THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 36, Issue of September 6, pp. 22262–22270, 1996

Printed in U.S.A.

C/EBP-β/LAP Controls Down-regulation of Albumin Gene Transcription during Liver Regeneration*

(Received for publication, January 25, 1996, and in revised form, April 22, 1996)

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Expression of the albumin gene in the liver is controlled by several liver-enriched transcription factors. However, the mechanisms which contribute to its regulation during pathophysiological states, such as liver regeneration, are still little understood. In the present study we found that during liver regeneration down-regulation of albumin mRNA expression is transcriptionally controlled through a minimal element (nucleotide −170 to +22) of the albumin promoter and is observed mainly during the G_1 phase of the cell cycle, while high levels of albumin expression are preserved at later time points. Decreased albumin mRNA levels correlate with a dramatic increase in nuclear expression of C/EBP-β/LAP, a protein known to bind to the D site of the albumin promoter and also to be involved in cell cycle control. In contrast, nuclear expression of other factors such as HNF-1 or C/EBP-α, which also have been shown to transcriptionally control albumin expression, is either unchanged or slightly decreased. We show that pre- and post-translational mechanisms are involved in the higher nuclear expression of C/EBP-β/LAP as early as 1 h after hepatectomy, which also leads to its increased binding toward the D site of the albumin promoter. Finally, *in vitro* transcription assays with liver nuclear extracts and recombinant C/EBP-β/LAP demonstrate that C/EBP-β/LAP can directly down-regulate transcription mediated by the minimal element of the albumin promoter. Additionally the inhibitory role of C/EBP-β/LAP on the albumin minimal promoter could be confirmed by transfection experiments in hepatoma cells. These results indicate that C/EBP-β/LAP, while enhancing transcription of cell cycle-related genes and controlling G_1/S phase checkpoint, down-regulates a major liver function, *i.e.* albumin synthesis, to prepare the hepatocyte for entry into the cell cycle.

Differentiated hepatocytes are important for the maintenance of several vital functions. They regulate carbohydrate and lipid metabolism and detoxification of exogenous and endogenous compounds, and they produce most of the plasma proteins, such as albumin, while resting in the G_0 phase of the cell cycle.

Albumin is a typical liver-specific gene which is important for the transportation of particles through the body and the preservation of serum colloid osmotic pressure in the blood. The albumin gene is highly expressed in the liver after birth, and its regulation has been shown to be transcriptionally controlled (1, 2). Several cis-acting elements in the albumin promoter exist (sites A–F). The B and D sites have been shown to be especially important as liver-specific transcription factors bind to these elements (3). HNF-1 binds to the B site (4, 5). Members of the C/EBP family which belong to the leucine zipper proteins of transcription factors interact at the D site of the albumin promoter (6–8).

Several events can influence the expression of the albumin gene. Under physiological conditions, the extracellular oncotic pressure controls albumin gene expression via HNF-1 (9, 10). During pathologic states, such as the acute phase response of the liver, albumin gene expression is down-regulated (11). In recent years several transcription factors have been cloned which are involved in the regulation of the acute phase genes. Stat 3, also known as APRF (acute phase response factor), has been shown to belong to the family of signal transducer and activators of transcription (12, 13). A second transcription factor involved in the regulation of the acute phase response has been cloned by several groups and named C/EBP-β, IL6-DBP, NF-IL6, LAP, or CRP2 (8, 14–18). It is a member of the C/EBP family and binds like the other members to the D site of the albumin promoter. Transcriptional and post-transcriptional mechanisms are important for the activity of LAP/NF-IL6 during the acute phase response (15). Interestingly, a specific role for C/EBP-β/LAP in cell cycle control has been proposed recently (19, 20).

Liver regeneration occurs after partial destruction or removal of parenchymal liver tissue. DNA synthesis in the parenchymal cells starts 12–16 h after a two-thirds hepatectomy and reaches a peak at around 24 h after surgery (21). Different factors are involved in regulating the transition from resting to dividing cells. However, the actual mechanisms mediating this process remain poorly understood (22). During liver regeneration, activation of several growth regulatory loops between hepatocytes and nonparenchymal cells occurs. While HGF has been shown to stimulate hepatocyte entry into the cell cycle, transforming growth factor-β seems to be involved in regulating the transition period from the proliferate state back to G_0 (22–24).

Reduction of functional liver mass has profound effects on the liver which has to discriminate between functions of immediate significance for survival and resources necessary for liver mass restoration. Thus mechanisms on the molecular level and the regulation of liver-specific transcription factors which are important in mediating the transcriptional control of the albumin gene, the major liver product, may be influenced by these

* This work was supported by Deutsche Forschungsgemeinschaft Grant Tr 285/3-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Part of this work was demonstrated at the AASLD meeting in Chicago, November 3–7, 1995.

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events. We show that down-regulation of albumin mRNA during liver regeneration is transcriptionally controlled and correlates with changes in nuclear expression and binding of C/EBP-β/LAP to the D site of the albumin promoter, a protein known to control cell cycle progression. As these regulatory mechanisms occur before entry into S phase, they could have an implication in G_0–G_1 phase transition by saving resources for progression of hepatocytes through the cell cycle.

MATERIALS AND METHODS

Two-thirds Hepatectomy and Preparation of Nuclear Extracts—6–9-week-old Sprague-Dawley rats were obtained from the animal facility of the Medizinische Hochschule Hannover. The animals were maintained on a 12-h night and 12-h hour light schedule. 12 h before surgery, food was withdrawn from the animals. Surgery was performed between 8 and 10 a.m. Anesthesia of animals was performed by an intraperitoneal injection of a combination of Rompun/ketamine. After a small subxyphoid incision, a two-thirds hepatectomy was performed essentially as described by Higgins *et al.* (25). Following surgery the abdominal cavum was closed by a suture. Sham surgery was performed exactly as indicated with the animals where resection of two-thirds of the liver was performed, except that the liver was only manipulated and not resected.

For each time point indicated, routinely four rats were used in parallel. The remaining livers were removed, pooled, and rinsed in ice-cold phosphate-buffered saline, and part of the livers were immediately frozen in liquid nitrogen. From the rest of the liver, nuclei were prepared essentially as described by Descombes *et al.* (8). All the steps were performed at 4 °C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Nuclear extracts were separated on a 10% SDS-polyacrylamide gel (26) and blotted onto a nitrocellulose membrane (Schleicher & Schuell, FRG) in 1% SDS, 20% methanol, 400 mM glycine, 50 mM Tris-HCl, pH 8.3, at 4 °C for 2 h at 200 mA. C/EBP-α was detected with an antibody purchased from Santa Cruz chemicals, the antibody generated against C/EBP-β was a generous gift from E. Ziff, the antibody against C/EBP-δ from G. Darlinghton, the antibody against cyclin A from G. Draetta, and the antibody against HNF-1 from R. Cortese. The antigen-antibody complexes were visualized using the ECL detection system as recommended by the manufacturer (Amersham Corp., FRG).

Recombinant C/EBP-β/LAP Protein—Recombinant protein was obtained using the pet-vector system for C/EBP-β/LAP as described earlier (27). The plasmid was transformed into the *Escherichia coli* strain BL 21/DE-3/pLys S, and the protein was induced and purified (27).

Gel Retardation Assays—For the gel retardation assays nuclear extracts or recombinant proteins were used as indicated. The binding reaction was performed for 20 min on ice. For binding assays an oligonucleotide spanning the D site of the albumin promoter was used as a ^{32}P-labeled probe. Free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel as described previously (27). For better resolution, some of the gels were run for 4 h at 300 V. "Supershift" experiments were performed with an antibody against C/EBP-α, β/LAP, and C/EBP-δ.

In Vitro Transcription Assays—*In vitro* transcription assays were performed as described by Gorski *et al.* (28). As DNA template we used the G-less cassette containing constructs Alb 400 and AdML 200 G-free which were a generous gift from U. Schibler. Nuclear extracts, template DNA, and recombinant proteins were preincubated on ice for 15 min in a total volume of 17 μl. Addition was made of 3 μl to the reaction mix containing 20 × NTP, RNasin, and 10 μCi of [^{32}P]UTP were added. The transcription was performed for 45 min at 30 °C. The reaction was terminated by adding the stop buffer (250 mM NaCl, 20 mM Tris/HCl, pH = 7.5, 5 mM EDTA, 1% SDS), 2 μl of 10 mg/ml tRNA, and 4 μl of 10 mg/ml proteinase K solution, and incubation was for 30 min at 37 °C. After phenol/chloroform extraction and ethanol precipitation, the RNAs were separated on a 6% polyacrylamide/urea gel.

Northern Blot Analysis—Northern blot analysis was performed as described before, according to standard procedures. Total RNA was isolated by the guanidium isothiocyanate method (29). 15 μg of total RNA were analyzed through a 1% agarose formaldehyde gel, followed by transfer to Hybond N membranes (Amersham). The C/EBP-α, C/EBP-β/LAP, C/EBP-δ, and GAPDH cDNA probes were labeled with [α-^{32}P]ATP according to random priming (Boehringer Mannheim, FRG). The hybridization procedure was performed as described elsewhere (29).

In Situ Hybridization—For the generation of ^{35}S-labeled RNA probes the plasmid pBS-LAP Δ21 (30) was digested with *Nco*I, and the sequences between the second and the third ATG of the LAP open reading frame were subcloned into pBS (LAP 22–152). To generate run-off transcripts of the "antisense" and "sense" strands, respectively, 1 μg of plasmids linearized with either *Hin*dIII or *Eco*RI restriction endonucleases and 10 units of T7 or T3 RNA polymerases were added to a 10-μl reaction mixture containing 100 μCi of ^{35}S-uridine 5′-α-thiotriphosphate (1250 Ci/mmol, Amersham, FRG), 1 mmol/L each adenosine, cytidine, and guanosine 5′-triphosphate, 10 mmol/L dithiothreitol, 25 units of human placental RNase inhibitor, 6 mmol/L $MgCl_2$, 40 mmol/L Tris-HCl, pH 7.5, 2 mmol/L spermidine, 10 mmol/L NaCl, and incubated 1 h at 37 °C, as described previously (31).

In situ hybridization analysis was performed on tissue frozen in liquid nitrogen and stored at −80 °C. Five-μm thick frozen tissue samples were collected onto 3-aminopropyltriethoxysilane-coated slides, air-dried, and fixed in 4% solution of paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) containing 5 mmol/L $MgCl_2$ for 15 min. Prehybridization, hybridization, and washings of slides were performed as described previously (32). Slides were first exposed to Kodak X-Omat AR films (Eastman Kodak Co.) at room temperature for 2–4 days, and then coated with Kodak NTB-2 emulsion and exposed at 4 °C for several weeks, typically 4–6 weeks. After development in Kodak D19 developer and fixation in Kodak Fixer, the slides were counterstained with hematoxylin-eosin, mounted, and viewed under a light microscope in the dark or by bright field.

Cell Culture, Transfection Experiments, and CAT Assays—HepG2 cells (ATCC) were cultured in minimal essential medium supplemented with 10% fetal calf serum. DNA transfection into HepG2 cells was carried out as described previously (30). The reporter construct used in these experiments represents the minimal albumin promoter linked to a CAT[1] reporter gene (ALB-CAT) (9). CAT assays were performed 48 h after transfection using thin layer chromatography for the separation of the reaction products. Quantification of the CAT results was carried out with a Fuji imager (30). Activation of the reporter construct is shown as fold activation compared to the condition when 2 μg of the ALB-CAT were transfected in HepG2 cells alone.

Quantification—Quantification of results was performed with a Fuji imager or by a densitometer as described previously (29, 30).

RESULTS

Transcriptional Control of Albumin mRNA Expression during Liver Regeneration—Following a two-thirds hepatectomy, the remaining liver cells enter the cell cycle, and after 7–14 days the original liver weight is reconstituted (33). Albumin mRNA expression was studied at different time points after hepatectomy and sham surgery by Northern blot analysis. As shown in Fig. 1*A*, a dramatic drop in albumin mRNA expression was detected starting 4 h after hepatectomy. Pretreatment levels were found again after 24 h. In contrast, only a very transient down-regulation was found in control animals following sham surgery (Fig. 1*B*).

Albumin mRNA expression can be regulated at the transcriptional and post-transcriptional level (3, 34). Thus, to study that transcriptional mechanisms may at least be in part involved in the down-regulation of albumin mRNA expression during liver regeneration, "*in vitro* transcription" analysis was performed. Nuclear extracts were tested before surgery and 6 h after hepatectomy. For albumin-specific transcription, a construct was used containing the albumin promoter combined with a G-less cassette which leads to a 400-bp transcription product (28). As an internal control the adenovirus major late (AdML) promoter was added to all the experiments. Fig. 1*C* shows that 6 h after hepatectomy transcription of the albumin promoter is down-regulated while transcription of the AdML promoter is unaffected. These results demonstrated that transcriptional events are crucial for the down-regulation of albumin mRNA expression during liver regeneration, as was also suggested by previous studies (35). However, for the first time these results show that the change in albumin mRNA expression after hepatectomy is mediated by a minimal promoter

[1] The abbreviations used are: CAT, chloramphenicol acetyltransferase; AdML, adenovirus major late.

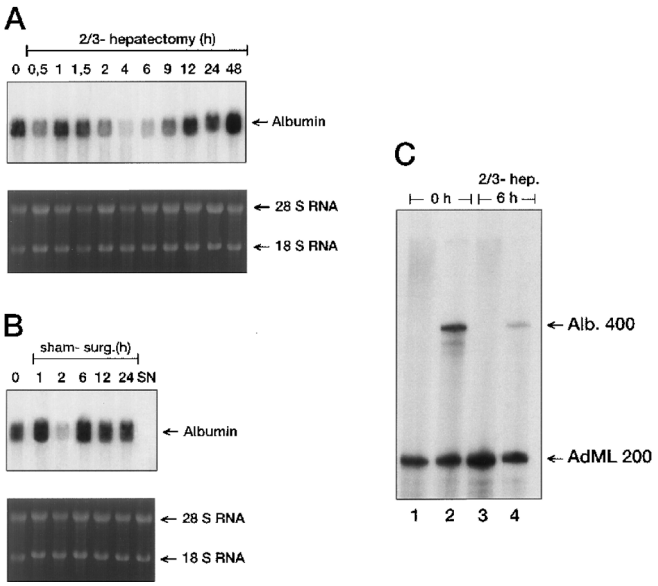

FIG. 1. **Transcriptional control of albumin gene expression during liver regeneration.** *A* and *B*, Northern blot analysis of total RNA (15 μg) at different time points following hepatectomy (*A*) or sham surgery (*B*). Membranes were hybridized with a ^{32}P-labeled cDNA for albumin. Ethidium bromide staining of total RNA was performed to monitor the loading of the gels. *C, in vitro* transcription assays of rat nuclear extracts were performed at time points before hepatectomy (*lanes 1* and *2*) and 6 h after hepatectomy (*lanes 3* and *4*). The construct containing adenovirus major late promoter (*AdML 200*) linked to a G-less cassette of 200 bp was used as a control and added in all incubation mixes. The construct containing the albumin promoter (*Alb. 400*) linked to a G-less cassette of 400 bp was added in *lanes 2* and *4*.

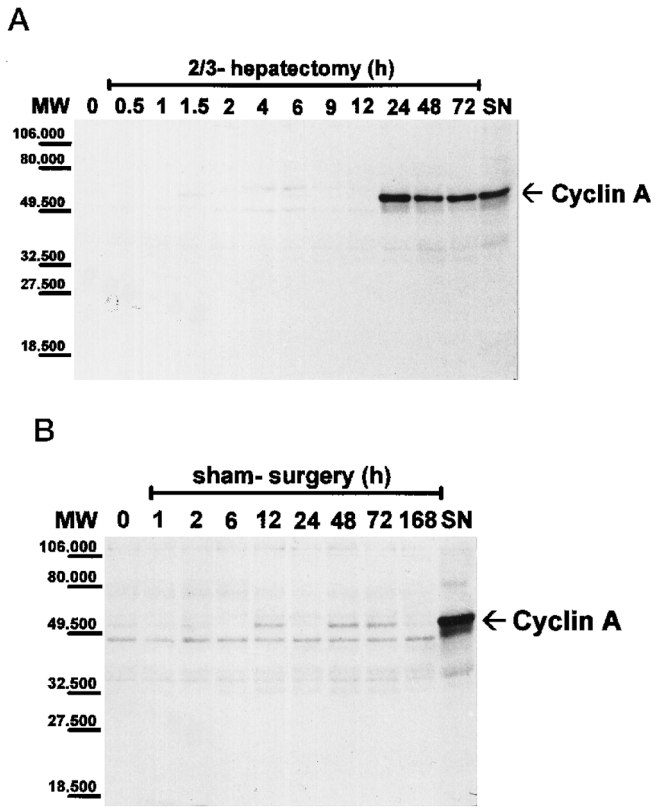

FIG. 2. **Nuclear expression of cyclin A indicates the start of DNA replication.** *A* and *B*, nuclear extracts from different time points following hepatectomy (*A*) and sham surgery (*B*) were separated by SDS-gel and blotted onto nitrocellulose. Expression of cyclin A was analyzed by anti-cyclin A antibody. Spleen nuclear extracts (*SN*) were used as a control.

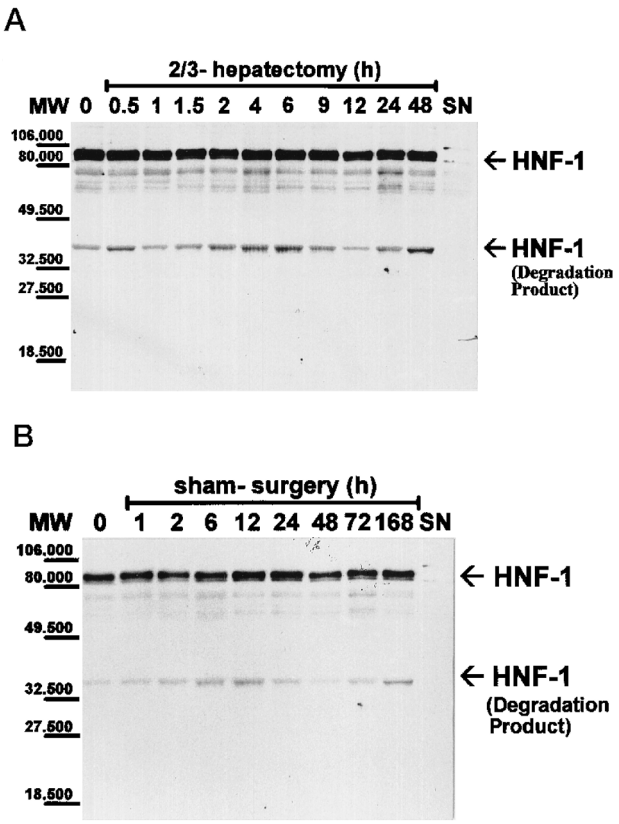

FIG. 3. **Nuclear expression of HNF-1, the major protein binding to the B site of the albumin promoter.** Nuclear extracts at different time points following hepatectomy (*A*) and sham surgery (*B*) were separated on a SDS-gel and blotted onto nitrocellulose. Spleen nuclear extracts were used as a control. HNF-1 was detected with an anti-HNF-1 antibody. The positions of HNF-1 and its degradation product are marked.

region of the albumin gene located between nucleotide −170 to +22.

Further experiments were performed to determine the time period of albumin down-regulation during cell cycle progression post-hepatectomy. Cyclin A is highly expressed in the nucleus during S phase, because the protein is required for G_1/S phase transition and control of S phase (36). Western blot analysis was performed at different time points after hepatectomy with an anti-cyclin A antibody. High nuclear expression of cyclin A started 24 h after hepatectomy, while nuclear cyclin A expression remained low after sham surgery (Fig. 2*B*). High nuclear expression of cyclin A was also evident in replicating splenocytes (Fig. 2, *A* and *B*).

Expression of Proteins Controlling Albumin Transcription during Liver Regeneration—After the results of the *in vitro* transcription analysis, which suggested that factors binding to the albumin promoter may control down-regulation of the gene post-hepatectomy, expression of the known transcription factors which bind to the albumin promoter were studied. HNF-1 binds to the B site and leucine zipper proteins of the C/EBP family namely C/EBP-α and C/EBP-β/LAP bind to the D site (5, 8). Time-dependent expression of HNF-1α and C/EBP family members was studied by Western blot analysis of nuclear extracts.

HNF-1α is strongly expressed before and during liver regeneration. In fact no difference in its nuclear expression could be detected after two-thirds hepatectomy or sham surgery (Fig. 3, *A* and *B*). C/EBP-α shows moderate down-regulation starting as early as 2 h after hepatectomy, while the 30 kDa C/EBP-α inhibitory protein was up-regulated. Normal C/EBP-α levels

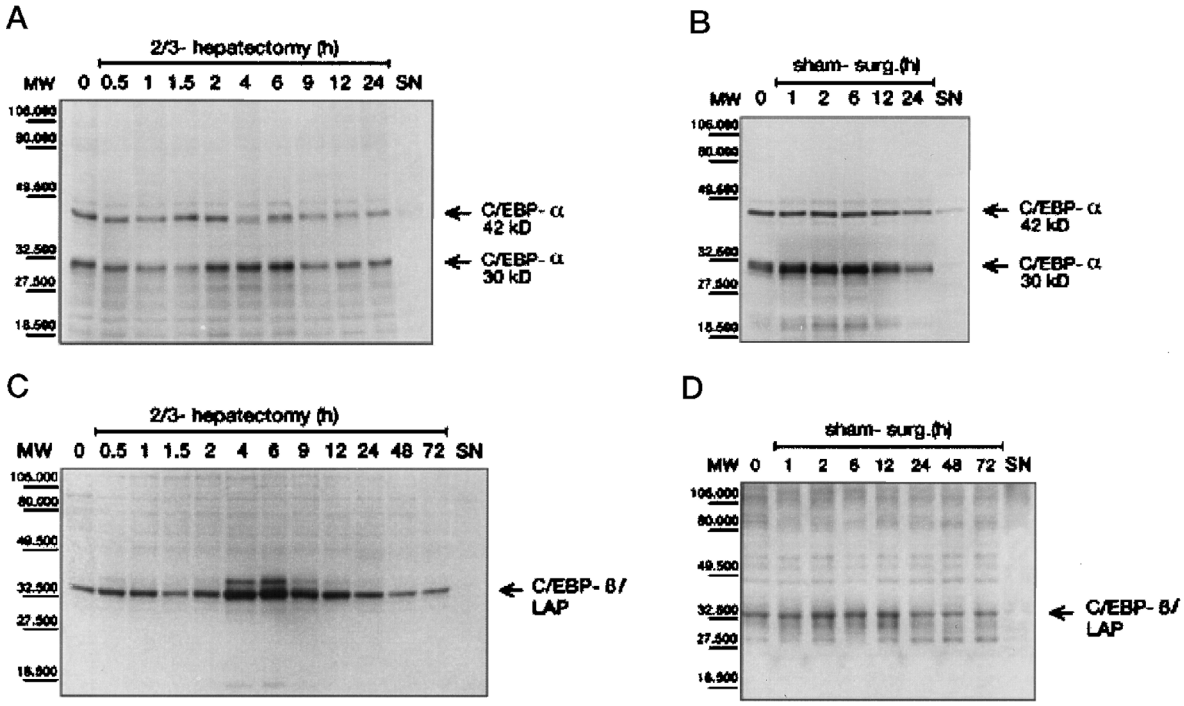

FIG. 4. **Nuclear expression of C/EBP-α and C/EBP-β/LAP, major binding proteins to the D site of the albumin promoter.** *A–D*, Nuclear extracts at different time points following hepatectomy (*A* and *C*) and sham surgery (*B* and *D*) were separated with a SDS-gel and blotted onto nitrocellulose. Spleen nuclear extracts (*SN*) were used as a control. C/EBP-α and C/EBP-β/LAP were analyzed by anti-C/EBP-α (*A* and *B*) and anti-C/EBP-β/LAP. The positions of C/EBP-α and C/EBP-β/LAP are indicated.

are reconstituted 12–24 h post-hepatectomy (Fig. 4*A*). In contrast, a more dramatic regulation was found for C/EBP-β/LAP. A more than 10-fold increase was apparent 4–6 h after hepatectomy (Fig. 4*C*). Additionally, a slower migrating band appeared at these time points and the band was especially prominent after 6 h. Comparison with other time points demonstrated that the ratio changes toward the slower migrating band. Pretreatment levels were found 24 h after hepatectomy. Loading of the gels was checked by Coomassie Blue staining which was performed in parallel to the Western blot analysis (data not shown). In contrast to hepatectomy, control rats showed no change in C/EBP-α expression and only a mild increase in C/EBP-β/LAP expression 2 h after surgery (Fig. 4, *B* and *D*).

Additionally, C/EBP-δ was studied by Western blot analysis. Neither before nor after surgery were specific signals detected using an anti-C/EBP-δ antibody (data not shown), indicating that C/EBP-δ was not induced in the liver following hepatectomy or sham surgery. Therefore, results of Western blot analysis suggest that decreased albumin transcription found during liver regeneration correlates best with changes in the nuclear expression of C/EBP-β/LAP.

C/EBP-α and C/EBP-β/LAP mRNA during Liver Regeneration—Earlier results showed that post-transcriptional mechanisms modulate the activity of C/EBP-β/LAP by intracellular pathways (37–39). In contrast, little is known about how C/EBP mRNA accumulation is regulated in the liver. Thus C/EBP-α and C/EBP-β/LAP mRNA expression was studied by Northern blot analysis. In parallel to the protein level, the signal for C/EBP-α slightly decreased following hepatectomy. After 2 h, 25% of the pretreatment level was found by Northern blot analysis (Fig. 5*A*). The time kinetics between C/EBP-α mRNA and protein expression indicates that they are directly linked to each other. Only moderate regulation was observed in control animals (Fig. 5*B*).

A constant increase of C/EBP-β/LAP mRNA was noticed starting immediately after hepatectomy and peak expression

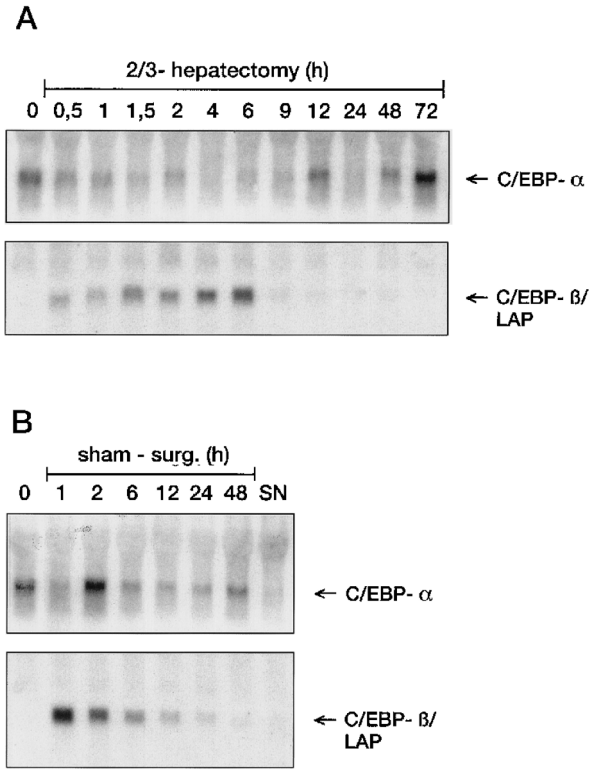

FIG. 5. **Northern blot analysis of C/EBP-α and C/EBP-β/LAP mRNA.** *A* and *B*, Northern blot analysis of total RNA (15 μg) at different time points following hepatectomy (*A*) or sham surgery (*B*). Membranes were probed with a ^{32}P-labeled cDNA for either C/EBP-α or C/EBP-β. The transcripts of C/EBP-α and C/EBP-β are marked.

was found 6 h after surgery (Fig. 5*A*). At later time points, mRNA levels dropped dramatically and returned to pretreatment values after 9 h. In control animals peak expression of

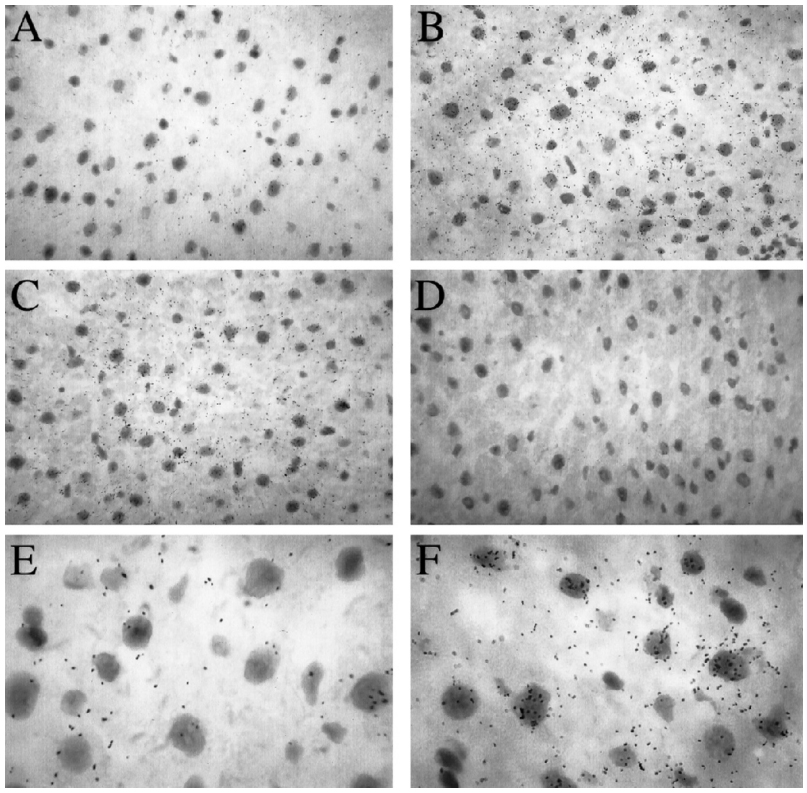

FIG. 6. ***In situ* hybridization for C/EBP-β/LAP transcripts.** *A–E*, localization of C/EBP-β/LAP transcripts in rats after partial hepatectomy or sham surgery. Liver tissue sections from control (*A* and *E*), 6 h after hepatectomy (*B*, *D*, and *F*) or 6 h after sham operation (*C*) were processed for *in situ* hybridization analysis with specific C/EBP-β/LAP ^{35}S-"antisense" (*A–C*, *E*, and *F*) or "sense" (*D*) cRNA probes synthesized *in vitro* using T7 or T3 RNA polymerases, respectively. The bright field microphotographs show higher accumulation of silver grains onto parenchymal cells throughout the lobule after hepatectomy (*B*) as compared to control liver (*A*), with minor accumulation of transcripts in the sham operated rat (*C*). No specific hybridization signal was detected with the sense probe on a serial section (*D*). Expression of C/EBP-β/LAP was confined to hepatocytes since no appreciable signal over the background level was found onto nonparenchymal cells (*F*). Magnification: *A–D*, × 628; *E* and *F*, × 1198.

C/EBP-β/LAP mRNA was evident as early as 1 h after sham surgery, and C/EBP-β/LAP mRNA constantly decreased thereafter, showing the opposite kinetics compared to the animals after hepatectomy (Fig. 5, *A* and *B*). Therefore we wondered whether the different kinetics in C/EBP-β/LAP mRNA expression between hepatectomy and sham surgery could be explained due to differences in its lobular distribution pattern. *In situ* hybridization studies with specific cRNA probes were performed to detect mRNA transcripts of C/EBP-β/LAP mRNA in different parts of the liver lobule. Fig. 6 shows that following hepatectomy a homogeneous distribution of hybridization signal for C/EBP-β/LAP mRNA can be found in hepatocytes throughout the liver lobule. Yet at 6 h after surgery the signal is dramatically enhanced as compared to that found for sham surgery, thus confirming the results of Northern blot analysis. Therefore in these animals, stimuli appear to activate C/EBP-β/LAP expression in hepatocytes regardless of their lobular position. No specific signal over background level for C/EBP-β/LAP was detected when the experiments were performed with the sense probe (Fig. 6*D*). Moreover, Fig. 6*F* shows that high amounts of C/EBP-β/LAP transcripts are specifically found in liver parenchymal cells.

Binding to the D Site of the Albumin Promoter during Liver Regeneration—We showed that differences in the nuclear expression of C/EBP-α and C/EBP-β do occur during liver regeneration, therefore we analyzed whether these changes affect DNA binding. Gel shift experiments were performed using the D site of the albumin promoter as a labeled probe. In order to detect minor differences in DNA binding, increasing amounts of nuclear extracts (obtained before surgery) were added to the incubation mix. All further experiments were performed with concentrations of nuclear extracts, where approximately 10% of the labeled probe is bound to proteins (see *lane 4* in Fig. 7*A*). The amount was equivalent to 1 μg of nuclear extract. C/EBP family members were identified by "supershift" experiments. Antibodies for C/EBP-α, C/EBP-β, and C/EBP-δ were added to the incubation mix. As shown in Fig. 7, *B* and *C*, specific bands corresponding to C/EBP-α and C/EBP-β/LAP were recognized and "supershifted." Other bands showed a light decrease in intensity which might be explained by heterodimer formation between C/EBP family members. Supershift experiments performed with antibodies for C/EBP-δ (Fig. 7*B*) indicated that C/EBP-δ is not present in nuclear extracts of Sprague-Dawley rats, thus confirming the results obtained by Western blot analysis.

Fig. 8*A* shows the time-dependent binding of nuclear extracts to the D site of the albumin promoter following hepatectomy. Increased binding of C/EBP-β/LAP starts as early as 0.5 h after surgery, and the strongest affinity toward the cognate DNA was found 4–6 h after hepatectomy. In contrast to the C/EBP-β/LAP band before surgery (*lane 1*) binding dramatically increased 4 and 6 h post-hepatectomy (*lanes 6* and *7*). This is especially evident in comparison to the three main bands which are unchanged after hepatectomy. After 4–6 h the intensity of the C/EBP-β/LAP band is comparable to these three bands, while before surgery the band is faint. In the nuclear extracts of rats which underwent sham surgery, only minor differences in binding of C/EBP-β/LAP to the D site of the albumin promoter were detected. This was also found at early time points when C/EBP-β/LAP mRNA was high. Changes in DNA-binding of C/EBP-α were less evident. Decreased DNA binding is found at 1 and 1.5 h post-hepatectomy. Later time points showed only minor changes compared to the binding affinity before surgery. These results indicate that the effect on DNA binding is especially prominent for C/EBP-β/LAP, while only minor differences can be detected for C/EBP-α.

C/EBP-β/LAP Down-regulates Transcription from the Albumin Promoter—We demonstrated that the decrease in albumin mRNA expression after hepatectomy is transcriptionally controlled through the albumin minimal promoter element. This drop of albumin gene expression is concomitant with an increase of C/EBP-β/LAP in its nuclear expression and its affinity toward the cognate DNA. Therefore we were interested to verify whether changes of C/EBP-β/LAP expression might di-

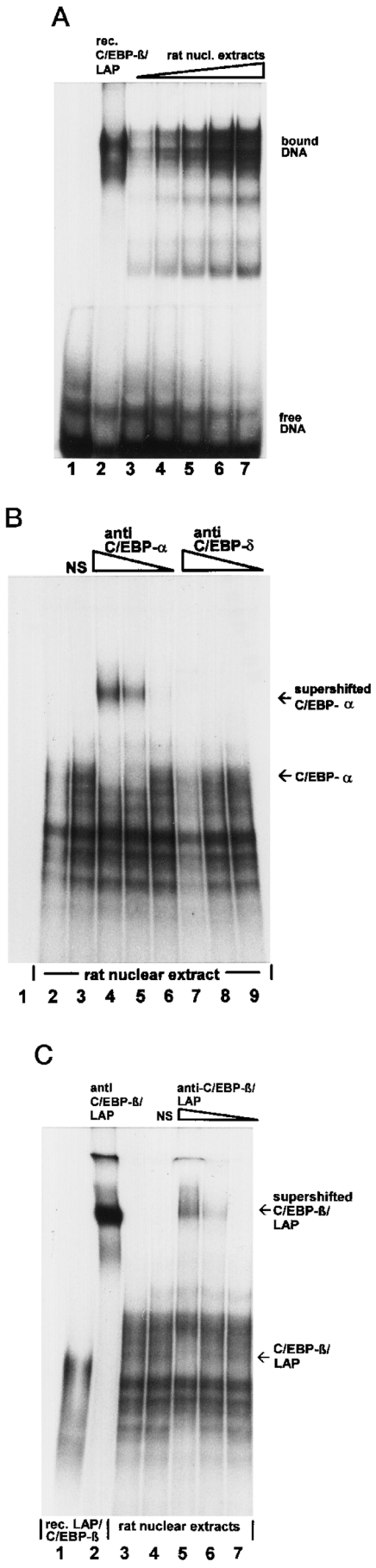

FIG. 7. **Identification of proteins binding to the D site of the albumin promoter.** Rat nuclear extracts before surgery or recombinant C/EBP-β/LAP were used for gel shift experiments. The nuclear proteins or recombinant protein were incubated for 15 min with the ^{32}P-labeled DNA representing the D site of the albumin promoter (25 pg) at 4 °C. The DNA-protein complexes were resolved on a 6% nondenaturating polyacrylamide gel. *A*, increasing amounts of nuclear extracts were added to the incubation mix. Further gel shift experiments were performed with the amount of nuclear extracts (1 μg) where 10% of the probe was bound to DNA. The positions of the free and bound DNA are indicated. *B* and *C*, specific bands corresponding to bound C/EBP-α and C/EBP-β were detected by adding increasing amounts of anti-C/EBP-a or anti-C/EBP-β/LAP antibodies to the incubation mix. No specific band was shifted with a specific antibody directed against C/EBP-δ. To get a better resolution of the different proteins binding to the D site of the albumin promoter, the gel was run at 300 V for 5 h. Therefore the free probe was run off the bottom of the gel. Recombinant C/EBP-β/LAP was used as a positive control.

rectly influence the transcriptional activity of the albumin promoter. The basal activity of the albumin promoter is known to be high in rat nuclear extracts, because several transcription factors are present and bind to the minimal promoter region (4–8). In contrast the activity is low in spleen nuclear extracts because liver-specific transcription factor like HNF-1 and C/EBP-α are missing. These two different assumptions offer the chance to study the effect of C/EBP-β/LAP on albumin transcription dependent on the initial activity of the promoter.

First, recombinant C/EBP-β/LAP was added to spleen nuclear extracts where the untreated activity of the albumin promoter is low (Fig. 9*A*). Small amounts of C/EBP-β/LAP increased albumin transcription, while higher amounts down-regulate promoter activity. In contrast, different kinetics was observed when C/EBP-β/LAP was added to liver nuclear extract, when the basal activity of the albumin promoter is high. Increasing amounts of C/EBP-β/LAP directly decreased transcription of the albumin promoter (Fig. 9*B*) mimicking the *in vivo* results as found after hepatectomy (compare Fig. 1*C*).

To further confirm that C/EBP-β/LAP may act as a repressor and activator of albumin gene transcription, transient transfection experiments were performed. Increasing amounts of C/EBP-β/LAP were transfected with a CAT-reporter construct linked to the minimal albumin promoter (ALB-CAT) (7). Transfection of the ALB-CAT alone lead only to a minor activation of the CAT reporter gene. However, cotransfection of 100 ng of the C/EBP-β/LAP expression vector resulted in a more than 10-fold increase in CAT reporter activity (Fig. 9*C*). Interestingly, a further increase of the C/EBP-β/LAP expression vector led to dose-dependent down-regulation of the ALB-CAT activity compared to the condition when 100 ng were used (Fig. 9*C*).

Next, experiments were designed to investigate the role of C/EBP-β/LAP on albumin transcription, when the initial activity of the promoter is high. Therefore increasing amounts of C/EBP-α were cotransfected with the ALB-CAT. An amount of 500 μg of C/EBP-α expression vector led to more than 10-fold activation of the ALB-CAT activity (Fig. 9*D*). This condition was used to study the effect of C/EBP-β/LAP on albumin gene transcription. As shown before by *in vitro* transcription analysis and in cell culture experiments, increasing amounts of C/EBP-β/LAP led to a direct and constant decrease of albumin gene transcription from the minimal albumin promoter. Thus *in vitro* transcription analysis and cell culture experiments conclusively offer an experimental explanation for a direct role of C/EBP-β/LAP in down-regulating albumin gene transcription. Therefore these results suggest that C/EBP-β/LAP may also be involved in the down-regulation of albumin gene transcription after two-thirds hepatectomy.

DISCUSSION

Following hepatectomy, quiescent hepatocytes enter the cell cycle and proliferate to restore the original liver mass. Stimu-

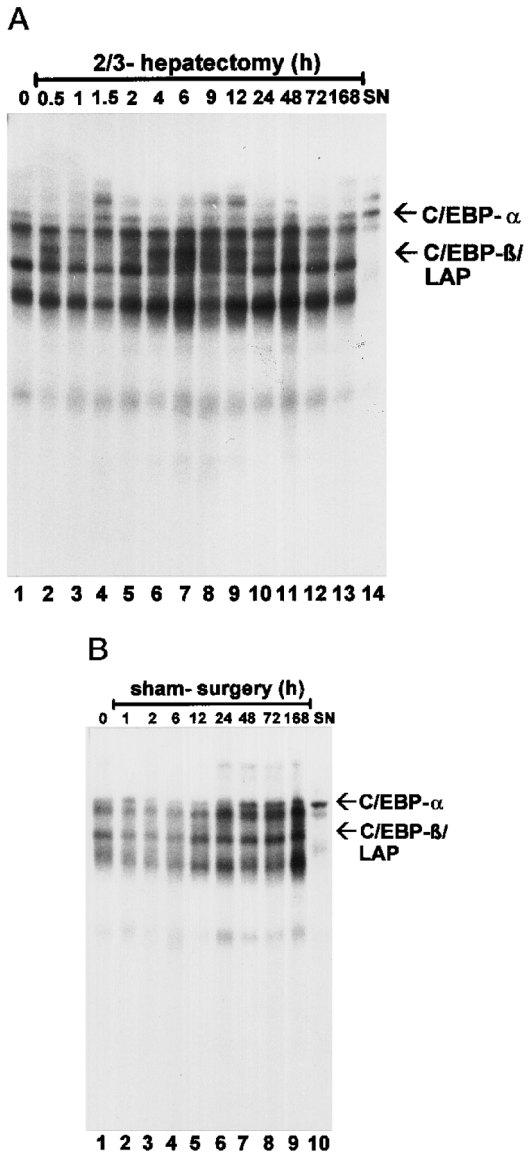

FIG. 8. **Binding to the D site of the albumin promoter during liver regeneration.** *A* and *B*, binding of rat nuclear extracts after two-thirds hepatectomy (*A*) and sham surgery (*B*) toward the D site of the albumin promoter was evaluated by gel shift experiments. 1 μg of rat nuclear extracts was used to study the time kinetic at each time point. The nuclear proteins were incubated for 15 min with the ^{32}P-labeled DNA representing the D site of the albumin promoter (25 pg) at 4 °C. The DNA-protein complexes were resolved on a 6% nondenaturating polyacrylamide gel. The bands which were supershifted due to the incubation with antibodies directed against C/EBP-α and C/EBP-β are indicated.

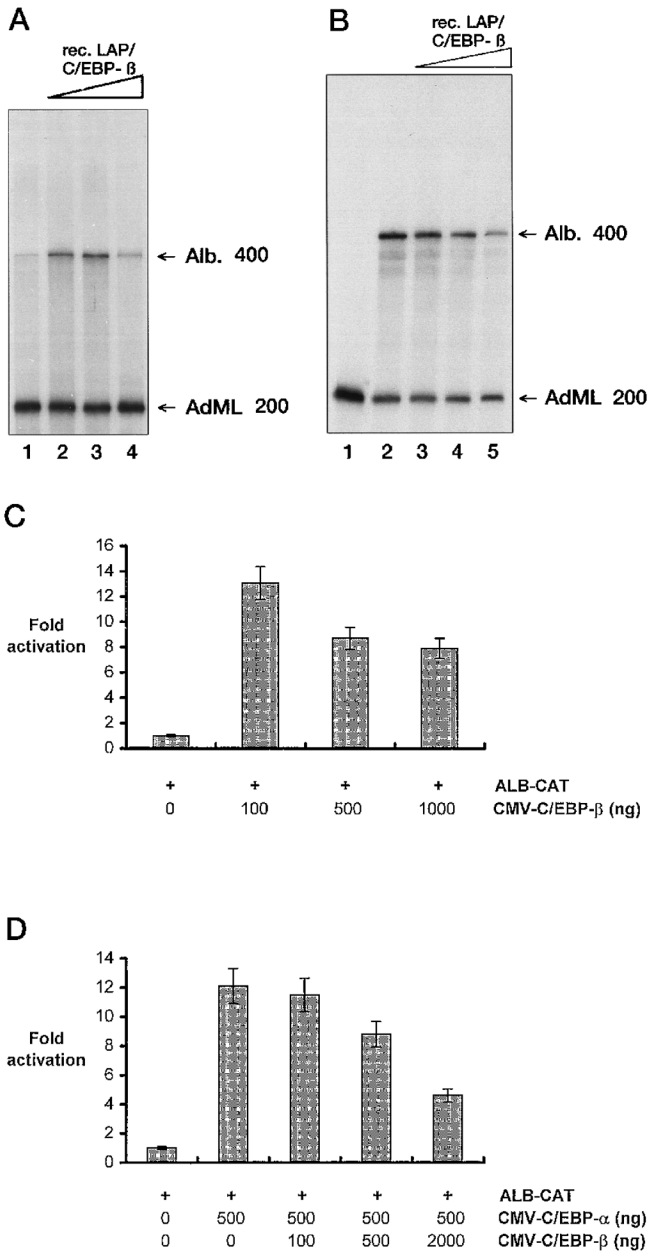

FIG. 9. **C/EBP-β/LAP down-regulates albumin gene transcription through the minimal promoter element.** *A* and *B*, *in vitro* transcription analysis was performed with spleen nuclear extracts (*A*) or rat nuclear extracts (*B*). The adenovirus major late promoter (*AdML 200*) construct was added in all incubation mixes as a control. The construct containing the albumin promoter (*Alb. 400*) was added in *lanes 2–5*. Increasing amounts of recombinant C/EBP-β/LAP were added in *lanes 3–5*. *C* and *D*, transfection experiments in HepG2 cells using the ALB-CAT as a reporter construct. Cotransfection experiments were performed with cytomegalovirus expression vectors for C/EBP-β/LAP and C/EBP-α. The concentrations of the plasmids were used as indicated. 2 μg of reporter construct were used in each experiment.

latory and inhibitory growth factors such as HGF and transforming growth factor-β mediate different regulatory loops from the cell surface to the nucleus and lead to the return from a proliferating to a resting state of the hepatocyte (22). The interest of this study was to investigate transcriptional mechanisms which are involved in regulating albumin gene expression during this process. Albumin mRNA is dramatically down-regulated in the first 24 h after hepatectomy but not at later time points, and the decrease is at least in part transcriptionally controlled as shown by *in vitro* transcription assay. HNF-1, one of the main transactivator of albumin gene transcription (3), does not contribute to the decrease in albumin expression as high nuclear expression remains unchanged after hepatectomy and no post-transcriptional mechanisms are known which may alter its DNA binding or transactivating function. Our data indicate that C/EBP-β/LAP, which binds to the D site of the albumin promoter *in vivo* and which is able to directly decrease its activity *in vitro*, is directly involved in down-regulating albumin gene transcription after partial hepatectomy. These events occur before transition into S phase and start of DNA synthesis.

Earlier results showed down-regulation of albumin gene expression during liver regeneration (35). In the later study, however, albumin gene expression was assayed by the "run-on" technique which does not allow direct evaluation of "*de novo*" RNA synthesis by a given promoter upon nuclear-protein bind-

ing and transcription machinery assembly. Therefore we performed *in vitro* transcription analysis and demonstrated that down-regulation of albumin mRNA post-hepatectomy depends on a minimal region (nucleotide −170 to +22) of the albumin promoter. Another study by Mueller *et al.* (7) did not observe any binding to the D site of the albumin promoter during liver regeneration after carbon tetrachloride intoxication. The discrepancy between this study and Mueller's report might be explained by the difference in the experimental systems. In fact, carbon tetrachloride leads to dramatic damage of the liver with prominent necroinflammatory phenomena and different timing of cell cycle progression (35). This may explain the total loss of nuclear protein binding toward the D site found in Mueller's study.

Before hepatocytes enter S phase, distinct genes have been shown to be up-regulated. This time period is especially important as a stepwise system of transcription factors exists which becomes activated following induction of hepatic regeneration. In an early "priming phase" immediate-early genes such as c-*fos*, c-*jun*, and c-*myc* and NF-κB-related proteins show elevated activity in the nucleus (40–42). This time period starts directly after liver resection. Peak expression of some of these genes is found as early as 10 min post-hepatectomy. The expression pattern in hepatocytes is very specific, and the assembly of these proteins most likely triggers G_0 escape (40, 43, 44). The period following the "priming phase," called the "progression phase," is marked by an increase of "delayed early genes" such as p53 (45, 46). During this time period C/EBP-β/LAP shows a maximal up-regulation on the protein and mRNA level.

Interestingly, many of the proteins which are involved in the "early priming phase" and in the later "progression phase" are strongly regulated by phosphorylation, and extracellular signals modulate the activity or the half-life of these factors (47). C/EBP-β/LAP is the target of several intracellular pathways (37–39). During liver regeneration C/EBP-β/LAP expression is strongly enhanced as shown by Western blot analysis, and a second slower migrating band appears which is most prominent after 4–9 h. This band is thought to be the result of a stronger phosphorylation of the protein (8).

Similar results in the timing and regulation of the C/EBP-β/LAP protein after hepatectomy was very recently shown by Greenbaum *et al.* (48) Additionally we show that post-hepatectomy this is due to increased C/EBP-β/LAP mRNA expression. Moreover in our study high mRNA expression was found after sham surgery with different kinetics compared to hepatectomy. Differences in the lobular distribution of C/EBP-β/LAP mRNA between sham surgery and hepatectomy, as investigated by *in situ* hybridization analysis, were not detected. Therefore an additional post-transcriptional mechanism is responsible for the higher expression of C/EBP-β/LAP protein after hepatectomy as compared to sham surgery. Thus, besides an acute phase-related activation of C/EBP-β/LAP (14, 15, 49), which is most likely responsible for higher mRNA expression after sham surgery, a post-translational pathways more strictly related to cell cycle control appear to regulate C/EBP-β/LAP expression after hepatectomy.

In fact, besides the acute phase genes, protooncogenes such as c-*fos* have binding motifs in their promoter and are induced by C/EBP-β/LAP (50). At present, it is unclear which signal(s) triggers C/EBP-β/LAP expression during liver regeneration, and the target genes which might be controlled for cell cycle progression are still little defined. Further studies with knock out mice or delivery systems which overexpress or down-regulate C/EBP-β/LAP during liver regeneration may help to define and understand the direct role of C/EBP-β/LAP on hepatocyte proliferation.

Therefore, during liver regeneration higher expression of C/EBP-β/LAP may have two different effects, it is involved in down-regulating albumin gene transcription, the major product of liver function, thus saving energy for cell division and liver mass restoration, and controls cell cycle progression by regulating S phase entry and expression of protooncogenes. Further cell cycle progression especially at the G_1/S phase check point is accompanied by a drop in the nuclear expression of C/EBP-β/LAP. Obviously, high C/EBP-β/LAP levels are not required when DNA starts to replicate. In fact down-regulation of C/EBP-β/LAP at this time point of the cell cycle may even be important during hepatocyte proliferation. Earlier results in cell culture systems by Buck *et al.* (19) and Barone *et al.* (20) show that high expression of C/EBP-β/LAP leads to growth arrest at the G_1/S phase check point.

Elegant studies by Das *et al.* (51) may explain why increased binding of C/EBP-β/LAP down-regulates the albumin gene. They demonstrated that differences in the arrangement around the TATA box or proximal sequence elements of a specific promoter lead to a specific assembly of the basal promoter elements. These specific arrangements account for the sensitivity toward a specific transactivation domain. Dependent upon the assembly of the basal machinery the interaction with a specific transactivation domain may have a positive or a negative effect on the specific promoter. Differences in the regulation of two genes in the same cell with binding sites for the same transcription factor could have their origin in this mechanism (50). These results could be important for the acute phase response in the liver or during liver regeneration. Basal promoter assembly, for instance of the C-reactive protein promoter, might be very sensitive for an up-regulation by C/EBP-β/LAP, while the opposite is true for the albumin promoter. Better understanding of sequence motifs which determine the assembly of the basal machinery at the albumin promoter and a better knowledge of the interaction between C/EBP-β/LAP and the basal machinery (30, 52, 53) could trace the molecular mechanism which is responsible for decreased transcription of the albumin gene following two-thirds hepatectomy. Results of Ossipow *et al.* (54) indicate that recombinant C/EBP-β/LAP cannot interact with the basal machinery when bound at the albumin promoter. Therefore their model could suggest that the post-hepatectomy increased binding of C/EBP-β/LAP interferes with maximal activation of the albumin gene, because it displaces C/EBP-α from binding to the D site of the albumin promoter.

Acknowledgment—We thank U. Schibler for his advice on the *in vitro* transcription assays and F. Horn, P. Straub, and U. Rüther for helpful discussions.

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