Growth hormone (GH), which is reduced with age, corrects the impaired proliferative capacity of livers of old animals. In this paper, we present a mechanism by which GH eliminates age-dependent negative control of proliferation and increases transcription of liver-specific genes in livers of old mice. The reduced proliferative capacities of the liver of old animals are associated with the CCAAT/enhancer-binding protein α (C/EBPα)-Brm complex, which inhibits E2F-dependent promoters. We found that a sequestration of C/EBPα into complexes with Brm leads to a weak interaction of C/EBPα with promoters of liver-specific genes, expression of which is reduced in old animals. Injection of either GH or the regulator of the amplitude of endogenous GH release, ghrelin, reduces the C/EBPα-Brm complex in livers of old mice, leading to a derepression of E2F targets, to increased interactions of C/EBPα with promoters of liver-specific genes, and to correction of their expression. GH-dependent elimination of the complex is mediated by the inhibition of cyclin D3-CDK4 activity and by elevation of a phosphatase, protein phosphatase 2A, which dephosphorylates C/EBPα and dissociates the complex.

C/EBPα family proteins regulate liver proliferation and differentiation. Two members of C/EBP family, C/EBPα and C/EBPβ, are involved in molecular pathways that cause an aging phenotype in tissue culture systems and in the liver (1–4). C/EBPα and C/EBPβ interact with the promoters of liver-specific genes and activate expression of corresponding proteins. In addition to activation of liver-specific promoters, C/EBPα plays a key role in the inhibition of liver proliferation (5–8). Examination of C/EBPα knock-out mice showed that C/EBPα is required for the inhibition of hepatocyte proliferation in newborn animals and after birth (7). Biological functions of C/EBPα during prenatal development of the liver depend on the cell type in which C/EBPα is expressed. For example, examination of proliferation in adult animals of C/EBP knockout mice at day 17 of gestation revealed that the deletion of C/EBPα causes a 3.5-fold increase of proliferation in hepatocytes, whereas biliary epithelial cells do not show significant change in the rate of proliferation (9). C/EBPα is also required for the inhibition of liver growth in adult mice. Experimental work from Wang’s group (6) demonstrated that growth-inhibitory activity of C/EBPα in adult animals protects livers from development of tumors and that this activity is mediated by inhibiting CDK2 and by an induction of protein levels of p21 in nuclei. Taken together, these studies revealed that liver-specific mechanisms of C/EBPα growth arrest in young animals involve direct interactions of C/EBPα with cell cycle-dependent kinases (10, 11) and with p21 (6, 7). Evaluation of C/EBPα animal models suggests a critical role for C/EBPα in aging. For example, deletion of C/EBPα in adult animals leads to the development of an aging phenotype in the liver (12, 13). Consistent with these findings, we and others have observed that aging changes pathways of C/EBPα growth arrest in young animals involve direct interactions of C/EBPα with cell cycle-dependent kinases (10, 11) and with p21 (6, 7). Evaluation of C/EBPα animal models suggests a critical role for C/EBPα in aging. For example, deletion of C/EBPα in adult animals leads to the development of an aging phenotype in the liver (12, 13). Consistent with these findings, we and others have observed that aging changes pathways of C/EBPα growth arrest in young animals involve direct interactions of C/EBPα with cell cycle-dependent kinases (10, 11) and with p21 (6, 7). Evaluation of C/EBPα animal models suggests a critical role for C/EBPα in aging. For example, deletion of C/EBPα in adult animals leads to the development of an aging phenotype in the liver (12, 13).
GH in the regulation of liver proliferation has been demonstrated by several studies, but the molecular pathways by which GH promotes proliferation are not well understood.

In this paper, we investigated the effects of GH on the C/EBPα-Brm complex and on the expression of liver-specific genes. In livers of old mice, the major portion of C/EBPα is associated with Brm and located on E2F-dependent promoters, where the complex represses transcription of cell cycle genes, such as c-Myc and FoxM1B. The sequestration of C/EBPα associated with Brm and located on E2F-dependent promoters, C/EBPα of PEPCK transcription. We have found that GH eliminates the reduced in livers of old mice, which correlate with a reduction of free C/EBPα capable of activating liver-specific promoters. Particularly, the interactions of C/EBPα with PEPCK and HNF6 promoters are reduced in livers of old mice, which correlate with a reduction of PEPCK transcription. We have found that GH eliminates the C/EBPα-Brm complex by a phosphorylation-dependent mechanism that involves alterations of at least two pathways: activation of PI3K-Akt-P2PA and a reduction of protein levels of cyclin D3, leading to a reduced activity of CDK4. The GH-mediated elimination of the complex leads to the derepression of E2F targets, to an increased association of C/EBPα with HNF6 and PEPCK promoters, and to correction of PEPCK expression. Ghrelin treatment of old mice, which provides a more physiological approach to GH replacement during aging, also restores the young liver phenotype in old mice.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies to cyclins D1, D2, and D3 and C/EBPα (14AA and N19); CDK6 and CDK4 (C-22); CDK2 (M2); Brm and Rb (C-15); HNF6 (H-100); peroxisome proliferator-activated receptor α (H-98); and glucokinase (H-88) were purchased from Santa Cruz Biotechnology Co. Antibodies to PP2A were from Signal Transduction Laboratories. Antibodies to PEPCK and Glycogen Synthase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to P2PA were from Signal Transduction Laboratories. Antibodies to PEPCK and Glycogen Synthase were from Cayman Chemical Co. and Chemicon International, respectively.

**Animals and Growth Hormone Studies**—Animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol AN-1439). In this paper, we have used young (4–6-month-old) and old (22–24-month-old) mice. Examination of C/EBPα-CDK and C/EBPα-Brm complexes was performed with five or six animals of each age group. Data for expression of liver-specific mRNAs and proteins in livers of young and old mice were obtained with 5–9 animals of each age group. Mouse recombinant growth hormone or ghrelin (1 mg/kg) was injected into old animals subcutaneously twice a day (at 8:30 a.m. and 8:30 p.m.) for 7 days. GH was purchased from the National Hormone and Peptide Program, and physiological saline (0.9% NaCl) was used as vehicle for GH and ghrelin and as the control. Twice a day injections provides relatively stable circulating GH levels during the whole time period. Under these conditions, injection of ghrelin causes biologically relevant release of GH (18). Examination of the C/EBPα-Brm complex, protein levels, and phosphorylation isoforms of C/EBPα were performed as described below. Data represent studies of four GH-treated, four ghrelin-treated, and four control animals.

**Protein Isolation and Western Blotting**—Nuclear extracts were isolated as described in previous papers (1, 4, 10). Proteins (50–100 μg) were added on gradient (4–20% or 8–16%) polyacrylamide gel, transferred on the membrane, and probed with antibodies to proteins of interest. To verify protein loading, each filter was reprobed with β-actin and then stained with Coomassie Blue. Levels of protein expression were calculated as ratios to β-actin.

**Gel Filtration Analysis of Protein-Protein Complexes in Mouse Liver**—The detailed procedure for the analysis of protein-protein complexes is described in our previous papers (1, 4). Nuclear extracts were isolated from livers of young and old mice as described (7) and fractionated by size exclusion column SEC450 (high pressure liquid chromatography; BioLogic HR; Bio-Rad). Locations of the proteins within gel filtration factions and C/EBPα complexes were examined by Western blotting and co-IP assays.

**Gel Shift Assay**—Examination of the interaction of E2F-Rb complexes with the FoxM1B promoter was performed as described in our previous papers (1, 19). DNA probes (see Fig. 5) were incubated with nuclear extracts from 3T3-L1 cells in the presence of salmon DNA as a competitor and loaded on a native 5% polyacrylamide gel.

**Examination of C/EBPα Phosphorylation by Two-dimensional Gel and Western Blotting with Ser(P)193-specific Abs**—To examine expression of the C/EBPα-Ser(P)193 isoform, C/EBPα was first immunoprecipitated from liver nuclear extracts with antibodies to total protein and then examined by Western blotting with antibodies to Ser(P)193. To confirm data of the IP-Western blot with Ser(P)193 Abs, we have also used a two-dimensional Western assay as described in our previous papers (4, 19).

**Real Time Quantitative Reverse Transcriptase-PCR**—Total RNAs were isolated from mouse livers using Trizol reagent (Invitrogen). The RNA was treated with RNase-free DNase (Invitrogen), and cDNA was synthesized using the oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). The real time PCRs contain 1× Brilliant SYBR Green QPCR master Mix (Stratagene), 200 nM each primer, 5% Me2SO, 1× ROX dye (Invitrogen), and synthesized cDNA. PCR amplification was performed in triplicate in a 96-well plate for each sample on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The relative expression of PEPCK was normalized to glyceraldehyde-3-phosphate dehydrogenase. The sequences of PCR primers are as follows: PEPCK, 5′-TCCCTGGACCTCAGT-GAAAGACAAA-3′ (forward) and 5′-TGTCTTTCCGGAGGCA-GTTGACAT-3′ (reverse), producing a 554-bp fragment; glyceraldehyde-3-phosphate dehydrogenase, 5′-AACCTGGCAT-TGTGGAAGGGCCTC-3′ (forward) and 5′-TGGAAGAGTGGGAGTTGCTGTGTA-3′ (reverse), producing a 382-bp fragment.

**Coimmunoprecipitation**—C/EBPα was immunoprecipitated from nuclear extracts with polyclonal antibodies (14AA; Santa Cruz Biotechnology), and the presence of Rb, Brm, E2F4, and C/EBPα IPs was examined by Western blotting with monoclonal antibodies to the mentioned proteins.

**Chromatin Immunoprecipitation (ChiP) Assay**—The chromatin immunoprecipitation assay was performed using the ChiP-IT kit (Active Motif). The chromatin solutions were isolated from livers of young and old animals, and DNA was sheared by enzymatic digestion according to the instruction.
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The size of DNA fragments produced averaged between 500 and 1000 bp in length. Antibodies against C/EBPα, E2F1, E2F4, and Rb were added to each aliquot of precleared chromatin and incubated overnight. Protein G beads were added and incubated for 1.5 h at 4 °C. After reversing the cross-linking, DNA was isolated and used for PCRs with primers specific for E2F binding sites within the c-Myc promoter and hydroxysteroid transferase (1) and FoxM1B promoter (see Fig. 5) as well as with primers to C/EBPα binding sites within the PEPC and HNF6 promoters. The sequences of the primers for these promoters are as follows: PEPC forward, 5′-GGCATCATCAGTCCGATGAGCGGG-3′; PEPC reverse, 5′-GATCGCGAGCTGGCATGGGAACTGCG-3′; HNF6 forward, 5′-GCTCGAGCGTGCGGCGGCAAGGC-3′; HNF6 reverse, 5′-AGAGGTCCAGCTTCACTAGCGGTCG-3′. As a control for the appropriate shearing of DNA, we have used primers for a region ~1.5 kb upstream of the C/EBPα site in each promoter. The sequences of these primers are as follows: PEPC-NP1-F, 5′-AACAACTGCGCTAACTCACAGA-3′; PEPC-NP2-R, 5′-GCTGACGTCCAGTATGAGCAGGAA-3′; HNF6-NP1-F, 5′-AGGCAAGCCATCTGGGTAAGAGAT-3′; HNF6-NP2, 5′-AGAGGCTTGTTGCTAGGTGCTT-3′. PCR mixtures were amplified for 1 cycle of 95 °C for 5 min, annealing temperature for primers (62 °C) for 5 min, and 72 °C for 2 min. Then PCR mixtures were amplified for 34 cycles of 95 °C for 1 min, annealing temperature for 2 min, and 72 °C for 1.5 min. PCR products were separated by 1.5% agarose gel electrophoresis or by 4% PAGE.

**Kinase Assay—Conditions for in vitro kinase assays are described in our previous publications (10, 11). Briefly, baculovirus-expressed purified CDK4-cyclin D1 and CDK6-cyclin D3 complexes or CDK4/Cdk6/cyclin D3 IPs were incubated with glutathione S-transferase-Rb or glutathione S-transferase-C/EBPα substrates in the kinase reactions with [γ-32P]ATP.**

**RESULTS**

Sequestration of C/EBPα into Complexes with Brm Reduces Association of C/EBPα with PEPC and HNF6 Promoters in Livers of Old Mice—A number of recent publications revealed that C/EBPα displays its functions via formation of multiple protein–protein complexes. Accordingly, we have performed a detailed examination of the amounts of C/EBPα in protein–protein complexes in livers of young and old mice. In livers of young animals, C/EBPα forms complexes with CDK2 and CDK4 (11). Therefore, we determined the relative amounts of C/EBPα complexes with CDKs and free C/EBPα using size exclusion chromatography/Co-IP. After separation of C/EBPα and C/EBPα–CDK complexes on the SEC450 column, CDK2 and CDK4 were simultaneously precipitated from each fraction, and C/EBPα was examined in CDK IPs and in supernatants. As seen in Fig. 1A, C/EBPα is observed in CDK complexes and in supernatant. Densitometric calculations demonstrated that 20–25% of C/EBPα is in complexes containing CDKs, whereas 75–80% of C/EBPα is CDK-free and is observed in the supernatant.

We next performed an examination of C/EBPα in livers of old mice, and the results of these studies are presented in Fig. 1B. After fractionation on a SEC450 column, Brm was immunoprecipitated from each fraction, and the amount of C/EBPα in IPs and in supernatants was determined by Western blotting. Consistent with previous studies, C/EBPα–Brm complex was observed in the high Mr region of gel filtration. Surprisingly, no C/EBPα was observed in supernatants after precipitation of Brm. Three independent experiments with protein extracts from livers of three old mice reproducibly showed no or very little C/EBPα in supernatants of the gel filtration fractions following immunoprecipitation of Brm complexes. Calculations of the amount of C/EBPα showed that around 94–95% of C/EBPα is associated with Brm within the high Mr, C/EBPα–Brm complex. Interestingly, the co-IP assay detected a small portion of C/EBPα (5–6%) in Brm IPs from gel filtration fractions with a molecular mass around 200 kDa. We suggest that this complex consists of C/EBPα and Brm only and might be a result of partial dissociation of the high Mr, C/EBPα–Rb-E2F4-Brm complex.

We next examined interaction of C/EBPα with E2F-dependent promoters (as a component of the C/EBPα–Brm complex) and the interactions of C/EBPα with PEPC and HNF6 promoters in livers from old mice. It has been previously shown that C/EBPα binds directly to the PEPC and HNF6 promoters (12, 13, 20). E2F1, E2F4, C/EBPα, and Rb were immunoprecipitated from chromatin solutions isolated from livers of young and old mice, and these IPs were used in PCRs with primers to c-Myc (E2F-dependent), HNF6, and PEPC (C/EBP-dependent) promoters. The results of these studies are shown in Fig. 1C. In agreement with previous observations, C/EBPα is not detectable on the c-Myc promoter in young livers, but only in liver it is associated with the E2F site of the c-Myc promoter as a component of the C/EBPα–Rb–E2F4–Brm complex. Examination of these IPs with primers to the HNF6 and PEPC promoters showed that only C/EBPα is associated with these promoters in livers of young mice. A weak association of Rb was also observed on the HNF6 promoter in livers from young mice. These data revealed that the C/EBPα–Brm complex does not interact with PEPC and HNF6 promoters in aging liver. Examination of C/EBPα complexes in livers from old mice by ChIP assays showed markedly different results. Within the sensitivity of our assays, we could not detect interactions of C/EBPα with HNF6 promoter in livers from old mice. Although C/EBPα is detected on the PEPC promoter in livers from old mice after 35 PCR cycles, a quantitative examination of the amounts of C/EBPα showed a significant difference. The PCR product of the PEPC promoter is detected after 22–24 PCR cycles in C/EBPα IPs from livers of young mice, whereas detection of the PEPC promoter in C/EBPα IPs from livers of old animals requires up to 28–30 cycles (Fig. 1D). To verify the quality of chip protocol, we have examined the association of C/EBPα complexes with hydroxysteroid transferase promoter. This promoter has been previously shown to interact with E2F1 and C/EBPα independently of E2F–Rb complexes (21) and age of mice (1). As can be seen in Fig. 1, C and D, the occupation of this promoter by E2F1 and C/EBPα is not altered in livers of old mice. Taken together, the ChIP results for the HNF6 and PEPC promoters indicate that the interaction of C/EBPα with these promoters is reduced in livers from old mice,
presumably due to sequestration of C/EBPα into the C/EBPα-Brm complex. Expression of Transcriptional Targets of C/EBPα in Old Livers—Given the reduced interaction of C/EBPα with promoters of HNF6 and PEPCK genes, we examined their expression in young and old livers. In addition to these proteins, we analyzed peroxisome proliferator-activated receptor α and glucokinase, since C/EBPα regulates their expression through direct binding to the promoters (13). Western blotting showed that protein levels of glucokinase and peroxisome proliferator-activated receptor α are not changed; however, protein levels of PEPCK are significantly reduced in old livers (Fig. 2). Examination of transcripts for these direct targets of C/EBPα showed that levels of PEPCK mRNA are also reduced to 25–30% compared with levels observed in livers of young mice. Since C/EBPα is a positive regulator of the PEPCK promoter (12, 13) and because free C/EBPα is reduced in old livers, these data suggest that a recruitment of C/EBPα into the C/EBPα-Brm complex might cause the reduction of PEPCK. To further test this hypothesis, we have examined whether elimination of the C/EBPα-Brm complex in livers of old mice corrects expression of direct targets of C/EBPα.

GH Eliminates C/EBPα-Brm Complex in Livers from Old Mice by D Dephosphorylation of C/EBPα—Considering possible mechanisms for elimination of the C/EBPα-Brm complex, we sought a pathway that eliminates the complex but does not change C/EBPα protein levels. Several observations suggested that GH signaling might be involved. First, GH treatment corrects liver proliferation in old animals (16), suggesting that GH might inhibit C/EBPα-Brm complex formation. Second, in mice, where GH action is blocked, the liver does not proliferate after PH in the absence of GH (17). Therefore, we tested whether GH treatment of old mice eliminates the C/EBPα-Brm complex in the liver. 24-month-old animals were treated with GH for 7 days, and livers were harvested from control (saline-injected) and GH-treated mice. We first examined the age-specific C/EBPα-Brm complex by size exclusion chromatography followed by examination of C/EBPα and Brm in the size exclusion fractions. Fig. 3A shows that in control animals, C/EBPα and Brm co-localize in high M, fractions; however, in liver
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Extracts from GH-treated animals, C/EBPα is no longer detectable in high \( M_1 \) fractions and is shifted to low \( M_1 \) complexes or free proteins. Interestingly, the position of Brm within fractions was not changed by GH treatment. The GH-mediated shift of C/EBPα is specific, since the position of cross-reactive (CRM) protein (on C/EBPα blot) is not altered by GH (Fig. 3A). Co-immunoprecipitation showed that Brm is present in C/EBPα IPs from high \( M_1 \) fractions of control livers, but Brm is not detectable in C/EBPα IP from size exclusion fractions from livers of GH-treated mice, showing that GH disrupts the C/EBPα-Brm complex. To confirm these observations, we performed direct co-immunoprecipitation studies. Fig. 3B shows that Rb, E2F4, and Brm are observed in C/EBPα IPs from control livers, whereas these proteins are not detectable by Western blotting in C/EBPα IPs from the livers of old animals treated with GH. Note that although the association of C/EBPα with Rb is reduced by GH, it might be detected after long exposure (see Fig. 7). Thus, these results demonstrate that treatment of animals with GH eliminates the C/EBPα-Brm complex.

To examine pathways by which GH neutralizes the complex, we determined protein levels of the components of the C/EBPα-Brm complex. Western blotting of nuclear extracts from control animals and from animals treated with GH showed no significant changes in the protein levels of Brm, C/EBPα, Rb, and E2F4 (Fig. 3C). Since our previous studies showed that phosphorylation of C/EBPα at Ser\(^{193} \) is critical for the interaction with Brm (4, 10), we next examined the phosphorylated status of C/EBPα in livers from old mice treated with GH. Two approaches were used for these studies: Western blotting with antibodies to the Ser\(^{193} \)-phosphorylated form of C/EBPα and two-dimensional gel separation. C/EBPα was immunoprecipitated with antibodies to total protein and then examined by Western blotting with Ser\(^{193} \) Abs and with Abs to total C/EBPα, as described in our previous paper (4). Fig. 3D shows that, although total amounts of C/EBPα are not altered in livers of GH-treated mice, the amounts of Ser\(^{193} \) isoform are dramatically reduced. In agreement with this observation, two-dimensional examination of C/EBPα showed no Ser\(^{193} \) isoform in old GH-treated animals (Fig. 3E). Thus, these results of two independent methods showed that GH eliminates the Ser\(^{193} \) isoform of C/EBPα, leading to a disruption of the age-specific complex.

**GH Reduces CDK4/CDK6 Activity in Old Livers by Down-regulation of Cyclin D3**—We have shown that the presence of a phosphate on Ser\(^{193} \) depends on the balance of activities of CDK4/CDK6 and PP2A and that aging increases phosphorylation of Ser\(^{193} \) by activation of CDK4/CDK6 through elevation of cyclin D3 (4). Therefore, we have next determined the effects of GH on expression of cyclin D3 and on kinase activities associated with cyclin D3. Western blotting with specific antibodies showed that protein levels of cyclin D3 are reduced in livers from old mice treated with GH (Fig. 4A). A series of multiple experiments showed a 3–4-fold reduction compared with levels in control animals. We then examined kinase activity associated with cyclin D3 in livers from old mice following GH treatment. Cyclin D3 was immunoprecipitated with specific Abs and examined in a kinase assay using glutathione S-transferase-Rb as substrate. Fig. 4B shows a typical result of these studies. GH treatment reduces activity of kinases associated with cyclin D3, which correlates with the reduction of protein levels of cyclin D3. Thus, these studies demonstrate that GH reduces cyclin D3-CDK4/6 activity in livers of old mice.

**GH Increases Nuclear Concentrations of PP2A, Which Dephosphorylates C/EBPα**—The phosphorylation of Ser\(^{193} \) of C/EBPα is also controlled by PI3K-Akt-PP2A (10). Therefore, we examined this pathway in livers from old mice treated with GH. Since Akt-1 is a target of PI3K (22), we performed Western blotting of protein extracts with antibodies specific to “active,” Ser\(^{473} \)-phosphorylated Akt1. These studies showed that the amount of phosphorylated Akt1 is increased in animals treated with GH (Fig. 4C). Since activation of PI3K-Akt pathway increases levels of PP2A in nuclei (10), we next examined PP2A in nuclear extracts of control and GH-treated mice by Western blotting with antibodies to a catalytic subunit of PP2A, 36-kDa subunit C. As can be seen in Fig. 4D, 36-kDa PP2A subunit C is mainly a cytoplasmic protein in control animals; however, in GH-treated mice, nuclear concentration of PP2A is increased.

To determine if GH-mediated accumulation of PP2A in nuclei dephosphorylates C/EBPα, PP2A was immunoprecipitated from livers of GH-treated mice and incubated with Ser\(^{193} \)-C/EBPα substrates obtained from different biological systems. The first substrate tested was C/EBPα, which was overexpressed in 3T3-L1 cells, where it is phosphorylated at...
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Ser193 (10). The second substrate was the C/EBPα-Brm complex purified from livers of old mice; C/EBPα-Ser(P)193 is the major isoform of C/EBPα within this complex (4). As can be seen in Fig. 4E, PP2A IP from livers of old GH-treated mice dephosphorylates these C/EBPα substrates at Ser193. These data are consistent with the hypothesis that GH-mediated elevation of PP2A disrupts the C/EBPα-Brm complex through dephosphorylation of C/EBPα at Ser193. Taken together, our data show that dephosphorylation of C/EBPα at Ser193 is mediated by a change in the balance of cyclin D3/PP2A (Fig. 4F).

C/EBPα-Brm Complex Inhibits Fox M1B Promoter—We next examined if elimination of the C/EBPα-Brm complex by GH leads to derepression of genes that control liver proliferation. Several key proteins are required for a proper proliferation of the liver after PH (23). Studies from Costa’s laboratory (24) demonstrated that transcription factor FoxM1B is a key positive regulator of liver proliferation after PH. Moreover, experiments with FoxM1B knock-out mice demonstrated that FoxM1B is involved in GH-mediated acceleration of liver proliferation in livers of old mice (16). Therefore, we hypothesized that expression of FoxM1B might be repressed in old animals by the C/EBPα-Brm complex. To test our hypothesis, we carefully investigated the mouse FoxM1B promoter. Since it had been shown that 300 bp of the FoxM1B promoter are sufficient to drive cell cycle-dependent expression of this gene (25), we cloned 783 bp of the mouse FoxM1B promoter into a reporter vector and examined its regulation by the C/EBPα-Brm complex. The nucleotide sequence of the proximal 300 nucleotides of the mouse FoxM1B promoter is shown in Fig. 5A. Because the C/EBPα-Rb-E2F4-Brm complex binds to E2F consensus sequences, we first performed a search for these sequences and found an element (located between −93 and −115) (Fig. 5A) that interacts with E2Fs. A typical gel shift assay with a DNA probe containing this E2F site is shown in Fig. 5B. WT and E2F mutant (CGC to TAT) (Fig. 5B, top) were incubated with nuclear extracts from 3T3-L1 cells that contained free E2F4 and E2F4-p130 complexes. The results show that E2F4 and E2F4-p130 complexes interact with the wild type FoxM1B promoter, since antibodies to E2F4 and to p130 supershifted the corresponding complexes. Mutation of the E2F site completely abolished these interactions.

We next examined if the identified E2F binding site of the FoxM1B promoter might serve as a negative regulator of transcription. In the course of these studies, we found that the surrounding region also contains a weak site which interacts with C/EBP family proteins and that this sequence acts as a positive element in cultured cells (see Fig. 5A). To distinguish the effect of the C/EBPα-Brm complex from that of direct binding of C/EBPα, we generated additional mutants of the FoxM1B promoter, in which C/EBP and E2F sites were mutated. Examination of a basal activity of the WT FoxM1B promoter and activities of these mutants in HEK293 cells is shown in Fig. 5B. FoxM1B promoter constructs

![Figure 3](image-url)

**FIGURE 3.** Growth hormone eliminates the age-specific C/EBPα-Brm complex by dephosphorylation of C/EBPα at Ser193. A, treatment of old animals with GH dissociates the age-specific C/EBPα-Brm complex. As can be seen in Fig. 4E, PP2A IP from livers of old GH-treated mice dephosphorylates these C/EBPα substrates at Ser193. These data are consistent with the hypothesis that GH-mediated elevation of PP2A disrupts the C/EBPα-Brm complex through dephosphorylation of C/EBPα at Ser193. Taken together, our data show that dephosphorylation of C/EBPα at Ser193 is mediated by a change in the balance of cyclin D3/PP2A (Fig. 4F).

C/EBPα-Brm Complex Inhibits Fox M1B Promoter—We next examined if elimination of the C/EBPα-Brm complex by GH leads to derepression of genes that control liver proliferation. Several key proteins are required for a proper proliferation of the liver after PH (23). Studies from Costa’s laboratory (24) demonstrated that transcription factor FoxM1B is a key positive regulator of liver proliferation after PH. Moreover, experiments with FoxM1B knock-out mice demonstrated that FoxM1B is involved in GH-mediated acceleration of liver proliferation in livers of old mice (16). Therefore, we hypothesized that expression of FoxM1B might be repressed in old animals by the C/EBPα-Brm complex. To test our hypothesis, we carefully investigated the mouse FoxM1B promoter. Since it had been shown that 300 bp of the FoxM1B promoter are sufficient to drive cell cycle-dependent expression of this gene (25), we cloned 783 bp of the mouse FoxM1B promoter into a reporter vector and examined its regulation by the C/EBPα-Brm complex. The nucleotide sequence of the proximal 300 nucleotides of the mouse FoxM1B promoter is shown in Fig. 5A. Because the C/EBPα-Rb-E2F4-Brm complex binds to E2F consensus sequences, we first performed a search for these sequences and found an element (located between −93 and −115) (Fig. 5A) that interacts with E2Fs. A typical gel shift assay with a DNA probe containing this E2F site is shown in Fig. 5B. WT and E2F mutant (CGC to TAT) (Fig. 5B, top) were incubated with nuclear extracts from 3T3-L1 cells that contained free E2F4 and E2F4-p130 complexes. The results show that E2F4 and E2F4-p130 complexes interact with the wild type FoxM1B promoter, since antibodies to E2F4 and to p130 supershifted the corresponding complexes. Mutation of the E2F site completely abolished these interactions.

We next examined if the identified E2F binding site of the FoxM1B promoter might serve as a negative regulator of transcription. In the course of these studies, we found that the surrounding region also contains a weak site which interacts with C/EBP family proteins and that this sequence acts as a positive element in cultured cells (see Fig. 5A). To distinguish the effect of the C/EBPα-Brm complex from that of direct binding of C/EBPα, we generated additional mutants of the FoxM1B promoter, in which C/EBP and E2F sites were mutated. Examination of a basal activity of the WT FoxM1B promoter and activities of these mutants in HEK293 cells is shown in Fig. 5B. FoxM1B promoter constructs
FIGURE 4. Growth hormone reduces activity of cyclin D3-CDK4/6 and increases PP2A. A, protein levels of cyclin D3 are reduced in livers of old mice after treatment with GH. Western blotting was performed with nuclear extracts isolated from livers of two control (injected with saline) and two GH-treated animals using antibodies against cyclin D3. Short (30 s) and long (2 min) exposures are shown. The membrane was reprobed with antibodies to β-actin and stained with Coomassie Blue to verify protein loading. B, growth hormone reduces the kinase activity associated with cyclin D3. Cyclin D3 was immunoprecipitated from livers of young, old, and old GH-treated animals. The kinase activity of cyclin D3 complexes was examined in an in vitro kinase assay with glutathione S-transferase-Rb substrate. Short (16-h) and long (36-h) exposures for Rb are shown. K6/d3, baculovirus-expressed, purified cyclin D3-CDK6 complex serves as a positive control. NS, a nonspecific band (molecular mass of 33 kDa) that appears in each reaction and serves as an additional control. C, GH activates the PI3K-Akt pathway in livers of old mice. Cytoplasmic extracts from livers of two control and two GH-treated mice were examined by Western blotting with phosphospecific Abs to Akt. The membrane was reprobed with antibodies to total Akt. D, nuclear concentration of a catalytic subunit C of PP2A is increased in livers of GH-treated mice. Cytoplasmic and nuclear extracts from livers of control and GH-treated mice were examined by Western blotting with antibodies to PP2A. The membrane was reprobed with Abs to β-actin. 3T3, a positive control; nuclear extracts from 3T3-L1 cells treated with insulin (10). E, PP2A precipitated from the liver of GH-treated mice dephosphorylates C/EBPα. PP2A was immunoprecipitated with antibodies to catalytic subunit C and incubated with C/EBPα expressed in 3T3-L1 cells and with C/EBPα-Brm complex isolated from livers of old mice. A half part of each substrate was incubated with a mock-agarose control. The samples were separated on two-dimensional gel and probed with antibodies to C/EBPα. The positions of C/EBPα isoforms are shown at the top. F, a hypothetical pathway by which GH eliminates the C/EBPα-Brm complex (see “Results”).

were used to distinguish specific effects of the C/EBPα-Brm complex (Fig. 5D, top): WT C/EBPα, which forms a C/EBPα-Brm complex and binds to DNA; C/EBPα-S193A, which does not form complexes with Brm; and C/EBPα-R290A mutant, which forms the C/EBPα-Brm complex but does not interact with DNA (4, 26). Fig. 5D (bottom) shows results of co-transfection of the WT FoxM1B promoter with C/EBPα constructs into Brm-positive HEK293 cells. In these cells, WT C/EBPα represses wild type FoxM1B promoter, whereas the C/EBPα-S193A mutant (which does not form a C/EBPα-Brm complex) activates the promoter presumably through a direct binding to the promoter. The mutation of R290A enhances the ability of C/EBPα to repress the FoxM1B promoter. These repressive effects of C/EBPα are consistent with the ability of the R290A mutant to form complexes with Brm.

To examine if Brm is required for the C/EBPα-mediated inhibition of the FoxM1B promoter, the C/EBPα-R290A mutant was co-transfected with the FoxM1B promoter into Brm-negative C33A cells. As seen in Fig. 5E, C/EBPα does not repress the FoxM1B promoter in C33A cells that lack functional Brm. Thus, these studies revealed that C/EBPα represses the FoxM1B promoter and that this repression requires both an intact E2F binding site within the promoter and expression of the functional Brm.

Livers of Old Animals Fail to Remove the C/EBPα-Rb-E2F4 Complex from FoxM1B Promoter after PH—Given inhibition of the FoxM1B promoter by E2F-Rb family proteins and by the C/EBPα-Brm complex in tissue culture systems, we next examined whether this inhibition occurs in the liver. For this goal, we examined occupation of the FoxM1B promoter by the E2F-Rb and C/EBPα-Brm complexes in livers of young and old mice using chip assays. Fig. 6A shows results of these studies. In quiescent livers from young mice, E2F1, E2F4, and Rb are observed on the FoxM1B promoter, but in livers of old mice, the pro-
moter is also occupied by the C/EBPα-E2F4-Rb complex. Although the FoxM1B promoter contains a weak binding site for C/EBPα, we could not detect association of C/EBPα with the FoxM1B promoter in young livers. The presence of C/EBPα on the promoter in livers of old mice seems to be mediated through the C/EBPα-E2F4-Rb complex, since elimination of the complex by GH (which does not change protein levels of C/EBPα) (see Fig. 3) removes C/EBPα from the promoter (see below).

Proliferation of the liver after PH is regulated by a complex cooperation of several networks (23). Since expression of FoxM1B after PH is reduced in livers of old mice (24) but is restored by GH treatment (16), we investigated whether the C/EBPα-E2F4-Rb complex might be involved in age-dependent repression of the FoxM1B after PH. Chromatin solutions were isolated from livers of young and old mice at 8 h after PH, and occupation of the FoxM1B promoter was examined by ChIP assay. Fig. 6A shows that in livers of young mice, Rb-E2F complexes are not detectable on the FoxM1B promoter 8 h after PH, whereas C/EBPα-Rbr complexes are observed on the FoxM1B promoter in livers of old mice after PH. Because the C/EBPα-Rbr complex inhibits the FoxM1B promoter in cultured cells, and in old livers transcription of FoxM1B is not increased following PH, these data suggest that the C/EBPα-Rbr complex represses the FoxM1B promoter in livers of old mice.

**GH-mediated Elimination of C/EBPα-Rbr Complex Releases C/EBPα from E2F Targets, Directs C/EBPα to Liver-specific Promoters, and Corrects Transcription of PEPCK**—We next asked if GH-mediated disruption of the C/EBPα-Rbr complex might lead to a redirection of C/EBPα to liver-specific promoters. To examine this hypothesis, ChIP assays were performed with E2F-dependent c-Myc and FoxM1B promoters and with C/EBPα-dependent PEPCK and HNF6 promoters. As seen in Fig. 6B, GH treatment releases C/EBPα from c-Myc and FoxM1B promoters and increases the amount of C/EBPα associated with PEPCK and HNF6 promoters. Interestingly, GH also releases Rb from the E2F-dependent promoters, whereas E2F1 and E2F4 remain associated with these promoters. The release of

*Figures 5 and 6.*
Growth Hormone Corrects Pathways in Livers of Old Mice

FIGURE 6. Growth hormone releases C/EBPα-Brm (C/EBPα-E2F4-Rb) complex from the E2F-dependent promoters and increases the interaction of C/EBPα with promoters of liver-specific genes. A, C/EBPα-E2F4-Rb complex occupies the FoxM1B promoter in quiescent livers and in livers from old mice after partial hepatectomy. Chromatin solutions were isolated from quiescent livers (0) and from livers at 8 h after PH from young and old animals. The ChIP assay was performed as described in the legend to Fig. 1. In quiescent young livers, E2F-Rb complexes are detected on the FoxM1B promoter. In old mice, the C/EBPα-E2F4-Rb complex occupies the FoxM1B promoter in quiescent livers and in livers after PH. B, GH treatment releases the C/EBPα-E2F4-Rb complex from E2F-dependent promoters. E2F1, E2F4, C/EBPα, and Rb were immunoprecipitated from chromatin solutions of the livers of young, old, saline-treated (control), and old GH-treated mice. These IPs were used for PCR with primers specific to FoxM1B, c-Myc, PEPCK, and HNF6 promoters. Ag, agarose-mock control. C, GH increases interactions of C/EBPα with the PEPCK promoter in livers of old mice. C/EBPα IPs from chromatin solutions of livers of young, old control, and old GH-treated mice were examined by 22–28 cycles of PCR with primers to the PEPCK promoter. D, GH increases transcription of PEPCK and repression of HNF6 in livers of old mice. Real time PCR was performed with RNA isolated from livers of young and old mice and from livers of animals treated with saline (control) or with GH.

both C/EBPα-E2F4-Rb and E2F4-Rb complexes from the E2F-dependent promoters suggests that GH removes a negative control of these E2F targets. To determine the amounts of C/EBPα on the PEPCK promoter in livers of young, old, and old GH-treated mice, chromatin IPs of C/EBPα from these livers were examined by a quantitative PCR. Fig. 6C shows that, whereas in control livers C/EBPα is detected on the PEPCK promoter after 28 PCR cycles, GH treatment increased association of C/EBPα with the PEPCK promoter so that C/EBPα is detectable on this promoter after 24 cycles. Examination of PEPCK and HNF6 transcripts in livers of old mice treated with GH by real time PCR showed a quite different result for these mRNAs. The GH-mediated redirection of C/EBPα to the PEPCK promoter correlates with the correction of its transcription (Fig. 6D). The GH-mediated induction of PEPCK transcription in livers of old mice was also detected by Northern blotting (data not shown). These data show that GH-mediated disruption of the C/EBPα-Brm complex directs C/EBPα to the PEPCK promoter and increases its transcription in livers of old mice. On the contrary, GH-mediated redirection of C/EBPα to the HNF6 promoter correlates with the 3–4-fold repression of the HNF6 transcripts. This observation is consistent with previous publications showing that C/EBPα negatively regulates the HNF6 promoter through a direct binding (9, 20). Recent work from Roesler’s group (27) suggested that this C/EBPα-mediated negative regulation involves a recruitment of CA150 protein to the HNF6 promoter. Taken together, the studies of effects of GH on the expression of liver-specific genes showed that GH restores transcriptional activities of C/EBPα.

Ghrelin-mediated Release of GH in Livers of Old Mice Reduces the C/EBPα-Brm Complex—Physiologically, GH is released episodically, with major pulses occurring at 3-h intervals. The amplitude of these pulses declines during aging but can be restored by treatment with ghrelin receptor agonists (18). Therefore, we asked whether treatment of old mice with ghrelin is sufficient to eliminate formation of the C/EBPα-Brm complex in the liver. The liver does not express the ghrelin receptor; therefore, any effect of ghrelin on the C/EBPα-Brm complex will be mediated indirectly by increased amplitude of endogenous GH release. Old mice were treated with ghrelin, and levels of Brm, Rb, E2F4, and C/EBPα and the C/EBPα-Brm complex were measured by Western blotting and by co-IP. Protein levels of Brm, Rb, E2F4, and C/EBPα were unaffected by ghrelin treatment, but like GH treatment, ghrelin reduces cyclin D3 and increases the nuclear concentration of P2PA (Fig. 7A). This result suggested that this ghrelin-dependent event might lead to dephosphorylation of C/EBPα at Ser193 and elimination of the C/EBPα-Brm complex. Co-IP studies showed that the interaction of C/EBPα with cyclin D3 is reduced similarly in ghrelin and GH-treated mice, whereas the interaction of C/EBPα with P2PA is increased. Indeed, these interactions caused dephosphorylation of C/EBPα at Ser193, as shown by Western blotting with Ser(P)193-specific Abs (Fig. 7B). Examination of C/EBPα-Brm complex by co-IP (Fig. 7C) and by gel filtration (Fig. 7D) showed that ghrelin treatment of old mice is sufficient to disrupt the C/EBPα-Brm complex. The results of real time PCR with RNA from livers of animals treated with ghrelin showed that, like GH treatment, ghrelin increases transcription of PEPCK to the levels observed in young livers (Fig. 7E). Taken together, these studies reveal that ghrelin-induced elevation of endogenous GH in old animals is sufficient to inhibit formation of the C/EBPα-Brm complex and to correct transcription of PEPCK.

DISCUSSION

Administration of GH or Injection of the Regulator of the Amplitude of Endogenous GH, Ghrelin, Eliminates the Age-specific C/EBPα-Brm Complex—In this paper, we have examined distribution of C/EBPα in protein complexes in livers from

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young and old mice and found that livers from old mice contain a very small portion of Brm-free C/EBPα/H9251. In complexes with Brm, C/EBPα/H9251 is mainly associated with E2F-dependent promoters, such as c-Myc and FoxM1B, and inhibits the activities of these promoters. Within the sensitivity of our assays, we could not detect the C/EBPα/H9251-Brm complexes on the promoters, to which C/EBPα/H9251 binds directly and activates transcription. Examination of direct targets of C/EBPα/H9251 showed that expression of PEPCK is reduced in livers of old animals. These observations are consistent with previous data showing the reduced expression of PEPCK in old hepatocytes (28).

In seeking pathways that could eliminate the C/EBPα-Brm complex but not change levels of C/EBPα, we examined whether GH might disrupt the complex. Our studies revealed that treatment of old animals with GH prevents the formation of the complex without changing production of the protein components of the complex; therefore, elimination of the complex is regulated by post-translational modifications. This observation is consistent with findings that components in the blood of young animals (which contains higher concentrations of GH) reduce the complex by a similar mechanism (2). Previous investigations demonstrated that the C/EBPα-Brm complex is supported in livers of old mice by a specific phosphorylation of C/EBPα at Ser193 by cyclin D3/CDK4–6 (4) and that a phosphatase PP2A dephosphorylates Ser193 and reduces the complex in liver tumors (10). Data in this paper show that both GH and ghrelin treatment of old mice reduces cyclin D3-associated kinase activities and activates the PI3K-Akt pathway, leading to accumulation of the catalytic subunit C of PP2A in nuclei. Although we have injected exogenous GH in concentrations close to endogenous levels of GH in young animals, this replacement of GH in old animals does not precisely mimic the episodic endogenous GH profile measured in plasma. A more physiological approach is to treat animals with an agonist of the GH secretagogue receptor (18, 29). Our results with ghrelin-injected mice show that physiologically relevant concentrations of GH restore the young liver phenotype in old mice. The restoration of the young liver proliferative molecular phenotype in old mice by

FIGURE 7. Ghrelin-mediated release of GH reduces C/EBPα-Brm complex in livers of old mice. A, expression of the components of C/EBPα-Brm complex, cyclin D3, and PP2A in livers of ghrelin-treated mice. Nuclear proteins were extracted from control and ghrelin-injected mice were fractionated by gel filtration, and the fractions were probed with Abs to C/EBPα. B, expression of C/EBPα, cyclin D3, and PP2A in livers of ghrelin-treated mice. C, expression of C/EBPα, cyclin D3, and PP2A in livers of ghrelin-treated mice. D, expression of C/EBPα, cyclin D3, and PP2A in livers of ghrelin-treated mice. E, expression of C/EBPα, cyclin D3, and PP2A in livers of ghrelin-treated mice. F, expression of C/EBPα, cyclin D3, and PP2A in livers of ghrelin-treated mice.
GH or ghrelin treatment probably extrapolates to human aging and is consistent with the hypothesis that deficits in endogenous ghrelin signaling contribute to the aging phenotype.

**GH-mediated Elimination of C/EBPα-Brm Complex Decreases E2F Targets and Increases Association of C/EBPα with Promoters of Liver-specific Genes**—Several key proteins are required for liver proliferation. Studies from the Costa laboratory (16, 24) demonstrated a crucial role for FoxM1B in proliferation of livers from young mice and in the reduced proliferative response of livers of old mice. We found that the FoxM1B promoter, which contains an E2F site, is a target of the C/EBPα-Brm complex and is occupied by the C/EBPα-Brm complex in livers of old mice. Experiments in cultured cells show that C/EBPα inhibits the FoxM1B promoter through this element in Brm-positive cells, but C/EBPα fails to inhibit the promoter in cells lacking functional Brm. Most important, mutation of the E2F binding site (which interacts with the C/EBPα-Brm complex) within the FoxM1B promoter abolishes C/EBPα-mediated inhibition of the promoter. Interestingly, the mutation of the E2F site also increases activity of the FoxM1B promoter in cells that do not express C/EBPα, suggesting that E2F-Rb family complexes also repress the promoter through this E2F site. Since c-Myc and FoxM1B are major initiators of liver proliferation after PH, we examined if GH-mediated correction of proliferation in livers of old mice might involve removal of the C/EBPα-Brm complex from the promoters of these genes. A ChIP assay showed that the C/EBPα-Brm complex is not detectable on the c-Myc and FoxM1B promoters in livers of old GH-treated mice. Interestingly, we observed that Rb is also removed from the c-Myc and FoxM1B promoters by GH treatment and that these promoters are occupied by free E2F1 and E2F4 (Fig. 6). Consistent with these observations, we could not detect Rb on several other E2F targets, such as dihydrofolate reductase and Cdc2, in livers of old mice after treatment with GH (data not shown). These findings suggest that GH also neutralizes negative control of proliferation mediated by E2F-Rb complexes. The elimination of the C/EBPα-Brm complex by GH does not change protein levels of C/EBPα but increases amounts of free protein. The ChIP assay demonstrated that the dissociation of the complex and the accumulation of free C/EBPα leads to removal of C/EBPα and Rb from the E2F-dependent promoters and increasing the amounts of C/EBPα associated with the PEPCK and HNF6 promoters. Based on results obtained in this paper, we propose a following model for the GH-mediated correction of proliferation and transcription in livers of old mice (Fig. 7D). Since the C/EBPα-Brm complex inhibits promoters of genes that are required for cell proliferation and because free C/EBPα regulates expression of liver-specific genes, the GH-mediated elimination of the complex derepresses the promoters of cell cycle genes and increases free C/EBPα, which interacts with C/EBP-dependent promoters, such as PEPCK and HNF6. Consistent with previously published effects of C/EBPα on PEPCK and HNF6, transcription of PEPCK is increased in livers of old GH-treated mice, whereas transcription of HNF6 is reduced. Although work in this paper is focused on the effects of GH on the C/EBPα protein, it is clear that GH might also affect other liver-specific transcription factors, such as C/EBPβ. In fact, our recent paper demonstrated that the elevation of cyclin D3 in livers of old mice activates translation of a dominant negative isoform of C/EBPβ, LIP, through the elevation of the CUBG1p1-Elf2 translational complex (30). We are now investigating if GH-mediated reduction of cyclin D3 in livers of old mice also changes expression of C/EBPβ.

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