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CREB Controls LAP/C/EBPβ Transcription

MONIKA NIEHOF, MICHAEL P. MANNS, AND CHRISTIAN TRAUTWEIN*

Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover, Hannover, Germany

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LAP/C/EBPβ is a member of the C/EBP family of transcription factors and is involved in hepatocyte-specific gene expression. Recently we showed that, besides its posttranscriptional regulation, LAP/C/EBPβ mRNA is modulated during liver regeneration. Therefore, in this study we investigated mechanisms which control LAP/C/EBPβ gene transcription. Deletion analysis of the 5′-flanking region, located upstream of the start site of transcription in the LAP/C/EBPβ gene, demonstrated that a small region in close proximity to the TATA box is important in maintaining a high level of transcription of the luciferase reporter gene constructs. In gel shift experiments two sites were identified which are important for specific complex formation within this region. Further analysis by cross-linking, super shift, and competition experiments was performed with liver cell nuclear extracts, hepatoma cell nuclear extracts, or recombinant CREB protein. These experiments conclusively demonstrated that CREB binds to both sites in the LAP/C/EBPβ promoter with an affinity similar to that with the CREB consensus sequence. Transfection experiments with promoter constructs where the CREB sites were mutated showed that these sites are important to maintain both basal promoter activity and LAP/C/EBPβ inducibility through CREB. Northern blot analysis and runoff transcription assays demonstrated that the protein kinase A pathway not only stimulated the activity of the luciferase reporter construct but also the transcription of the endogenous LAP/C/EBPβ gene in different cell types. Western blot analysis of rat liver cell nuclear extracts and runoff transcription assays of rat liver cell nuclei after two-thirds hepatectomy showed a functional link between the induction of CREB phosphorylation and LAP/C/EBPβ mRNA transcription during liver regeneration. These results demonstrate that the two CREB sites are important to control LAP/C/EBPβ transcription in vivo. As several pathways control CREB phosphorylation, our results provide evidence for the transcriptional regulation of LAP/C/EBPβ via CREB under different physiological conditions.

Tissue-specific gene expression is frequently organized by a set of transcription factors which are expressed in a certain combination in differentiated cells. These transcription factors bind to different regulatory sites within the promoter or in more distant regions of a given gene. Interaction of these transcription factors with the RNA polymerase activates gene transcription in a cell-specific manner, and thus, these genes are considered tissue-specific. In contrast, tissue-specific expression of transcription factors is less frequently observed. Many of the transcription factors which contribute to the expression of specific genes in differentiated cells are found in more than one tissue.

C/EBP proteins belong to the leucine zipper family of transcription factors from which several members have been characterized (5, 46). LAP/C/EBPβ was first described by several groups (5, 9) and has also been named NF-IL6 (1), IL6-DBP (31), AGP/EBP (7), and CRP2 (46). The protein was linked to hepatocyte-specific gene regulation, because it shows high expression in liver cells (9) and binds to several control elements of liver-specific genes (10). Additionally, it was suggested that LAP/C/EBPβ contributes to regulating the acute-phase response of the liver (1, 31). Meanwhile, it has become clear that LAP/C/EBPβ also plays a role in other tissues. Results derived from experiments in cell culture and with knockout mice demonstrated that LAP/C/EBPβ is important in the process of lymphocyte (17, 27, 34, 37) and adipocyte (5, 48, 50) differentiation and that it is also highly expressed in neuronal tissue (9).

Posttranscriptional modification, i.e., phosphorylation, is important in changing the activity of LAP/C/EBPβ (26, 28, 39, 45). Inhibitory parts in the protein block the activation domain and the DNA-binding domain by intramolecular protein interaction. The inactivated state can be reverted by phosphorylation, which leads to inhibition of interprotein binding and thus activates the protein by changing its tertiary structure (18, 47). Consequently, LAP/C/EBPβ is more active in enhancing transcription of its target genes.

In recent experiments it became evident that several events, during liver regeneration (42) and during adipocyte differentiation (5, 48, 50), can modulate the mRNA level of LAP/C/EBPβ. These results suggested that, besides the posttranslational pathways, additional mechanisms exist which control the expression of LAP/C/EBPβ. Therefore, we became interested in investigating the transcriptional control of the LAP/C/EBPβ gene itself.

Deletion studies with a region located upstream of the transcriptional start site were performed by the use of luciferase reporter gene assays. These studies demonstrated that LAP/C/EBPβ expression is directly linked to a small region in its promoter located 60 to 120 bp upstream of the start site of transcription. Interestingly, the rate of LAP/C/EBPβ transcription can be modulated by extracellular pathways through these promoter elements. Therefore, we provide direct evidence that LAP/C/EBPβ, besides its posttranslational regulation, can be controlled by extracellular pathways at a transcriptional level.

MATERIALS AND METHODS

Cell culture, transfection experiments, and luciferase assays. HepG2 cells (American Type Culture Collection [ATCC]), NIH 3T3 cells (ATCC), and Neuro 217 cells (kindly provided by M. Wegner) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). DNA transfection was performed by a modified calcium phosphate precipitation method as described previously (40). HepG2 or Neuro 217 cells were grown on 60-mm dishes to about 50% confluence when used for transfection experiments. The amount of reporter and expression vectors is indicated in the figure legends.

* Corresponding author. Phone: 49-511-532-3865. Fax: 49-511-532-4896.

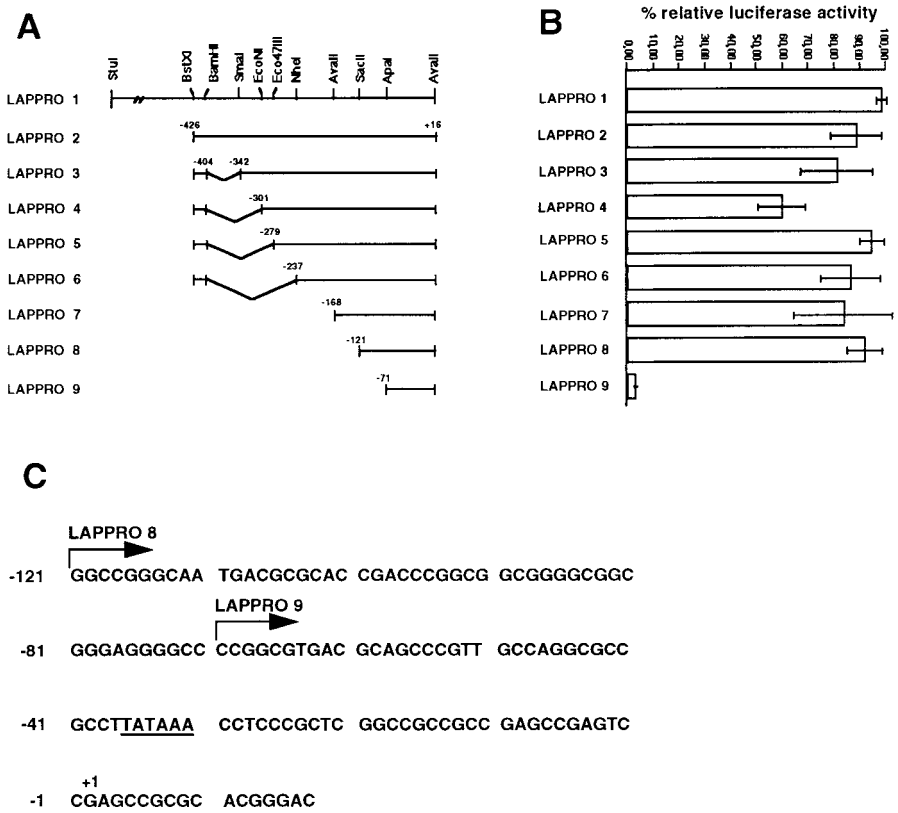

FIG. 1. LAP/C/EBPβ transcription is mediated by a region in proximity to the TATA box. (A) Increasing deletions were introduced in the 5′-flanking region of the LAP/C/EBPβ open reading frame. LAPPRO 1 corresponds to the whole 1.4-kb 5′ region located between the *Stu*I and *Ava*II sites. The *Ava*II site is located 16 bp downstream of the start site of transcription in the LAP/C/EBPβ gene. Restriction sites in the 1.4-kb fragment were used to create increasing 5′-terminal deletions as indicated (LAPPRO 2 to 9). All fragments were linked to a luciferase reporter gene. (B) Transfection experiments with the deletion constructs of the 5′-flanking region of the LAP/C/EBPβ gene. HepG2 cells were transfected with 2 μg of the respective LAPPRO 1 to 9 luciferase reporter constructs. Cells were harvested 48 h after transfection, and the luciferase activity was measured. Error bars indicate standard deviations. (C) Nucleotide sequence of the LAPPRO 8 fragment (first arrow) corresponding to −121 to +16 of the 5′-flanking region of the LAP/C/EBPβ gene. The start of the LAPPRO 9 fragment at nucleotide −71 is marked with an arrow. The TATA box is underlined. The transcription start site is indicated as +1.

The total amount of DNA was kept constant in each transfection experiment by adding pBSK$^+$ DNA (Stratagene) to a final mass of 6 μg. All transfections contained 0.2 μg of the β-galactosidase reporter pRSVβGal as an internal standard. Forty-eight hours after transfection, cells were harvested for luciferase assays.

For stimulation experiments cells were cultured in DMEM supplemented with 5% FCS immediately after transfection. After 24 h cells were stimulated with 10 μM forskolin (Sigma) or with 300 μM chlorophenylthio-cyclic AMP (CPT-cAMP; Sigma) for 24 h.

To measure luciferase activity, cells were washed twice with phosphate-buffered saline (PBS) and lysed by adding 350 μl of extraction buffer (25 mM Tris-H_3PO_4 [pH 7.8], 2 mM EDTA, 10% [vol/vol]) glycerol, 1% [vol/vol] Triton X-100, and 2 mM dithiothreitol [DTT]) for 10 min. The lysates were cleared by centrifugation. Fifty microliters of the supernatant was assayed by addition of 300 μl of measuring buffer (25 mM glycylglycine, 15 mM $MgSO_4$, and 5 mM ATP). The light emission was measured in duplicate for 10 s in a Lumat LB 9501 (Berthold) by injecting 100 μl of 250 μM luciferin. Each experiment was performed in duplicate and repeated at least three times. The data show the specific luciferase activity and represent the average of three independent experiments.

Plasmid construction. A genomic DNA fragment containing the 5′, 3′, and coding regions of rat LAP/CRP2/C/EBPβ (pBSR CRP2 5.0) was kindly provided by Peter Johnson. The single restriction site *Stu*I, located approximately 1,400 bp 5′ upstream of the transcription start site, and the restriction site *Ava*II, located immediately downstream of the transcription start site, were used to transfer the 5′-terminal 1,400-bp fragment into the p19Luc vector in front of the luciferase gene (LAPPRO 1). The first 550 bp upstream of the transcription start site were sequenced. Increasing 5′-terminal deletions with single restriction sites as outlined in Fig. 1A were introduced in the luciferase reporter constructs (LAPPRO 2 through 9).

The deletions −121 to −112 and −103 to −76 were introduced in the LAP PRO 8 WT construct (−121 to +16) by ligation of an oligonucleotide, corresponding to −111 to −104, to the *Apa*I site (Fig. 1A and C). The resulting LAPPRO 8 WTΔ construct was confirmed by sequencing.

The mutations −109 to −107 (ACG to GTT) and/or −65 to −61 (TGACG to GATCC) were introduced in the LAPPRO 8 WT construct (−121 to +16) by recombinant PCR and verified by sequencing. The LAPPRO 8 MUT I construct corresponds to the mutation −109 to −107, the LAPPRO 8 MUT II construct corresponds to the mutation −65 to −61, and the LAPPRO 8 MUT I + II construct corresponds to the mutations −109 to −107 and −65 to −61.

The pRSVβGal vector was used as an internal standard. The pCMV/SP1 expression vector and the p2xCRE-Luc, with two CREB binding sites linked to the prolactin minimal promoter in front of the luciferase gene, which served as the positive control, were both kindly provided by Michael Wegner. The pGEM3/ATF1 vector (15) was digested with *Sal*I and *Eco*RI, and the resulting ATF1 insert was cloned in the pBK/CMV expression vector (Stratagene). The CREB, PKAwt and PKAmut expression vectors have been described previously (4, 13, 25). The pmcCREBΔα vector was kindly provided by Wolfgang Schmid.

Nuclear extracts and gel retardation assays. HepG2 nuclear extracts were prepared by the modified Dignam C method (39), and rat liver cell nuclear extracts were prepared as previously described (41). For gel retardation assays nuclear extracts were used as indicated in the figure legends. Binding buffer consisted of 25 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 34 mM KCl, 2 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of poly(dI-dC)/μl, and 2 μg of bovine serum albumin/μl. The binding reaction was performed for 30 min on ice. Free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel.

The region −127 to −68 of the LAP promoter named oligonucleotide I was generated as a PCR fragment (5′-TCC CGC GGC CGG GCA ATG ACG CGC ACC GAC CCG GCG GCG GGG CGG CGG GAG GGG CCC CGG-3′). The oligonucleotides were purchased from Eurogentec (Seraing, Belgium) and used as ^{32}P-labeled probes. Oligonucleotide II corresponds to −123 to −95, oligonucleotide III corresponds to −123 to −107, oligonucleotide IV corresponds to −110 to −87, and oligonucleotide V corresponds to −102 to −75 (Fig. 2A). WT

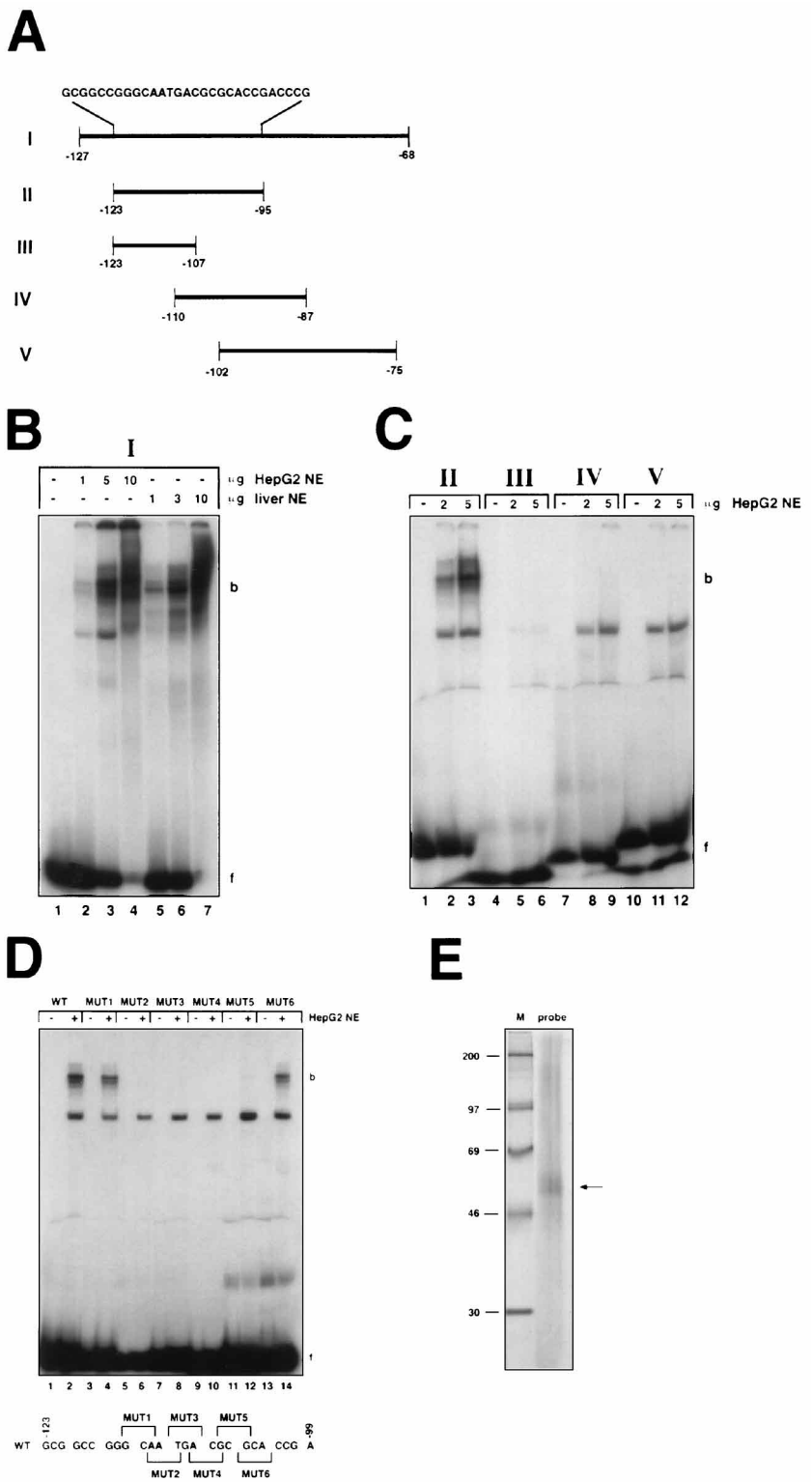

FIG. 2. A single motif is important for protein binding in the region between nucleotides −121 and −71 in the LAP/C/EBPβ promoter. (A) Oligonucleotides I to V, derived from the LAP/C/EBPβ promoter and used for gel shift experiments, are shown. (B) Gel shift experiments with oligonucleotide I (−127 to −68) as the ^{32}P-labeled probe. The binding reaction was performed either without (lane 1) or with the indicated amounts (lanes 2 to 7) of HepG2 or rat liver cell nuclear extracts (NE). The positions of the bound protein-DNA complexes (b) and the free probe (f) are marked. (C) Gel shift experiments with oligonucleotides II (−123 to −95; lanes 1 to 3), III (−123 to −107; lanes 4 to 6), IV (−110 to −87; lanes 7 to 9), or V (−102 to −75; lanes 10 to 12) as the ^{32}P-labeled probe. The binding reaction was performed without NE (lanes 1, 4, 7, and 10) and with either 2 μg (lanes 2, 5, 8, and 11) or 5 μg (lanes 3, 6, 9, and 12) of HepG2 NE. (D) Different overlapping mutations were introduced in the DNA binding motif of the LAP/C/EBPβ promoter sequence (WT; −123 to −99). The resulting oligonucleotides (MUT 1 to 6) were used for gel shift analysis. The binding reaction was performed either without (lanes 1, 3, 5, 7, 9, 11, and 13) or with (lanes 2, 4, 6, 8, 10, and 12) 2 μg of HepG2 NE. (E) UV cross-linking analysis. HepG2 NE was incubated with ^{32}P-labeled WT oligonucleotide (−123 to −99) in a fivefold upscale of panel D, lane 2, separated on a 6% nondenaturing polyacrylamide gel, and irradiated with UV light. The retarded protein-DNA complexes were excised and separated on a 10% SDS-polyacrylamide gel. The resulting signal is marked with an arrow. Molecular mass markers (M) are shown in kDa.

and MUT 1 to 6 oligonucleotides correspond to −123 to −99: WT, 5′-GCG GCC GGG CAA TGA CGC GCA CCG A-3′; MUT1, 5′-GCG GCC GGT TGA TGA CGC GCA CCG A-3′; MUT2, 5′-GCG GCC GGG CGG AGA CGC GCA CCG A-3′; MUT3, 5′-GCG GCC GGG CAA GTG CGC GCA CCG A-3′; MUT4, 5′-GCG GCC GGG CAA TGG TTC GCA CCG A-3′; MUT5, 5′-GCG GCC GGG CAA TGA CTT TCA CCG A-3′; and MUT6, 5′- GCG GCC GGG CAA TGA CGC TTG CCG A-3′.

WT2 corresponds to −71 to −43 (5′-CCG GCG TGA CGC AGC CCG TTG CCA GGC GC-3′).

The CREB WT consensus oligonucleotide corresponds to the sequence 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′, and the CREB MUT oligonucleotide corresponds to 5′-AGA GAT TGC CTG TGG TCA GAG AGC TAG-3′. The antibodies against CREB1, ATF1, ATF2, and ATF4 were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif. Super shift experiments were also performed with a CREB1 antibody kindly provided by Wolfgang Schmid. In vitro transcription of CREB was performed with a *Xho*I linearized template with T3 RNA polymerase. In vitro translation of CREB was performed as instructed by the supplier (Promega).

UV cross-linking experiments. A sample in the gel retardation assay was scaled up five times (25 μg of nuclear extract and 1.5×10^5 cpm of the labeled oligonucleotide) and separated on a 6% polyacrylamide gel. The wet gel was placed on ice, irradiated with UV light at 254 nm for 10 min at 250 mJ in a Stratalinker UV cross-linker (Stratagene) and exposed for 2 h for autoradiography. The retarded protein-DNA complexes were excised, soaked in sodium dodecyl sulfate (SDS)-sample buffer for 10 min at 25°C, boiled for 2 min, placed in the bottoms of the wells of a denaturing SDS–10% polyacrylamide gel, and separated. Gels were dried and exposed for autoradiography.

Northern blot analysis. Cells were grown to subconfluency, washed twice with PBS, and incubated with DMEM supplemented with 5% FCS for 48 h. Cells were stimulated with 10 μM forskolin for different times as indicated in the figure legends. Total cellular RNA was prepared with an RNeasy kit (Qiagen) according to the manufacturer's instructions. Fifteen micrograms of total RNA was analyzed through a 1% agarose formaldehyde gel, transferred to Hybond N^+ membranes (Amersham), and fixed by heating at 80°C for 1 h. The LAP/C/EBPβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were labeled with [α-^{32}P]CTP according to the instructions for Rediprime (Amersham). The hybridization procedure was performed as described previously (38). Blots were exposed for autoradiography and exposed to an image plate (Fuji) for quantification. The counts of the LAP signal were distributed through the counts of the GAPDH signal and set to 1 for untreated cells. The values for forskolin-stimulated cells were shown as fold activation and represent the average of triplicate experiments.

Two-thirds hepatectomy and preparation of nuclei and nuclear extracts. Sprague-Dawley rats were maintained and anesthetized as previously described (41). After a small abdominal incision, two-thirds hepatectomy was performed (38). Following surgery, the abdominal cavum was closed by a suture. At different time points, the remaining livers were removed, pooled, and rinsed in ice-cold PBS and liver cell nuclei and liver cell nuclear extracts were prepared (9). For each time point, four rats were used in parallel.

Nuclear runoff transcription assays. Cells were grown to subconfluency, washed twice with PBS, and incubated with DMEM supplemented with 5% FCS for 48 h. Stimulation was carried out with 10 μM forskolin for different times. Cells were harvested in reticulocyte standard buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM DTT), incubated for 10 min on ice, and disrupted with 25 strokes in a Dounce homogenizer B. Nuclei were collected by centrifugation at $400 \times g$ for 5 min.

For runoff transcription assays, 5×10^7 nuclei (from cell culture or from rat liver cells) were incubated in reaction buffer (5 mM Tris-HCl [pH 8.0], 2.5 mM $MgCl_2$, 0.15 mM KCl, and 1 mM DTT) containing 10 μl of 100 mM ATP, 10 μl of 100 mM CTP, 10 μl of 100 mM GTP, and 10 μl of 10 mCi of [α-^{32}P]UTP/ml for 30 min at 30°C. RNA was extracted and precipitated as described previously (41).

For hybridization, 6 μg of cDNA was denatured and fixed on nylon membrane for 1 h at 80°C. The cDNA was preincubated for 1 h at 65°C in prehybridization buffer (20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [TES] [pH 7.4], 10 mM EDTA, 400 mM NaCl, 0.2% SDS, 2× Denhardt's solution, 5 mM DTT, and 200 μg of carrier RNA/ml). Hybridization was performed with 2×10^6 cpm of RNA/ml in 1 ml of hybridization buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 400 mM NaCl, 0.2% SDS, 2× Denhardt's solution, 5 mM DTT, and 200 μg of carrier RNA/ml) in 15-ml tubes for 36 h at 65°C. After extensive washing (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.2% SDS), samples were digested with RNase (8 ml of 2× SSC and 80 μg of RNase A) for 30 min at 37°C. Blots were first exposed for autoradiography and then exposed to an image plate (Fuji) for quantification. The ratio between the counts of the LAP signal and of the GAPDH signal was calculated and set to 1 for untreated cells (see Fig. 8) or for rat liver cell nuclei prepared before two-thirds hepatectomy (see Fig. 9). The values for forskolin-stimulated cells (see Fig. 8) or for different times after two-thirds hepatectomy (see Fig. 9) were shown as fold stimulation.

SDS-polyacrylamide-gel electrophoresis and Western blot analysis. Rat liver cell nuclear extracts were separated on a 10% SDS-polyacrylamide gel (21) and blotted onto nitrocellulose membrane (Schleicher and Schuell) in a solution containing 1% SDS, 20% methanol, 400 mM glycine, and 50 mM Tris-HCL (pH 8.3) at 4°C for 2 h at a constant current of 200 mA. The phospho-specific CREB (Ser133) antibody was a generous gift from Wolfgang Schmid. The antigen-antibody complexes were visualized with the enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham).

RESULTS

LAP/C/EBPβ transcription is mediated by a region in proximity to the TATA box. LAP/C/EBPβ is highly expressed in liver cells, and we have shown that its mRNA expression is modulated during liver regeneration. However, until now little has been known about the transcriptional control of the gene itself. Therefore, transient transfection experiments were performed in the human hepatoma cell line HepG2 to study the influence of the 5′-flanking region on the control of the LAP/C/EBPβ gene. Luciferase reporter constructs were cloned from a genomic fragment, which includes the LAP/C/EBPβ open reading frame as well as the 5′- and 3′-flanking regions. The LAP/C/EBPβ gene comprises three in-frame ATGs. The TATA box is located 82 bp 5′ upstream of the first ATG, and the major start site of transcription is located 49 bp 5′ upstream of the first ATG. The whole 5′-flanking region starting 16 bp downstream from the start site of transcription and comprising approximately 1.4 kb was linked to a luciferase reporter gene. Increasing 5′-terminal deletions were introduced in this promoter fragment (LAPPRO 1 to 9) (Fig. 1A).

The different reporter constructs were tested by transient transfection experiments in HepG2 cells for their ability to activate the luciferase reporter gene (Fig. 1B). Strong activation of the luciferase reporter construct was found when the whole 1.4-kb 5′ region was used (LAPPRO 1). The activity of this fragment was set to 100%, and all the deletion mutants were compared to this construct. The level of luciferase activation was slightly lower when the first two deletion constructs (LAPPRO 2 and 3) were used. Sixty percent of the LAPPRO 1 activity was reached with the construct LAPPRO 4. Luciferase activity increased again when the constructs LAPPRO 5 to 8 were used, but without exceeding the activity of LAPPRO 1. However, a dramatic decrease in luciferase activity was observed when the LAPPRO 9 construct (nucleotides −71 to +16) was transfected. The activity of LAPPRO 9 was less than 5% of that of the LAPPRO 1 construct. The transient transfection experiments suggested that the region located between nucleotides −121 and −71 (Fig. 1C) is important for controlling transcription of the LAP/C/EBPβ gene. Thus, this region was investigated in further experiments.

A single motif is important for protein binding in the region between nucleotides −121 and −71. In the search for proteins which might bind to this region, gel shift experiments were performed with a fragment which included nucleotides −127 to −68 (oligonucleotide I) (Fig. 2A). Nuclear extracts from HepG2 cells and from rat liver cells showed complex formation with similar binding patterns (Fig. 2B). These experiments indicated that complex formation occurred in a region which was linked to high luciferase activity in the transfection experiments.

To further characterize the potential DNA-binding sites, overlapping oligonucleotides spanning positions −123 to −75 of the 5′ region were used for gel shift experiments (Fig. 2A). Only oligonucleotide II (−123 to −95) showed specific binding resembling the pattern observed when the whole region (oligonucleotide I) was used for complex formation. In contrast, none of the other oligonucleotides showed specific binding. Further analysis of oligonucleotides III (−123 to −107) and IV (−110 to −87) suggested that the region in which both oligonucleotides overlap could be important for the complex for-

mation detected with oligonucleotide II. Therefore, overlapping point mutations were introduced in this region and gel shift experiments with the different mutated oligonucleotides (MUT1 to 6) were performed (Fig. 2D). Complete DNA binding was associated with an intact motif comprising the sequence AATGACGCG. We obtained the same result when nuclear extracts derived from rat liver cells were used (data not shown).

The complex was associated with the appearance of a major and a minor band. UV cross-linking analysis was performed to further differentiate the potential factors which bind to this element. Cross-linking experiments identified one potential factor with a molecular mass of approximately 50 kDa binding to this element (Fig. 2E).

CREB binds to its consensus DNA sequence and to the element in the LAP/C/EBPβ promoter with the same affinity. Next we tried to identify proteins which might bind to the motif characterized by gel shift analysis. Computer-assisted analysis revealed only CREB as a possible candidate. However, the sequence was an incomplete CREB DNA-binding motif, as only five out of eight nucleotides were conserved compared to the CREB consensus sequence. To compare DNA binding between the motif in the LAP/C/EBPβ promoter and the CREB consensus sequence, gel shift experiments were performed with an oligonucleotide corresponding to the CREB consensus site (CREB WT) as the competitor. For complex formation HepG2 nuclear extracts and the oligonucleotide comprising the binding site in the LAP/C/EBPβ promoter (WT; −123 to −99) (Fig. 2D) were used. Interestingly, even low concentrations (10-fold excess) of the CREB WT oligonucleotide competed for binding of HepG2 nuclear extracts while higher concentrations completely abolished the binding (Fig. 3A). The same concentration (10-fold excess) of the mutant CREB oligonucleotide (CREB MUT) had only a minimal effect on DNA binding (Fig. 3A). A higher concentration (1,000-fold excess) of CREB MUT also competed for complex formation.

The same competition experiments were performed with the unlabeled sequence of the LAP/C/EBPβ promoter (WT). As was shown for the CREB WT sequence, low amounts of the WT promoter motif also competed for complex formation (Fig. 3B). However, competition experiments with the mutant oligonucleotide (MUT4) (Fig. 2D) did not influence binding (Fig. 3B). The results with the mutant oligonucleotides (MUT4 and CREB MUT) used in the competition experiments indicated that CREB MUT still has a low affinity for the proteins found by complex formation.

To further support these observations, the same experiments performed with HepG2 nuclear extracts were repeated with in vitro translated CREB protein (Fig. 3C and D). As was shown with the nuclear extracts, these experiments confirmed that the sequence of the LAP/C/EBPβ promoter (WT) competed DNA binding of in vitro translated CREB as efficiently as the consensus CREB oligonucleotide (CREB WT) did. Again, for the in vitro translated CREB, the MUT4 oligonucleotide did not have any effect on DNA binding while the CREB MUT oligonucleotide did compete DNA binding when high concentrations (1,000-fold excess) of the unlabeled oligonucleotide were used (Fig. 3C and D). Therefore, we revealed the same competition pattern with the mutant oligonucleotides whether we used HepG2 nuclear extracts or in vitro translated CREB protein for complex formation with the sequence of the LAP/C/EBPβ promoter (WT).

As a final proof that the sequence in the LAP/C/EBPβ promoter binds CREB-related proteins as efficiently as the CREB WT consensus sequence does, competition experiments were performed in which the CREB WT site was used for complex formation with in vitro translated CREB protein. The experiments shown in Fig. 3E demonstrated that the sequence in the LAP/C/EBPβ promoter (WT) competes for binding of in vitro translated CREB as efficiently as CREB WT does. Therefore, our gel shift experiments conclusively indicate that the sequence found in the LAP/C/EBPβ promoter binds CREB as efficiently as the consensus CREB WT motif does.

CREB binds to two elements in the LAP/C/EBPβ promoter. The gel shift experiments suggested that the protein which binds to the LAP/C/EBPβ promoter might belong to the CREB family of transcription factors. Thus, gel shift experiments were performed with antibodies directed against different members of the CREB family. As indicated in Fig. 4A, an anti-CREB1 antibody supershifted the major band of the complex formed between HepG2 nuclear extracts and the motif found in the LAP/C/EBPβ promoter. Antibodies directed against other CREB family members had no effect on complex formation of the major band. In HepG2 nuclear extracts the lower, minor band seemed to disappear when the anti-ATF1 antibody was used. However, we found no supershifted bands. Therefore, in hepatoma cell lines CREB is the major factor binding to the motif located between nucleotides −114 and −104 in the LAP/C/EBPβ promoter.

The expression of CREB-related proteins may differ in differentiated hepatocytes. Therefore, the antibodies were also used to investigate complex formation with rat liver cell nuclear extracts (Fig. 4B). In contrast to HepG2 nuclear extracts, where a major and a minor band were found, in liver cell nuclear extracts binding of two major bands was evident. Anti-CREB1 completely supershifted the upper band, which corresponds to the results obtained with HepG2 nuclear extracts. Binding of the second band seemed inhibited by the anti-ATF1 antibody without creating a new band. So far, we have not further analyzed the nature of the lower band. Our experiments indicate that in liver cell nuclear extracts as well as in the hepatoma cell line CREB itself seems to be the main member of the CREB family which binds to the motif located between −114 and −104 in the LAP/C/EBPβ promoter.

Because the reporter construct analysis suggested that the sequence between −121 and +16 has a major effect on the transcriptional control of LAP/C/EBPβ expression and our experiments showed that an incomplete CREB site can also efficiently bind CREB, the whole region was screened for additional incomplete CREB sequences. Unexpectedly, a second incomplete motif was found in close proximity to the first site, directly located at the 5′ end (nucleotides −69 to −61) of the LAPPRO 9 luciferase construct. Therefore, this motif was used to perform gel shift experiments with HepG2 and rat liver cell nuclear extracts and in vitro translated CREB. Interestingly, as was already shown for the first CREB site in the LAP/C/EBPβ promoter, this second site also binds nuclear extracts and CREB as efficiently as the first CREB site and the CREB consensus sequence do (Fig. 4C and data not shown).

PKA stimulates LAP/C/EBPβ expression via CREB. The DNA-binding experiments showed that two CREB sites do exist in the LAP/C/EBPβ promoter. Signals at the cell membrane inducing the protein kinase A (PKA) cascade lead to enhanced transcription of PKA-dependent target genes via phosphorylation of CREB. To examine the importance of this mechanism for controlling LAP/C/EBPβ transcription, we performed transient cotransfection experiments in the HepG2 cell line. In these studies the two luciferase reporter constructs LAPPRO 8 (with two CREB sites) and LAPPRO 9 (without the first CREB site) (Fig. 1A and C) were used. Cotransfection experiments with an expression vector for CREB and the LAP

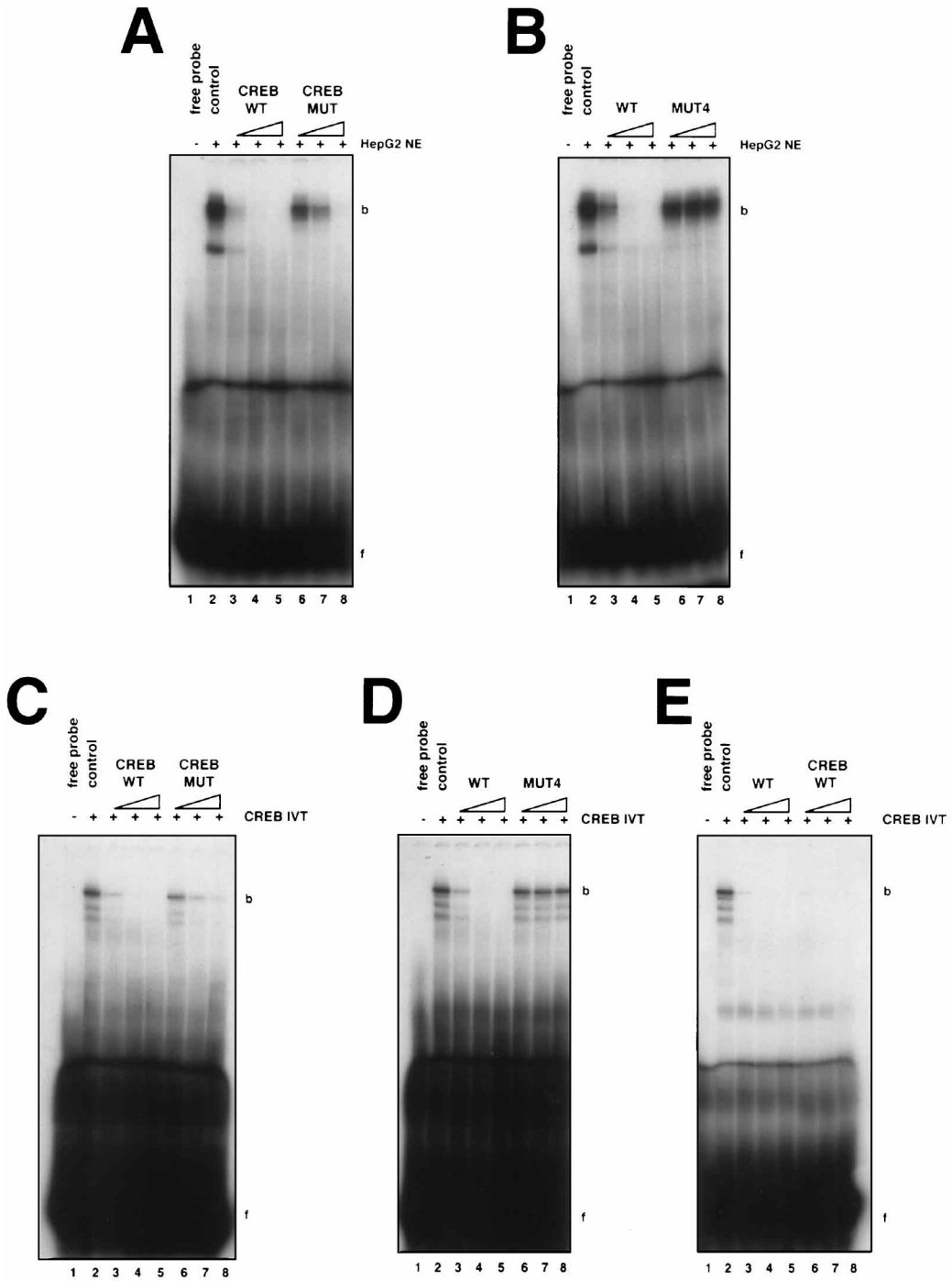

FIG. 3. CREB binds to its consensus DNA sequence and to the element in the LAP/C/EBPβ promoter with the same affinity. (A) Competition experiments with the identified binding site (−123 to −99) in the LAP/C/EBPβ promoter (WT) as the ^{32}P-labeled probe and the CREB consensus (CREB WT) or mutant (CREB MUT) oligonucleotide as the competitor. Complex formation was performed without nuclear extract (NE) (free probe; lane 1) or with 2 μg of HepG2 NE (lanes 2 to 8). Either the unlabeled CREB WT (lanes 3 to 5) or CREB MUT (lanes 6 to 8) oligonucleotide was used as the competitor in 10- (lanes 3 and 6), 100- (lanes 4 and 7), or 1,000-fold (lanes 5 and 8) excess. The positions of the bound protein-DNA complexes (b) and the free probe (f) are marked. (B) Competition experiments with WT oligonucleotide as the ^{32}P-labeled probe and unlabeled WT and its mutant 4 (MUT4; Fig. 2D) oligonucleotide as the competitor. HepG2 NE was used as indicated for panel A. Unlabeled WT (lanes 3 to 5) and MUT4 (lanes 5 to 8) oligonucleotides were used in 10- (lanes 3 and 6), 100- (lanes 4 and 7), or 1,000-fold (lanes 5 and 8) excess as the competitors. (C and D) Competition experiments were performed as indicated for panels A and B, respectively, except that 2 μl of in vitro translated CREB protein (CREB IVT) was used for complex formation instead of HepG2 NE. (E) Competition experiments with CREB WT oligonucleotide as the ^{32}P-labeled probe and unlabeled WT and CREB WT oligonucleotides as the competitors. Complex formation was performed with 2 μl of CREB IVT as indicated (lanes 2 to 8). Unlabeled WT (lanes 3 to 5) or CREB WT (lanes 6 to 8) oligonucleotide was used in 10- (lanes 3 and 6), 100- (lanes 4 and 7), or 1,000-fold (lanes 5 and 8) excess as the competitor.

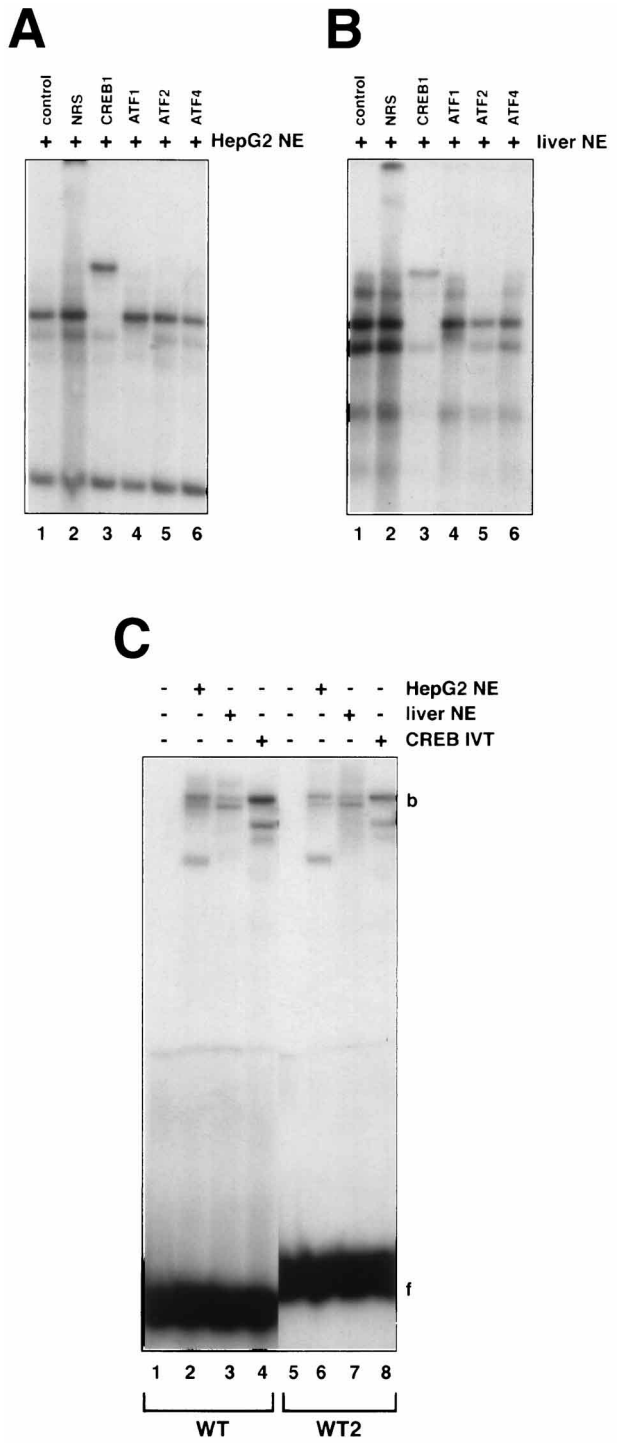

FIG. 4. CREB binds to two elements in the LAP/C/EBPβ promoter. (A and B) Super shift analysis with different antibodies directed against CREB family members. Complex formation was performed with the ^{32}P-labeled WT oligonucleotide (−123 to −99 in the LAP/C/EBPβ promoter) and either 2 μg of HepG2 nuclear extract (NE) (A) or 2 μg of rat liver cell NE (B). For super shift analysis, antibodies against four different CREB family members (lanes 3 to 6) or normal rabbit serum (NRS) (lane 2) were added. Incubation without antibodies is shown in lane 1 (control). (C) Gel shift experiment with ^{32}P-labeled WT oligonucleotide (−123 to −99; lanes 1 to 4) or WT2 oligonucleotide (−71 to −43; lanes 5 to 8) derived from the LAP/C/EBPβ promoter. The binding reaction was performed without NEs (lanes 1 and 5), with 2 μg of HepG2 NE (lanes 2 and 6), with 2 μg of rat liver cell NE (lanes 3 and 7), or with 2 μl of in vitro translated CREB protein (CREB IVT) (lanes 4 and 8). The positions of the bound protein-DNA complexes (b) and the free probe (f) are marked.

PRO 8 construct showed twofold-higher reporter activity than for the LAPPRO 8 construct alone (Fig. 5A). Cotransfection of increasing amounts of an ATF1 expression vector (50 ng) (Fig. 5A and data not shown) did not stimulate reporter gene activity. Interestingly, more than a 10-fold increase was found when the catalytic subunit of PKA was cotransfected with the LAPPRO 8 construct. When we used both CREB and the catalytic PKA subunit, transcription was enhanced even more than 20-fold (Fig. 5A). In contrast, cotransfection with the mutated catalytic PKA subunit did not modulate reporter gene activity. No regulation by CREB or a PKA-dependent mechanism was found when the LAPPRO 9 luciferase reporter gene was transfected (Fig. 5A). Control experiments were performed with a luciferase reporter gene which contains two consensus CREB sites (CRE-LUC). The pattern of activation after cotransfecting CREB and/or the catalytic subunit of PKA was similar to that found for the LAPPRO 8 construct (Fig. 5B). Thus, these results suggested that the CREB binding sites in the LAP/C/EBPβ promoter mediate PKA inducibility comparably to those in a promoter comprising the consensus CREB sequence.

The GC-rich sequences flanking the first CREB site could influence the basal activity or the PKA inducibility of the LAPPRO 8 WT luciferase reporter gene. Therefore, we created the deletion mutant LAPPRO 8 WTΔ (Fig. 5C). Transfection experiments with the LAPPRO 8 WTΔ reporter construct showed no difference in basal activity compared to that of the LAPPRO 8 WT construct. Additionally, cotransfection of the catalytic subunit of PKA with the LAPPRO 8 WTΔ construct still enhanced transcription comparably to the LAP PRO 8 WT construct (Fig. 5D). Cotransfection of increasing amounts of the transcription factor SP1 did not stimulate the activity of the LAPPRO 8 WT or the LAPPRO 8 WTΔ constructs (Fig. 5D). These data indicated that the flanking sequences of the first CREB site neither influenced the basal promoter activity nor stimulated the LAP/C/EBPβ promoter via PKA.

To further evaluate the effect of PKA on LAP/C/EBPβ gene activation through CREB, we were interested in studying the effect of activating internal PKA on LAPPRO 8 transcription. Both reporter constructs, LAPPRO 8 and CRE-LUC, showed only weak activation in the HepG2 cell line (less than twofold) after stimulation by forskolin (data not shown). Therefore, stimulation experiments were performed in neuronal cells, where strong activation of PKA through forskolin has been shown before. Transfection experiments with the LAPPRO 8 luciferase reporter plasmid and the CRE-LUC reporter plasmid were performed in the neuronal cell line Neuro 217. Additionally, we created LAPPRO 8 reporter constructs with mutations either in the first CREB site (LAPPRO 8 MUT I), in the second CREB site (LAPPRO 8 MUT II), or in both sites (LAPPRO 8 MUT I + II) (Fig. 6A). In the Neuro 217 cell line the background level of the LAPPRO 8 WT reporter construct was much higher than that of the CRE-LUC reporter construct (Fig. 6B and C). However, cotransfection with the catalytic subunit of PKA still enhanced transcription of the LAPPRO 8 WT construct 2.5-fold. Stimulation with forskolin had a comparable effect to cotransfection with the catalytic subunit. Stimulation with the membrane-permeable cAMP analog CPT-cAMP had an even better effect, showing threefold-greater activation of the LAPPRO 8 WT reporter plasmid than of the unstimulated (control) cells. Activation of the CRE-LUC reporter plasmid after either cotransfection with the catalytic PKA subunit or stimulation with forskolin or CPT-cAMP was 10- to 20-fold greater than that of the unstimulated cells (Fig. 6C). Mutation of both CREB sites in the LAPPRO 8 MUT I +

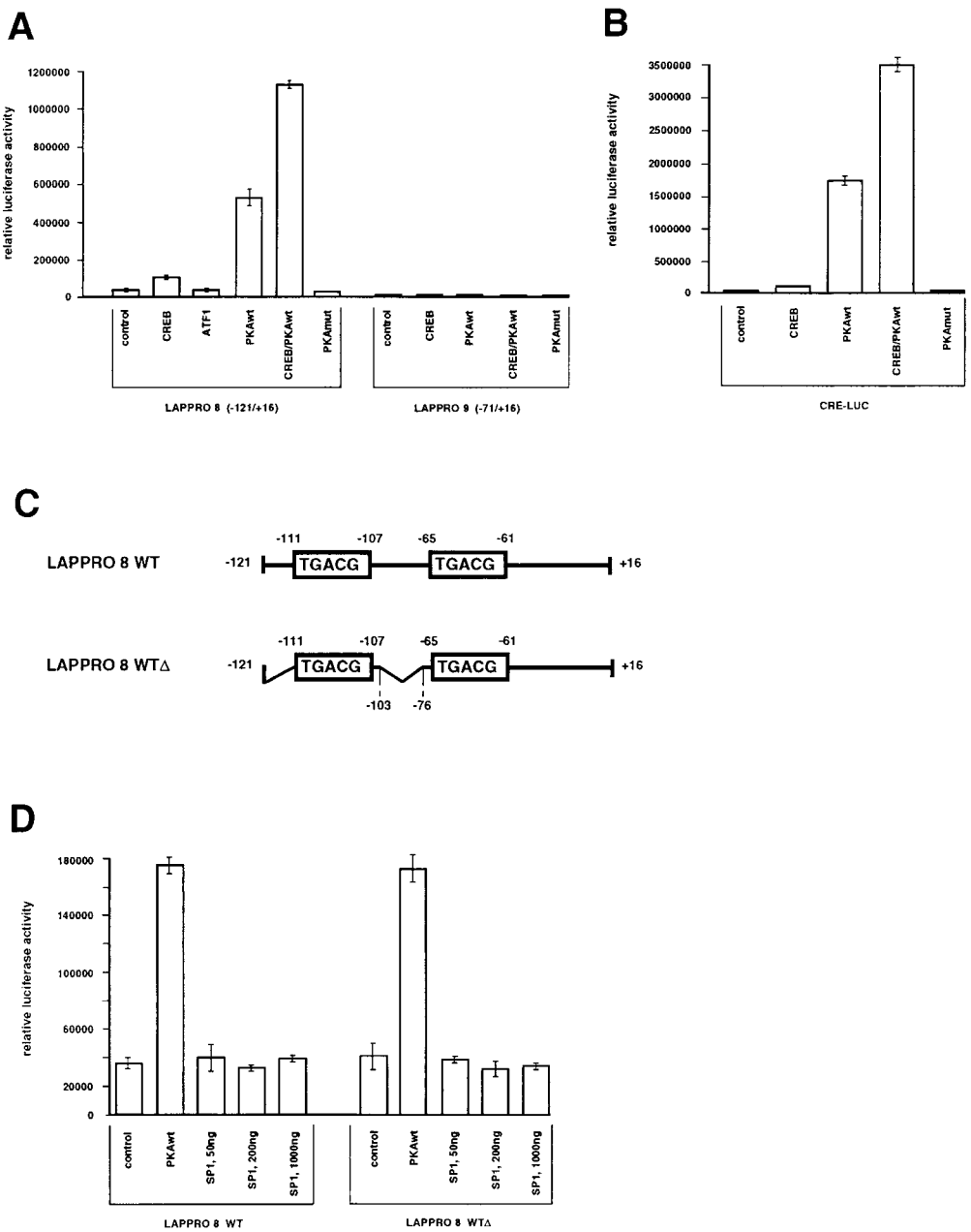

FIG. 5. PKA stimulates LAP/C/EBPβ expression. (A and B) Luciferase reporter gene assays. HepG2 cells were transfected with 2 μg of DNA from the LAP/C/EBPβ promoter construct LAPPRO 8 or LAPPRO 9 (A) (Fig. 1A) or from the CRE-LUC construct (B). DNA from the plasmids was cotransfected as follows: control, none; CREB, 50 ng of CREB; ATF1, 50 ng of ATF1; PKAwt, 1 μg of PKAwt; CREB/PKAwt, 50 ng of CREB and 1 μg of PKAwt; or PKAmut, 1 μg of PKAmut. Cells were harvested 48 h after transfection and measured for luciferase activity. (C) The LAPPRO 8 construct (LAPPRO 8 WT) (Fig. 1A and C) and the deleted LAPPRO 8 construct (LAPPRO 8 WTΔ) are shown. In the LAPPRO 8 WTΔ construct the flanking sequences of the first CREB site are deleted. The fragments were linked to a luciferase reporter gene. (D) Luciferase reporter gene assays. HepG2 cells were transfected with 2 μg of DNA from the LAP/C/EBPβ promoter construct LAPPRO 8 WT or LAPPRO 8 WTΔ. DNA from the plasmids was cotransfected as follows: control, none; PKAwt, 1 μg of PKAwt; or the indicated amount of SP1. Error bars indicate standard deviations.

II construct led to a dramatic drop in basal activity and to loss of inducibility by PKA (Fig. 6B). Mutation of only one site (LAPPRO 8 MUT I or LAPPRO 8 MUT II) also abolished basal activity but retained twofold PKA induction (Fig. 6C). These data indicate that the two CREB sites −111 to −107 and −65 to −61 in the LAP/C/EBPβ promoter act synergistically and are important for high basal promoter activity. While activation by PKA was completely blocked by mutations in both CREB sites (Fig. 6B), mutation of only the first or the second CREB site resulted in residual activation by PKA (Fig. 6C). These results indicate that, besides mediating basal activity, both CREB sites in the LAP/C/EBPβ promoter are required for full PKA inducibility.

PKA induces the endogenous LAP/C/EBPβ gene. Our next interest was to prove whether the observation made in promoter studies is relevant to the regulation of the endogenous

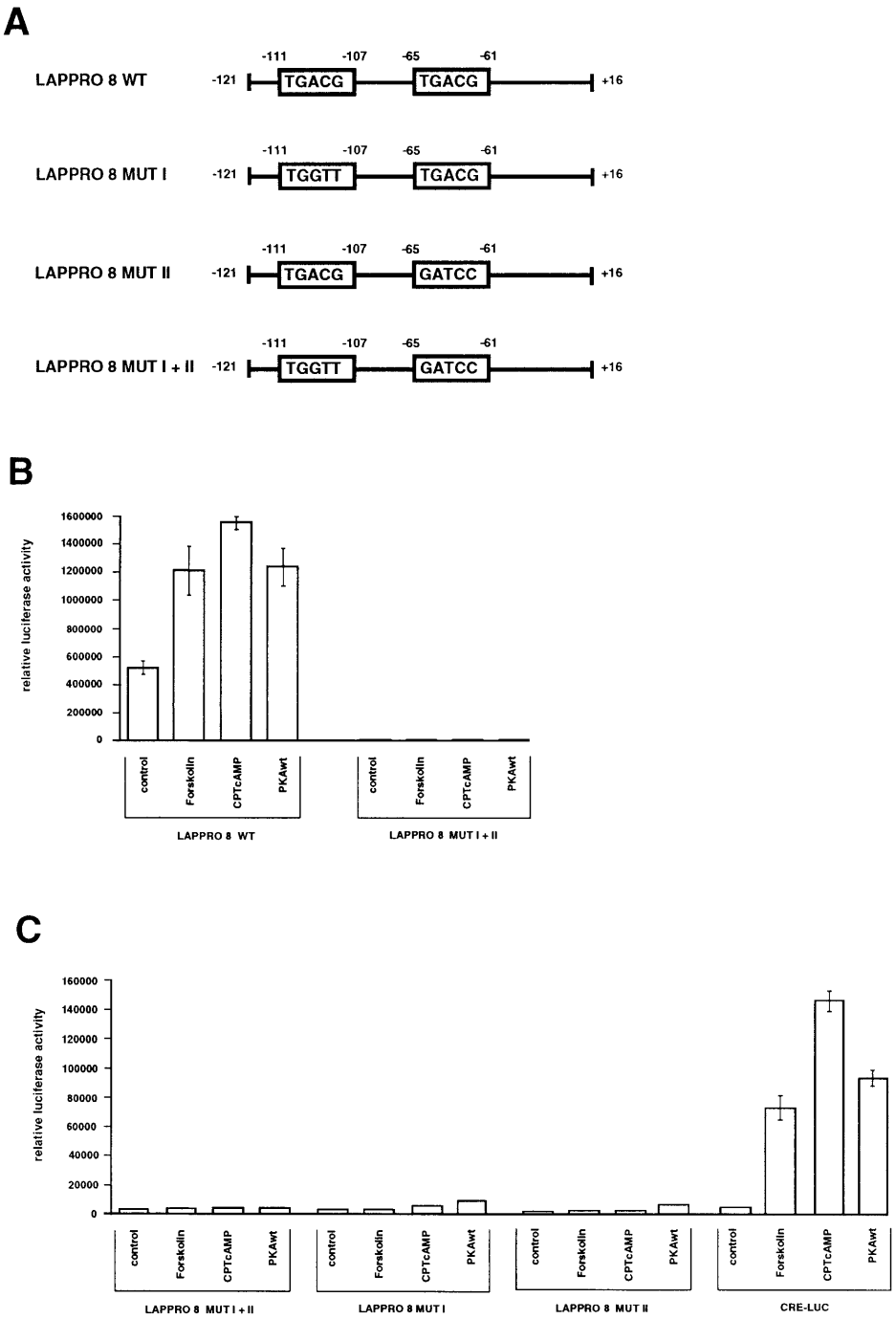

FIG. 6. Forskolin and CPT-cAMP stimulate LAP/C/EBPβ expression via the two incomplete CREB motifs. (A) The LAPPRO 8 construct (LAPPRO 8 WT) (Fig. 1A and C) and the mutant LAPPRO 8 constructs (LAPPRO 8 MUT I, II, and I + II) are shown. The first CREB site in the LAPPRO 8 MUT I construct and the second CREB site in the LAPPRO 8 MUT II construct were mutated, while in the LAPPRO 8 MUT I + II construct both sites were mutated. The fragments were linked to a luciferase reporter gene. (B and C) Transfection and PKA stimulation experiments with LAPPRO 8 WT, LAPPRO 8 MUT I, II, and I + II, and CRE-LUC. Neuro 217 cells were transfected with 2 μg of the LAP/C/EBPβ promoter constructs LAPPRO 8 WT (B), LAPPRO 8 MUT I, II, and I + II (B and C), or with 2 μg of CRE-LUC DNA (C). Twenty-four hours after transfection, cells were either not treated (control) or 10 μM forskolin or 300 μM CPT-cAMP was added. A cotransfection experiment was performed with 1 μg of PKAwt. Cells were harvested 48 h after transfection and measured for luciferase activity. Error bars indicate standard deviations.

LAP/C/EBPβ gene. Therefore, we used the neuronal cell line Neuro 217 to study LAP/C/EBPβ mRNA expression after stimulation with forskolin. As shown in Fig. 7A and B, stimulation of PKA led to nearly threefold-greater induction of the LAP/C/EBPβ mRNA level after 4 h. At later time points the LAP/C/EBPβ mRNA level dropped, and after 24 h the LAP/C/EBPβ mRNA level was only approximately 1.5 times the pretreatment level. To show that our observation has a general

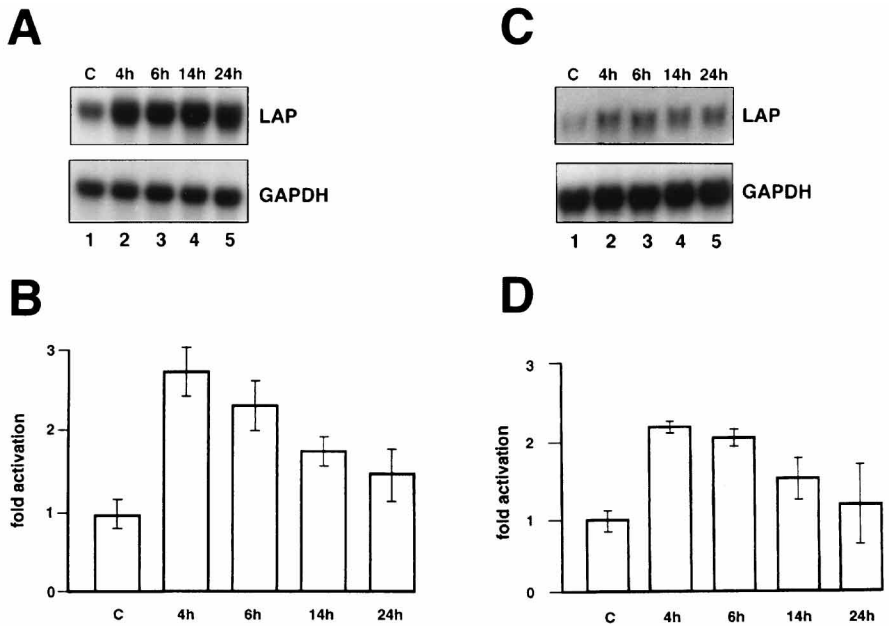

FIG. 7. Forskolin increases the endogenous LAP/C/EBPβ mRNA expression in NIH 3T3 and Neuro 217 cells. (A and C) Total RNA of Neuro 217 (A) or NIH 3T3 (C) cells was isolated at the indicated time points before (c; lane 1) and after (lanes 2 to 5) forskolin treatment. Northern blot analysis was performed. The blots were hybridized with ^{32}P-labeled DNA for LAP/C/EBPβ and GAPDH. (B and D) The ratio between the LAP/C/EBPβ and the GAPDH signals was calculated and set to 1 for untreated (c) Neuro 217 (B) or NIH 3T3 (D) cells. Changes after forskolin treatment are shown as fold activation. Error bars indicate standard deviations.

relevance to the transcriptional control of the LAP/C/EBPβ gene, stimulation experiments were also performed in NIH 3T3 cells. As was shown for the neuronal cell line, in NIH 3T3 cells PKA stimulation through forskolin also led to more than a twofold increase of the LAP/C/EBPβ mRNA level after 4 h. At later time points the LAP/C/EBPβ mRNA level decreased. After 24 h the LAP/C/EBPβ mRNA level was 30% higher than before forskolin stimulation (Fig. 7C and D).

To determine whether the regulation of LAP/C/EBPβ mRNA is transcriptionally mediated, we performed nuclear runoff transcription assays. In the Neuro 217 cell line more than a fivefold-higher transcription rate of the LAP/C/EBPβ mRNA was observed 30 min after forskolin stimulation (Fig. 8A and B). LAP/C/EBPβ transcriptional rates normalized 2 h after stimulation (data not shown). These results suggest that the increased transcriptional rate of the LAP/C/EBPβ gene is only a transient event during the first hour after forskolin stimulation. Runoff experiments were also performed in the NIH 3T3 cell line. As was shown for the neuronal cells, in NIH 3T3 cells higher transcription rates were also found 30 min and 1 h after stimulation (Fig. 8C and D). Therefore, the results in both cell lines strongly suggest that transcription of the endogenous LAP/C/EBPβ gene is enhanced by activating the PKA pathway mediated by CREB binding to its promoter.

CREB Ser133 phosphorylation is associated with increased LAP/C/EBPβ gene transcription during liver regeneration. Finally, we were interested in investigating whether CREB phosphorylation is functionally linked to increased LAP/C/EBPβ gene transcription in vivo. In earlier experiments we have shown by Northern blot analysis that higher LAP/C/EBPβ mRNA levels were found during liver regeneration after two-thirds hepatectomy (42). Therefore, we studied whether an increase in CREB Ser133 phosphorylation is associated with increased LAP/C/EBPβ gene transcription.

Western blot analysis with rat liver cell nuclear extracts was performed before and at different time points after hepatectomy. CREB Ser133 phosphorylation was detected with a phospho-specific CREB antibody. Strong phosphorylation of CREB Ser133 was found only 4 h after hepatectomy but not before or 12 h after surgery (Fig. 9A).

To study whether the increase in LAP/C/EBPβ mRNA levels during liver regeneration is mediated by a transcriptional mechanism, we performed runoff transcription assays with rat liver cell nuclei prepared before and at different time points after hepatectomy. As demonstrated in Fig. 9B and C, a nearly 2.5-fold increase in LAP/C/EBPβ gene transcription was found 4 h after hepatectomy. In contrast, 12 h after surgery, when no increase in CREB Ser133 was detected, LAP/C/EBPβ gene transcription was comparable to the pretreatment level. Therefore, these results show that CREB Ser133 phosphorylation is functionally linked to increased LAP/C/EBPβ gene transcription during liver regeneration. These experiments indicate that phosphorylated CREB may contribute to the induction of LAP/C/EBPβ gene transcription under different physiological conditions.

DISCUSSION

During embryonal development tissue differentiation occurs while an increasing number of transcription factors—important for maintaining tissue-specific gene regulation—are expressed. This controlled process urges the search for a transcriptional hierarchy between the different transcription factors. One of the mechanisms which is involved in controlling the differentiation process is direct transcriptional control at the promoter level. Earlier studies demonstrated that HNF-4 binds in the promoter of HNF-1, which directly regulates HNF-1 transcription in the liver (19). Thus, during the time course of embryogenesis HNF-4 is expressed earlier than HNF-1, while in a later stage of liver development members of the C/EBP protein family are found. LAP/C/EBPβ is already expressed before birth, but around and after birth its nuclear

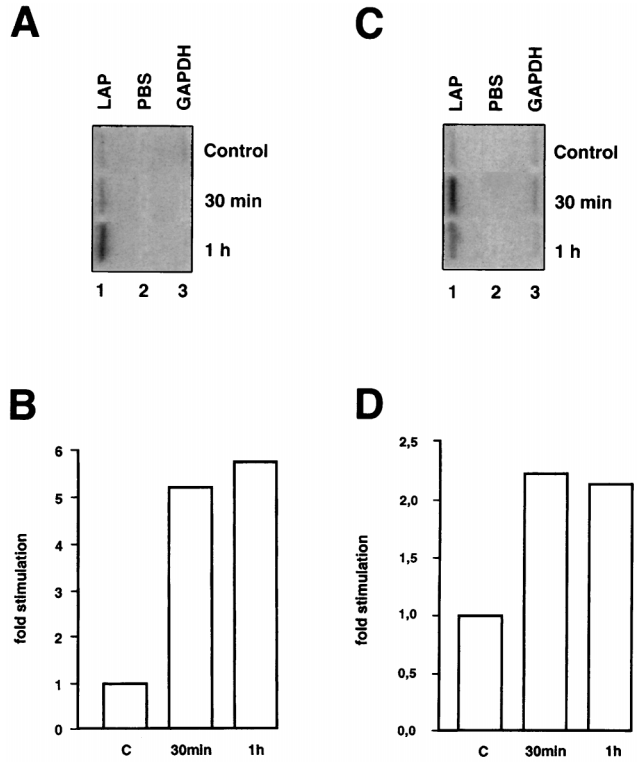

FIG. 8. Forskolin stimulates transcription of the endogenous LAP/C/EBPβ gene. (A and C) Neuro 217 (A) or NIH 3T3 (C) cells were treated with forskolin. Before (Control) or 30 min and 1 h after treatment the nuclei were isolated and runoff transcription assays with [α-^{32}P]UTP were performed. RNA was extracted and hybridized with DNA for LAP/C/EBPβ (lane 1), pBluescript (PBS; lane 2), or GAPDH (lane 3). (B and D) The ratio between the LAP/C/EBPβ and GAPDH signals was calculated and set to 1 for untreated (c) Neuro 217 (B) or NIH 3T3 (D) cells. The changes after forskolin stimulation are shown as fold stimulation.

expression is dramatically enhanced (10). Therefore, different regulatory mechanisms could be important in controlling LAP/C/EBPβ expression in the liver at a transcriptional and post-transcriptional level.

In the present study we show that CREB directly controls LAP/C/EBPβ transcription by binding to two elements in its promoter. Only five out of eight nucleotides in each of these two elements are conserved compared to eight of eight in the consensus DNA-binding site of CREB. However, gel shift experiments showed that both sites in the LAP/C/EBPβ promoter bind CREB with the same affinity as those in the CREB consensus oligonucleotide.

In a human hepatoma cell line and in the Neuro 217 cell line (data not shown) CREB is the main factor which binds to these two elements in the LAP/C/EBPβ promoter. In rat liver cell nuclear extracts a second factor besides CREB binds to both elements with a similar affinity to that of CREB. The second factor which binds to both elements is presently unknown. However, our experiments demonstrated that CREB binds to both elements even when nuclear extracts are derived from liver cells, suggesting that CREB binding in the LAP/C/EBPβ promoter may also be relevant in vivo.

Several physiological stimuli lead to phosphorylation of CREB at Ser133 and therefore regulate its ability to activate transcription. Phosphorylation occurs in response to cAMP (13), increased intracellular Ca^{2+} (35), and growth factors like nerve growth factor (12). Phosphorylation of CREB Ser133 increases binding to a spacer protein called CBP (CREB binding protein), which mediates the link to the basal transcription machinery and therefore stimulates transcription of CREB-dependent genes (3, 20). The most-studied example leading to CREB phosphorylation at Ser133 is the PKA pathway (13).

Our experiments demonstrated that in HepG2 cells the LAPPRO 8 construct mediates PKA inducibility comparably to the CRE-LUC construct, which contains two wild-type CREB consensus sites linked to a luciferase reporter gene. However, in the Neuro 217 and the HuH7 cell lines (data not shown) the increase in reporter activity after PKA stimulation was less prominent. An obvious explanation for this finding is the difference in the basal activity of the LAPPRO 8 reporter construct, which was observed in the cell lines included in this study. In the HepG2 cell line reporter activity is low and comparable to that of the CRE-LUC reporter construct. In contrast, in the Neuro 217 and the HuH7 (data not shown) cell lines basal reporter activity of the LAPPRO 8 construct is high. The stimulation after PKA induction is only three- to fourfold higher, whereas the absolute luciferase activity is approximately 10-fold higher than the activity of the CRE-LUC con-

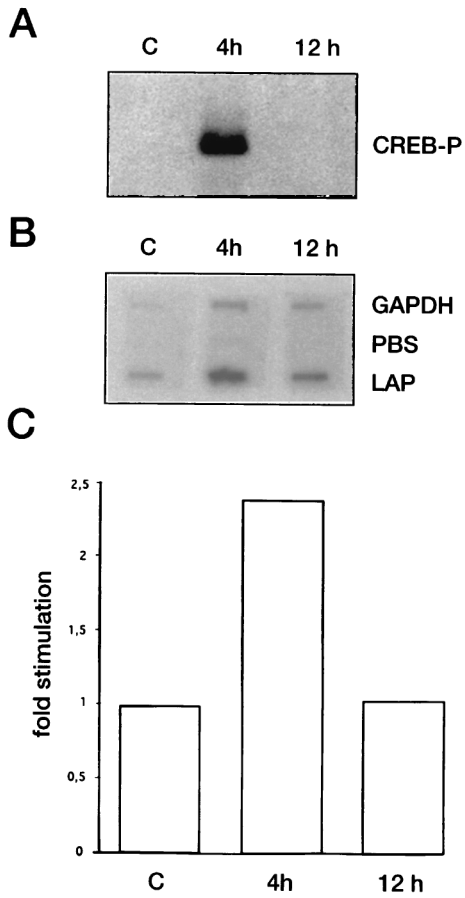

FIG. 9. CREB Ser133 phosphorylation is associated with increased LAP/C/EBPβ gene transcription during liver regeneration. (A) Western blot analysis of rat liver cell nuclear extracts was performed with a phospho-specific (Ser133) CREB antibody before (C) or 4 or 12 h after two-thirds hepatectomy. (B) Rat liver cell nuclei were isolated before (C) or 4 or 12 h after two-thirds hepatectomy, and runoff transcription assays with [α-^{32}P]UTP were performed. RNA was extracted and hybridized with DNA for LAP/C/EBPβ (LAP), pBluescript (PBS), or GAPDH. (C) The ratio between the LAP/C/EBPβ and the GAPDH signals was calculated and set to 1 for control rat liver cell nuclei. The changes after two-thirds hepatectomy are shown as fold stimulation.

struct after stimulation. Additionally, in the Neuro 217 and the HuH7 cell lines basal activity in the LAPPRO 8 MUT construct drops dramatically when both CREB sites are mutated, and its activity is similar to that of the vector control alone. Therefore, the difference found between these cell lines indicates that there might be an additional, more cell-type-specific mechanism which changes basal promoter activity.

In several promoters at least two CREB sites are found in close proximity (12, 30). In the LAP/C/EBPβ promoter the mutation of one site abolishes the basal promoter activity and PKA inducibility is only twice the promoter activity without stimulation. Therefore, the assembly of two CREB sites in the LAP/C/EBPβ promoter is functionally synergistic. A possible explanation for the synergistic effect is that binding of CREB at two sites in close proximity in the LAP/C/EBPβ promoter stabilizes the bridging protein CBP in the complex, and thus, interaction with the basal machinery is more effective, which leads to higher gene transcription.

Our experiments indicated that the two CREB sites are necessary for basal and PKA-mediated transcription of the LAP/C/EBPβ gene. To further exclude the possibility that the GC-rich flanking regions around the first CREB site may also influence basal activity or PKA inducibility, we constructed the deletion mutant LAPPRO 8 WTΔ. Additionally, cotransfection experiments with an SP1 expression vector were performed. The results indicated that only the two CREB sites are important to maintain basal activity and PKA inducibility of the LAP/C/EBPβ promoter.

The gel shift experiments with rat liver cell nuclear extracts suggested that a second factor may bind to both CREB sites in the LAP/C/EBPβ promoter. Therefore, we studied the role of another CREB/ATF family member in promoter activity. Cotransfection experiments with an ATF1 expression vector showed no effect on the LAP/C/EBPβ promoter. At present, the second factor is not further characterized and we have not yet identified the factor which regulates the basal transcription.

A wide variation in the functional response of CREB sites has been observed. This variation might be due either to differences in the immediate flanking sequences or to interactions between protein-protein or DNA-protein complexes. The possibility that other factors might be important in modulating the effect of CREB on LAP/C/EBPβ promoter activity is interesting. It has been demonstrated that the different C/EBP family members, especially, might play an important role in mediating the PKA inducibility in different, also liver-specific, promoters. Interestingly, cooperation of CREB with C/EBP family members seems important even for mediating tissue-specific inducibility by PKA (16, 32). At least two sites in the LAP/C/EBPβ promoter, located 5′ upstream of the two CREB sites, seem functionally relevant for mediating an autoregulatory loop (8). Therefore, cooperation of CREB with a C/EBP family member(s) could be important in modulating the PKA effect on LAP/C/EBPβ transcription.

Northern blot analysis and runoff transcription assays demonstrated that PKA inducibility of the LAP/C/EBPβ gene, which was suggested by the promoter experiments, is physiologically relevant, because the endogenous LAP/C/EBPβ gene is also transcriptionally controlled by the PKA pathway. Induction of LAP/C/EBPβ mRNA transcription was rapid and transient, and after as little as 2 h, pretreatment transcription levels were found. The kinetics of increased LAP/C/EBPβ gene transcription mediated through CREB are not cell type specific, as stimulation experiments were performed in two cell lines derived from different tissues. Relatively high mRNA levels persisted for several hours after the transcription rate returned to pretreatment level. The estimated half-life of the mRNA after forskolin stimulation was around 7 h. The half-life of the LAP/C/EBPβ mRNA in the Neuro 217 cell line, determined by actinomycin D transcriptional inhibition experiments, was approximately 3.5 h (data not shown). This difference raises the possibility that an additional mechanism may stabilize LAP/C/EBPβ mRNA after forskolin treatment.

Our final experiments showed the functional link between CREB Ser133 phosphorylation and increased LAP/C/EBPβ gene transcription during liver regeneration. Western blot analysis of rat liver cell nuclear extracts with a phospho-specific (Ser133) antibody showed strong Ser133 phosphorylation of CREB 4 h after two-thirds hepatectomy. Runoff transcription assays with rat liver cell nuclei resulted in a nearly 2.5-fold increase in LAP/C/EBPβ transcription at the same time point. These experiments indicate that the two CREB sites in the LAP/C/EBPβ promoter are important in increasing LAP/C/EBPβ mRNA during liver regeneration. The cAMP-PKA signal cascade leads to the phosphorylation of CREB at Ser133 and therefore to enhanced transcription of CREB-dependent target genes (13). An increase in hepatic cAMP is described in the early transition phase during liver regeneration when hepatocytes are triggered by several growth-regulatory mechanisms to leave the G_0 state and enter the cell cycle (11, 43). The exact pathway which leads to high cAMP levels is not yet defined, but high levels of growth factors like hepatocyte growth factor (23) or cytokines, e.g., transforming growth factor α (29, 33) and interleukin 6 (IL-6) (41) are found. The increase in CREB Ser133 phosphorylation shown by Western blot analysis correlates with the rise in cAMP levels (11). These observations show that during liver regeneration the cAMP-PKA signal pathway may regulate CREB phosphorylation and thereby increase LAP/C/EBPβ transcription.

CREB has been postulated to regulate genes which are involved in energy metabolism, like, e.g., the PEPCK [phosphoenolpyruvate carboxykinase (GTP)] gene (30). Gluconeogenesis does not occur until birth, and subsequently a rise in hepatic cAMP, activation of PKA, and increased phosphorylation of CREB Ser133 occurs, which initiates transcription of the PEPCK gene in the liver (24). A similar time course as for PEPCK regulation was observed for LAP/C/EBPβ. Only minor LAP/C/EBPβ concentrations were found before birth, but during and after birth high expression was evident (10). Therefore, the two described CREB sites in the LAP/C/EBPβ promoter might contribute to the time course of LAP/C/EBPβ gene regulation after birth.

In addition to its role in cAMP-dependent gene regulation, a role for CREB has been suggested in many processes involved in growth control and differentiation (12). Growth factor-induced phosphorylation of CREB Ser133 contributes to the activation of immediate early genes. Recently, growth factor activation of CREB via the RAS/MAPK pathway was described and RSK2, a member of the ribosomal S6 kinase family, was identified as the responsible CREB kinase (49). During the early phase of liver regeneration, where high levels of growth factors are found, CREB phosphorylation could therefore be mediated by the MAPK as well as by the PKA pathway. Further studies are necessary to analyze the signal transduction pathway(s) which leads to enhanced LAP/C/EBPβ transcription via CREB during liver regeneration.

Mediators of the acute-phase response, like IL-6, have been reported to increase LAP/C/EBPβ transactivation at the posttranslational level via phosphorylation (26, 28, 51). Moreover, they have been shown to stimulate LAP/C/EBPβ gene transcription (1, 2, 8). The contribution of CREB binding sites in regulating the cytokine response is becoming more obvious. CREB and ATF1 phosphorylation via the stress-induced p38

MAP kinase-RK pathway was recently observed (36). ATF2 shows stress kinase-dependent activation which is induced by tumor necrosis factor alpha or UV radiation (14, 22, 44). In the IL-1 gene a CREB binding site is important in conferring lipopolysaccharide (LPS) inducibility (6). In the cytokine response, most likely mediated through the stress pathway, CREB could be important in mediating transcriptional activation of LAP/C/EBPβ.

Several pathways control CREB phosphorylation, and therefore, our results provide evidence for the transcriptional regulation of LAP/C/EBPβ via CREB under different physiological conditions.

ACKNOWLEDGMENTS

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