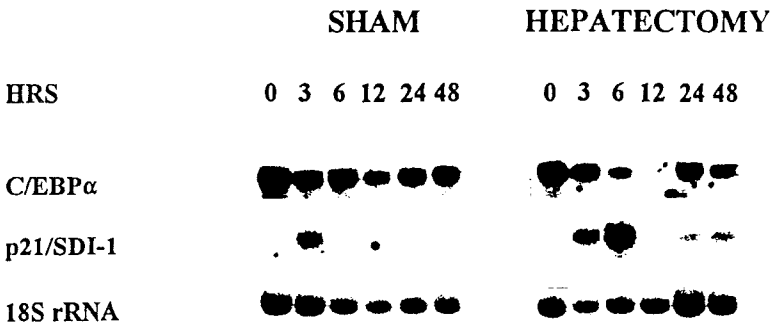
HRS AFTER PARTIAL HEPATECTOMY

Fig. 4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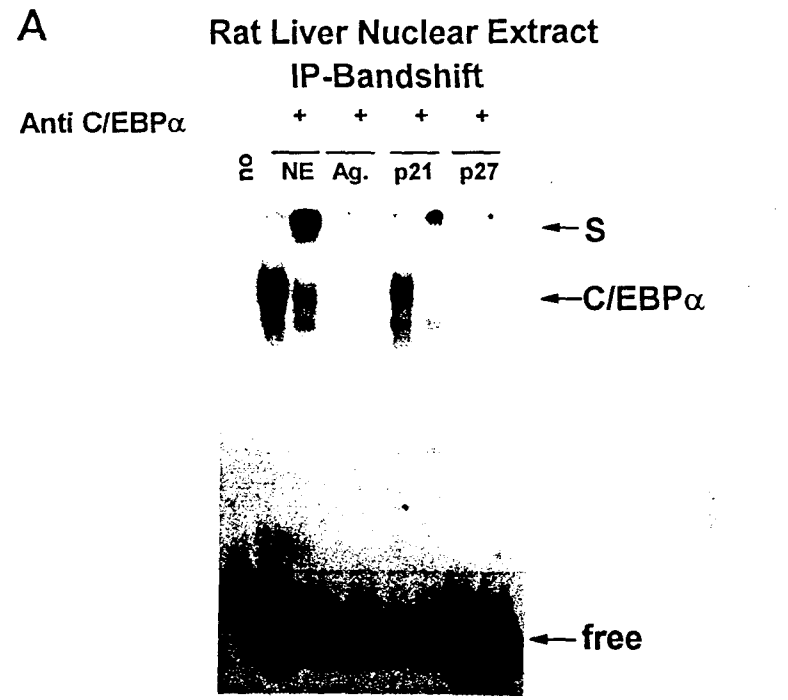
p21/SDI-1
18S rRNA

Fig. 5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Anti C/EBPα
+ + + +
PE
NE
Ag.
p21
p27
←S
←C/EBPα
←free
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Rabbit Reticulocyte Lysate
Immunoprecipitation
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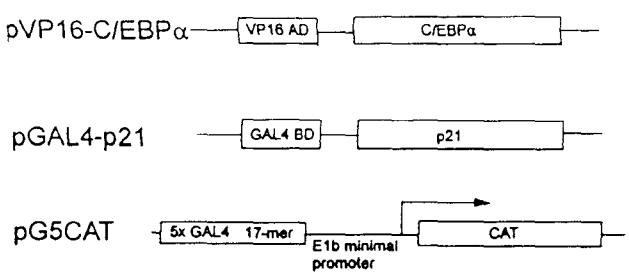
←NF-kB
C/EBPα

Fig. 6.

A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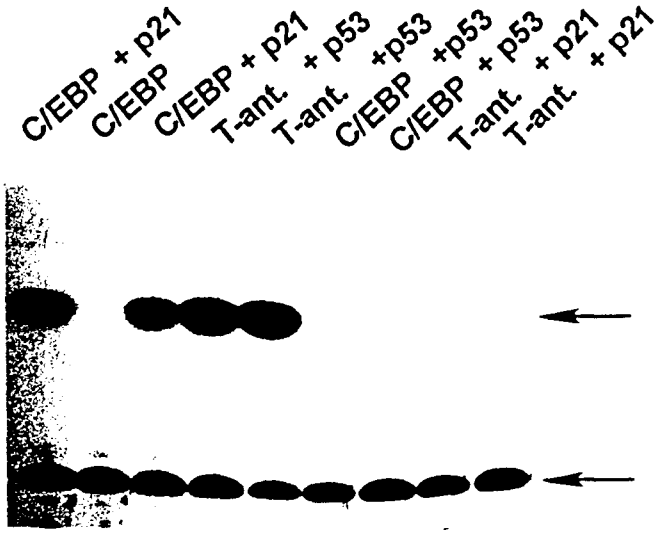
CAT

B


C/EBP + p21
C/EBP
C/EBP + p21
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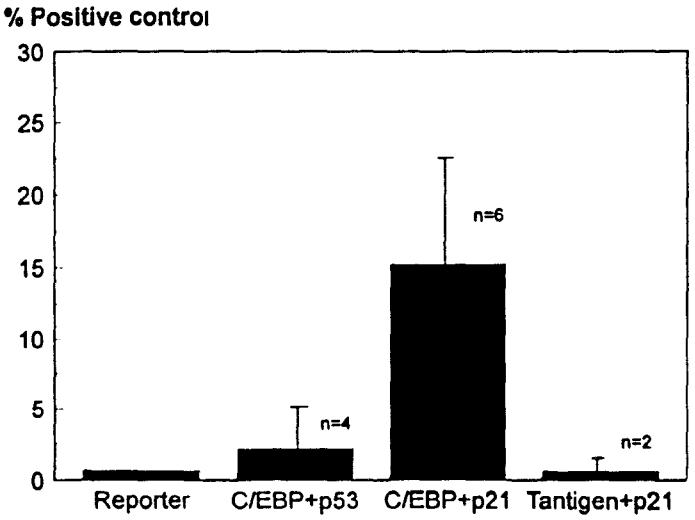
T-ant. + p21
T-ant. + p21

C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25
20
15
10
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C/EBP+p53
C/EBP+p21
Tantigen+p21

Fig. 7.

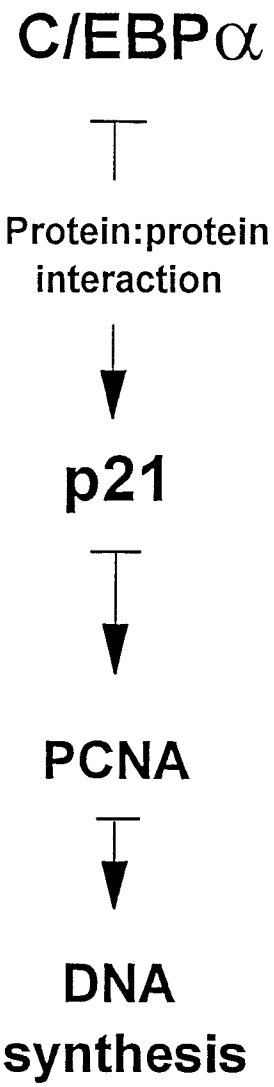

Fig. 8