C/EBPβ Activates E2F-regulated Genes in Vivo via Recruitment of the Coactivator CREB-binding Protein/P300*1

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The E2F transcription factors play an essential role in regulating the G1- to S-phase transition of the cell cycle. Previous studies have identified the importance of interactions between E2Fs and other transcription factors as a mechanism for transcriptional control of a subset of E2F regulated target genes. However, the mechanisms responsible for E2F target gene specificity remain incompletely understood. Here we report that in a mammalian in vivo model of synchronized proliferation, C/EBPβ occupancy on the promoters of E2F-regulated growth-related genes increases as a function of cell cycle progression. C/EBPβ binding to these promoters is associated with recruitment of the coactivator CBP/p300, histone H4 acetylation, and maximal activation of E2F target genes. Moreover, binding of CBP/p300 to E2F targets is markedly reduced in C/EBPβ null mice, resulting in reduced expression of E2F regulated genes. These findings identify C/EBPβ as a direct activator of E2F target genes in mammalian cell cycle progression through a mechanism that involves recruitment of CBP/p300. The demonstration of a functional link between C/EBPβ and CBP/p300 for E2F target gene activation provides a potential mechanism for how coactivators such as CBP/p300 can be selectively recruited to E2F target genes in response to tissue-specific growth stimuli.

E2F target genes including cyclins, cyclin-dependent kinases, and the activator E2F proteins themselves are induced in mid to late G1 phase and are important for the G1/S transition. In G0 and early G1 phase, the promoters of the pro-proliferative E2F genes (E2F1, -2, and -3) are transcriptionally silenced as a result of binding of the repressive E2Fs, a pocket protein such as pRB or p130, and chromatin remodeling proteins (1, 2). As cells reenter the cell cycle, sequential activation of cyclin-dependent kinases with their partner cyclins results in phosphorylation of the pocket proteins, relieving the inhibition of activator E2F gene transcription (3). Previous studies have shown that physical interactions between TFE3, NF-Y, B-myb, and YY1 and individual E2F proteins are important for determining E2F target gene specificity and temporal control of transcription of a subset of E2F target genes (4–7). However, the mechanisms responsible for E2F target gene specificity remain incompletely understood (8).

The partial hepatectomy model in rodents has been extensively used to investigate the mechanisms responsible for hepatic growth and proliferation and is one of only a few models for mammalian in vivo cell cycle entry (9, 10). After surgical removal of 70% of the liver, the majority of the remaining, normally quiescent, hepatocytes and nonparenchymal cells synchronously reenter the cell cycle and proliferate, restoring the original liver mass in 10–14 days. Immediately after surgery, cytokine and growth factor signals emanating from Kupffer and endothelial cells in the liver rapidly activate a number of transcription factors including NF-κB, STAT3 (signal transducers and activators of transcription 3), and C/EBPβ via posttranslational modifications resulting in the transcriptional activation of immediate-early growth genes including myc, fos, and jun (9, 10). Neither hepatoma cells lines nor primary hepatocytes recapitulate the regulatory events and synchronous cell cycle entry and progression that occur in hepatocytes in vivo. The bZIP transcription factors, CCAAT enhancer-binding proteins α and β, regulate diverse functions in the liver and other organs including control of cellular proliferation, differentiation, and metabolism (11). For instance, in the livers of young mice, C/EBPα blocks cell cycle progression of quiescent hepatocytes via inhibition of cyclin-dependent kinases (12). C/EBPβ can directly repress E2F transcription (13), and the ability of C/EBPβ to repress E2F is required for cell cycle exit and terminal differentiation of both adipocytes and granulocyte lineages through a mechanism that is independent of its ability to bind directly to DNA (13, 14). In aged mice, in which proliferation is impaired, C/EBPα is thought to repress E2F promoters via formation of a complex with E2F4, pRB, and the chromatin remodeling protein Brm (15).

The transcription factor C/EBPβ is an important effector of growth signals in experimental models of physiologic and neoplastic growth (16–22). However, the downstream targets of C/EBPβ that mediate its proliferative function have not been elucidated. The importance of C/EBPβ for hepaticocyte proliferation is highlighted by the fact that regeneration is impaired in C/EBPβ−/− mice after partial hepatectomy and is associated with a significant reduction in hepatocyte DNA synthesis (16). The majority of growth-related genes are not dependent on C/EBPβ with the exception of cyclins including cyclin E, A, and B. Evidence from other experimental systems indicates that cyclins are often regulated by E2Fs. Based on the previously identified regulation of E2F genes by C/EBPα described above,

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.
TABLE 1
Expression of E2F-regulated genes in quiescent and 40-h posthepatectomy wild type and C/EBPβ−/− livers posthepatectomy

| Gene       | Fold change | 40 h WT/0 h WT | WT S.E. | KO S.E. | 40 h WT/40 h KO | p Value |
|------------|-------------|----------------|---------|---------|---------------|---------|
| cdc45L     | 2.6         | 0.9            | 9.4     | 0.3     | 2.8           | 0.03    |
| topBP-1    | 12.7        | 0.8            | 8.1     | 2.4     | 1.6           | 0.63    |
| p107       | 16.7        | 0.7            | 18.7    | 0.1     | 0.9           | 0.11    |
| Lamin B1   | 2.1         | 0.6            | 1.7     | 0.4     | 1.2           | 0.63    |
| mlh1       | 1.6         | 0.3            | 1.7     | 0.4     | 1.0           | 0.89    |
| Cdc25a     | 3.6         | 0.4            | 9.9     | 2.1     | 0.4           | 0.17    |
| DNA pol ep58| 6.0      | 0.3            | 9.8     | 1.7     | 0.6           | 0.15    |
| DNA pol α p49| 9.3     | 0.2            | 14.7    | 2.4     | 0.6           | 0.25    |
| cdc25b     | 13.0        | 3.3            | 3.9     | 1.8     | 3.3           | 0.05    |
| cdc25c     | 6.2         | 1.8            | 1.0     | 0.4     | 5.9           | 0.01    |
| cdk2       | 2.4         | 0.7            | 2.7     | 0.7     | 0.9           | 0.84    |
| cdk4       | 2.8         | 0.8            | 3.8     | 1.1     | 0.7           | 0.51    |
| cks2       | 3.8         | 0.8            | 2.3     | 0.9     | 1.7           | 0.33    |
| Hdg5a      | 2.6         | 0.8            | 2.6     | 0.7     | 1.0           | 0.99    |
| Thymidine synthase | 6.2 | 0.7 | 13.8 | 1.9 | 0.4 | 0.12 |
| lmg2       | 17.3        | 4.1            | 10.1    | 1.1     | 1.7           | 0.23    |
| lmg4       | 1.5         | 0.7            | 2.5     | 0.4     | 0.6           | 0.08    |
| lmg17      | 2.9         | 0.3            | 3.4     | 0.5     | 0.9           | 0.53    |
| lprt       | 0.7         | 0.0            | 1.6     | 0.0     | 0.5           | 0.00    |
| mcm3       | 10.3        | 1.3            | 3.8     | 1.3     | 2.7           | 0.02    |
| mcm6       | 91.2        | 3.5            | 60.6    | 17.5    | 1.5           | 0.23    |
| mcm2       | 15.2        | 1.1            | 16.5    | 1.6     | 0.9           | 0.55    |
| mcm4       | 16.3        | 4.9            | 31.7    | 2.9     | 0.5           | 0.11    |
| mcm5       | 43.5        | 6.1            | 31.2    | 8.2     | 1.4           | 0.35    |
| mcm6       | 91.2        | 3.5            | 60.6    | 17.5    | 1.5           | 0.23    |
| mcm7       | 9.5         | 2.6            | 18.8    | 1.2     | 0.5           | 0.08    |
| mcm10      | 0.2         | 0.0            | 0.2     | 0.0     | 0.9           | 0.22    |
| nput       | 2.3         | 0.1            | 2.6     | 0.1     | 0.9           | 0.16    |
| fen-1      | 8.0         | 6.1            | 4.2     | 3.3     | 1.9           | 0.64    |
| B-Myb      | 2.3         | 1.2            | 12.7    | 3.6     | 0.2           | 0.11    |
| orc6L      | 3.2         | 1.7            | 3.7     | 1.2     | 0.9           | 0.83    |
| orc3L      | 3.2         | 1.8            | 3.6     | 0.9     | 0.9           | 0.84    |
| bim        | 0.9         | 0.5            | 0.5     | 0.1     | 1.8           | 0.50    |
| msh2       | 5.3         | 1.7            | 0.7     | 0.3     | 7.9           | 0.03    |
| msh5       | 2.5         | 0.4            | 1.1     | 0.3     | 2.3           | 0.05    |
| cdt1       | 6.2         | 1.5            | 0.8     | 0.2     | 7.5           | 0.03    |
| cdc6       | 17.3        | 2.9            | 10.1    | 0.8     | 1.7           | 0.23    |
| cdc7       | 128.8       | 27.9           | 4.7     | 2.1     | 27.6          | 0.02    |
| tfdp-1     | 3.1         | 0.9            | 5.2     | 0.5     | 0.6           | 0.17    |
| rrm1       | 9.5         | 2.7            | 18.7    | 5.6     | 0.5           | 0.27    |
| rrm2       | 36.3        | 6.4            | 56.4    | 11.5    | 0.6           | 0.26    |
| pCNA       | 9.1         | 5.8            | 3.0     | 1.9     | 3.1           | 0.43    |

We hypothesized that changes in the occupancy of E2F-dependent promoters by C/EBPα and C/EBPβ could be important for activation of E2F-regulated target genes and provide a mechanistic explanation for the role of C/EBPβ in the cell cycle.

EXPERIMENTAL PROCEDURES

Animals—The derivation of mice homozygous for the C/EBPβ mutation has been previously described (16). Partial hepatectomies were performed as described (16). All animal studies were performed in accordance with the University of Pennsylvania animal care committee guidelines (Institutional Animal Care and Use Committee).

RNA Analysis—Total liver RNA was prepared from wild type and C/EBPβ−/− mice at 0, 16, 24, 40, and 48 h post-partial hepatectomy by guanidine isothiocyanate and cesium chloride ultracentrifugation (23) or using the RNAeasy kit (Qiagen). 5 μg of RNA was used as a template to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen). Oligo(dT)18 was used for priming. cDNA was diluted 15-fold in H2O. 1 μl of diluted cDNA was used as template in quantitative real time PCR analysis using the Brilliant® SYBR Green QPCR Master Mix (Stratagene), 10 μM primers, and the reference dye at a 1:200 dilution according to the manufacturer’s instructions using the Mx4000 PCR system (Stratagene). All genes were normalized to TATA box-binding protein and shown as fold induction over wild type 0-h post-partial hepatectomy expression level. Primer sequences are available upon request.

Transcription Factor Binding Sites—PCR and real-time PCR primers for chromatin immunoprecipitation (ChIP) assay analysis were designed to flank E2F binding sites within E2F-regulated gene promoters. These E2F binding sites were found by entering the promoter sequences of genes 1000 bp upstream of the transcription start site and the 3’-untranslated regions obtained from the UCSC Genome Browser (24) into the transcription factor search program TFSEARCH (25). Binding site matches with scores less than 90 (of 100) were ignored.

The abbreviations used are: ChIP, chromatin immunoprecipitation; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; HEK, human embryonic kidney; HPRT, hypoxanthine-guanine phosphoribosyltransferase.
Chromatin Immunoprecipitation—ChIPs were performed as previously described (26) with the following modifications; 25 μg of chromatin DNA was used in each immunoprecipitation as determined by analyzing an aliquot of purified DNA from each sample using 260-nm UV absorption. To ensure that salt concentrations were identical across all immunoprecipitations, each 25-μg DNA chromatin sample was brought up to a total volume of 250 μl in nuclear lysis buffer and then diluted 1:1 in ChIP dilution buffer. Adjusted ChIPs were then precleared, immunoprecipitated with 2 μg of antibody or 2 μl of antiserum, and washed as previously described (27). Anti-C/EBPα (sc-61), anti-C/EBPβ (sc-50), anti-E2F1 (sc-193), anti-E2F2 (sc-633), anti-CBP (sc-369), and anti-p300 (sc-585) antibodies were purchased from Santa Cruz Biotechnology. Anti-acetyl-histone H4 antiserum (06-866) was purchased from Upstate Biotechnology. Anti-rabbit IgG (100-NC) antibody was purchased from Lab Vision. Immunoprecipitated DNA was purified using a QiaQuick PCR purification kit (Qiagen) in a final elution volume of 60 μl. Purified total input DNA from each chromatin preparation was diluted to a concentration of 5 ng/ml to be used as control template in all conventional and quantitative PCR analyses.

Conventional PCR analysis was performed by amplifying 1 μl of immunoprecipitated DNA or 1 μl of diluted total input DNA for 35 cycles. 10 μl of each PCR reaction was run on a 1% agarose gel stained with ethidium bromide or SYBR green (Sigma). All reactions were performed with at least three biological replicates. Primer sequences are available upon request. Quantitative PCR analysis was performed using the Brilliant SYBR Green QPCR master Mix, 10 μM primers, and the reference dye at a 1:200 dilution according to manufacturer’s instructions using the Mx4000 PCR system. 1 or 2 μl (E2F1 only) of immunoprecipitated DNA or diluted total input DNA was used in each reaction. All reactions were performed with two to five biological replicates and three technical replicates with reference dye normalization. The median cycle threshold (Ct) value was used for analysis. ChIP QPCR reactions were normalized to total input by expressing the ChIPs as a percentage of the -fold differential between Input and immunoprecipitation (IP) for the test gene normalized to the -fold differential between Input and IP for 28 S ribosomal control gene. The following formula was used to make this calculation: IP signal = \( \frac{2^{\text{(28S gene - test gene)}}}{(\text{input 28S gene - input test gene)}} \).

Plasmid Constructs—pcDNA3-LAP and pcDNA3-E2F-1 encoding full-length rat C/EBPβ and E2F-1 coding regions were gifts from Dr. David T. Kurtz, Medical College of South Carolina (13). pCMV-CBP-HA expression plasmid was obtained from Richard Goodman.

Expression of Recombinant Proteins—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 1-glutamine, penicillin, and streptomycin at 37 °C, 5% CO₂. Cells were transfected with pcDNA3-LAP, pcDNA3-E2F1, pcDNA3-CBP, singly and in combination using Lipofectamine 2000 (Invitrogen). For single and double transfections, pcDNA3 backbone plasmid was added to maintain equal amounts of transfected plasmid DNA per 60-mm plate. Twenty-four hours post-transfection cells were prepared in lysis...
buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1.0% Nonidet P-40, 0.5 mM EDTA, plus protease inhibitor mixture (Roche Diagnostics). Cell lysates were sonicated on high setting using the

FIGURE 2. Histone H4 acetylation of E2F-regulated genes is reduced during liver regeneration in C/EBPβ−/− mutant livers. A, chromatin extracted from wild type and C/EBPβ−/− livers at the indicated hours post-partial hepatectomy (PHX) was immunoprecipitated with antibody specific for acetylated histone H4 or rabbit IgG as a negative control. Immunoprecipitated chromatin was analyzed by PCR with primers specific for cdc6 and mcm3. B–F, quantification of acetylated histone H4 bound to the cdc6, mcm3, msh2, msh5, and hprt promoters. Chromatin was prepared and immunoprecipitated as described in A and analyzed by real-time PCR. Acetylation was analyzed by real-time PCR using a sequence from 28 S ribosomal RNA promoter as a reference for unbound DNA (see "Experimental Procedures"). Error bars indicate S.E. n = 2–5. *, p value < 0.05.

FIGURE 3. C/EBPβ occupancy on cdc6, mcm3, msh2, and msh5 promoters during liver regeneration. A, chromatin extracted from wild type (Control) and C/EBPβ−/− (Mutant) livers at 0 and 40 h post-partial hepatectomy (PHX) was immunoprecipitated with antibody specific to C/EBPβ, C/EBPβ, or rabbit IgG as a negative control. ChIP assay efficacy was determined by subjecting immunoprecipitates and input chromatin to PCR using primers specific for phosphoenolpyruvate carboxykinase pck1 and hprt, a positive and negative control, respectively. Note the absence of a band for phosphoenolpyruvate carboxykinase in the C/EBPβ lanes from mutant mice, indicating that the C/EBPβ antibody used is monospecific. C/EBPβ occupancy on cdc6, mcm3, msh2, msh5, and hprt (B–F) promoters was quantified by real-time PCR as described in Fig. 2. Error bars indicate S.E. n = 2–5. (*, p value < 0.05).
BioRuptor (Diogene) for three 30-s pulses, and debris was pelleted by centrifugation at 13,000 rpm for 20 min. Lysates were then precleared with protein G-agarose and directly subjected to immunoprecipitation (see below).

Preparation of Cell Lysates from Mouse Liver—Livers were removed from C/EBPβ/H9252/H11001/H11001 and C/EBPβ/H9252/H11002/H11002 mice 40 h posthepatectomy, snap-frozen in liquid nitrogen, and resuspended using a Dounce homogenizer in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM NaF, 7.5 mM EGTA pH 8.0, 7.5 mM MgCl2) to which protease inhibitor mixture was added (Sigma).

Immunoblot Analysis—A portion of lysates obtained from each transfection condition or regenerating liver lysates was subjected to 10% SDS-PAGE and electroblotted onto an Immobilon-P membrane (Millipore, Billerica MA). Membranes were blocked with phosphate-buffered saline (PBS) with 0.1% (v/v) Tween 20 (PBS-T) containing 5% (w/v) nonfat dry milk for 3 h at room temperature. Incubation with anti-C/EBPβ (Santa Cruz, sc-50, 1:1000), E2F1, (Santa Cruz, sc-193, 1:1000), CBP (Santa Cruz, sc-369, 1:1000) was performed overnight in PBS-T supplemented with 5% nonfat dry milk. Horseradish-conjugated secondary antibodies (Invitrogen, 1:10,000) were used to detect antibody-antigen complexes by chemiluminescence (Lumi-Light PLUS Western blotting Substrate, Roche Diagnostics).

Immunoprecipitation—Immunoprecipitation was performed with protein G-agarose (Invitrogen). In brief, 500 μg of either

![Image](image-url)
Regulation of E2F Target Genes by C/EBPβ

A. Cdc6

B. Mcm3

C. Msh2

D. Msh5

E. HPRT

F. Schematic representation of the regulation of E2F target genes by C/EBPβ.
Regulation of E2F Target Genes by C/EBPβ

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In liver extracts or 2 mg of 40-h posthepatectomy liver lysate were pre-cleared and then incubated with 1 μg of one of the following antibodies: C/EBPβ (Santa Cruz, sc-50), E2F1 (Santa Cruz, sc-193), IgG (Neomarkers, NC-100-P1), or 2 μg of CBP (Santa Cruz, sc-369). For detection of endogenous C/EBPβ-E2F interactions in liver lysates, immunoprecipitates were washed with lysis buffer. Immunoprecipitates were separated by SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted as described above (immunoblot analysis).

Statistical Analysis—Student’s t test with equal variance and two-tailed distribution was used to determine the significance of differences between groups (Microsoft Excel statistical analysis software, Redmond, WA). Results where indicated are expressed as means ± S.E.

RESULTS

To identify the C/EBPβ-dependent E2F target genes that are responsible for the defect in DNA synthesis in C/EBPβ−/− livers, we assessed the expression of 40 E2F target genes previously implicated in either the G1/S transition, DNA replication, or DNA repair (28–33) (Table 1). Activation of eight E2F-regulated target genes encoding proteins involved in the licensing of DNA replication, (mcm3, cdc6, cdh1, cdc45l1), DNA mismatch repair (msh2 and msh5), and the G1/S transition (cdc25b, cdc25c), was reduced in C/EBPβ−/− livers posthepatectomy (Fig. 1 and Table 1). E2F-mediated activation of DNA replication licensing factors has particular relevance to normally quiescent cells such as hepatocytes that must be induced to reenter the cell cycle in response to a growth stimulus. In continuously cycling cells, the activity of these proteins is regulated primarily by changes in their activity and/or chromatin occupancy (34, 35). However, in quiescent cells these proteins are degraded completely, presumably as an additional barrier to prevent cells from entering the cell cycle inappropriately (36). As a result quiescent cells are highly dependent upon de novo transcription of DNA replication licensing factors by E2F regulatory pathways. Therefore, decreased expression of these factors likely contributes to the impaired DNA synthesis in C/EBPβ−/− livers posthepatectomy, providing a mechanistic explanation for the C/EBPβ−/− phenotype (37–40).

Having demonstrated that several E2F-regulated genes are dependent on C/EBPβ for their full activation in the regenerating liver, we next explored potential mechanisms through which C/EBPβ might exert this effect. pRB family proteins (“pocket proteins”) target histone deacetylases to promoters, and transcriptional activation of E2F target genes is associated with hyperacetylation of histones (41). We, therefore, investigated whether C/EBPβ was important for histone acetylation of E2F target genes during the hepatic cell cycle. ChIP was performed on four of the C/EBPβ-dependent E2F genes identified in Table 1 (mcm3, cdc6, msh2, and msh5), and occupancy of acetylated histones on their promoters was determined by quantitative PCR. Although there was no significant induction of histone H3 acetylation of these E2F-regulated promoters during liver regeneration (data not shown), acetylated histone H4 binding was increased 40 h posthepatectomy. Histone H4 acetylation was reduced ~2-fold in C/EBPβ−/− livers (Fig. 2), in close correlation to the attenuated mRNA expression of E2F targets in the C/EBPβ−/− livers 40 h posthepatectomy (See Fig. 1).

We next investigated whether C/EBPβ directly activates these E2F-dependent genes. To test the antibodies against C/EBPβ, we first performed ChIP assays with primers for the phosphoenthionylpyruvate carboxykinase (pck1) promoter, a well known C/EBPβ target (42). Fig. 3A shows that C/EBPβ occupancy is abolished in C/EBPβ−/− livers as expected. Quantitative real time PCR analysis of chromatin immunoprecipitated from wild type and C/EBPβ−/− livers during quiescence and 40 h posthepatectomy was used to examine C/EBPβ binding to cdc6, mcm3, msh2, and msh5 promoters (Fig. 3, A–E) and to a negative control promoter hprt (Fig. 3F). In wild type livers, C/EBPβ occupancy on the msh2 and msh5 promoters was similar at both 0 and 40 h posthepatectomy, with C/EBPβ occupancy increased about 2–3-fold at the 40th time point on cdc6 and mcm3 promoters. This increase in C/EBPβ recruitment to these E2F-regulated gene promoters corresponds to both the peak of DNA synthesis during liver regeneration (16) and the peak of E2F regulated gene transcription in wild type animals (Fig. 1). In contrast, C/EBPβ occupancy on the msh2 and msh5 promoters was similar at both 0h and 40h posthepatectomy, suggesting that posttranslational modifications of C/EBPβ and/or recruitment of other transcription factors contribute to activation of these genes by C/EBPβ. We considered the possibility that unopposed action of C/EBPα in C/EBPβ−/− hepatocytes could prolong C/EBPα occupancy on the cdc6 and mcm3 promoters posthepatectomy, producing the delay in cell cycle progression that occurs in these mice. However, C/EBPα binding was not increased on the cdc6 and mcm3 promoters in C/EBPβ−/− livers 40 h posthepatectomy relative to wild type livers (Fig. 3A and supplementary Fig. 1). Taken together these data suggest that C/EBPβ regulates E2F gene activation in the regenerat-

FIGURE 6. C/EBPβ enhances CBP and/or p300 recruitment to cdc6, msh2, and msh5 promoters. Chromatin extracted from wild type (wt, control) and C/EBPβ−/− (ko, mutant) livers at 0 and 40 h post partial hepatectomy was immunoprecipitated with antibody specific to CBP, p300, or rabbit IgG as a negative control. Quantification of CBP and p300 occupancy to the cdc6, mcm3, msh2, msh5, and hprt promoters. Primers surrounding the E2F site in the promoters of the cdc6, msh2, msh5, and hprt genes were used for PCR to assess the enrichment of this site in DNA immunoprecipitated by either CBP or p300. Occupancy on cdc6, mcm3, msh2, msh5, and hprt promoters (A–E) was quantified by real-time PCR as described in Fig. 2 (see “Experimental Procedures”). Error bars indicate S.E. **p < 0.01, *p < 0.05, F, control of E2F-regulated transcription by C/EBPβ. This model, based on E2F data and published work, shows C/EBPα and C/EBPβ involved in a complex that regulates the transcription of genes required for S-phase through E2F consensus binding sites contained within their promoters. Left, in G0, E2F4 and C/EBPβ contribute to maintaining transcriptional silence of E2F-regulated genes. It is possible that C/EBPα contributes to repression of E2F target promoters by recruiting a histone deacetylase (HDAC) or SWI/SNF factor. Right, during the transition from G0 to S phase, the binding of C/EBPα to E2F-regulated promoters decreases, with a concomitant release of E2F4, allowing access for the pro-proliferative E2Fs, E2F-1 and 2. Simultaneously, occupancy of the E2F regulated promoters by C/EBPβ increases and C/EBPα decreases coincident with maximal acetylation of histone H4 and E2F target gene expression. Recruitment of CBP and/or p300 by C/EBPβ enhances E2F target gene transcription by one of several mechanisms including connecting tissue-specific transcription factors to the basal transcriptional apparatus, acting as a protein scaffold for the assembly of multiprotein complexes or via their ability to acetylate histones or transcription factors including E2Fs. Pol II, polymerase II.
Regulation of E2F Target Genes by C/EBPβ

...ing liver through a mechanism that involves binding to E2F target gene promoters.

The transcriptional coactivator proteins CBP (CREB-binding protein) and p300 enhance transcription of target promoters (43). C/EBPβ binds to the adenosine E1A-interacting domain of CBP and p300, and recruitment of CBP or p300 by C/EBPβ to promoters leads to co-activator phosphorylation and enhanced transcriptional activity of C/EBPβ target genes (44, 45). Furthermore, recruitment of CBP/p300 to E2F target gene promoters has been implicated in transcriptional activation and cell cycle progression (46, 47). Although previous studies have demonstrated that CBP and p300 can bind to either C/EBPβ or E2Fs, it has not been established whether C/EBPβ binds to E2Fs or if all three proteins form a complex. To investigate these possibilities, C/EBPβ, CBP, and E2F1 were expressed in HEK 293 cells, immunoprecipitated with CBP or E2F1 antibodies, and analyzed by immunoblot analysis. As shown in Fig. 4A, CBP is able to associate with E2F1 and C/EBPβ consistent with previously published reports demonstrating CBP/E2F1 (46) and CBP/C/EBPβ (45) interaction. Our ability to detect immunoprecipitate E2F1 and C/EBPβ in the absence of transfected CBP (Fig. 4) most likely reflects the presence of endogenous CBP protein in 293 cells, which has been described previously (48). We consistently detected decreased amounts of co-immunoprecipitated E2F1 protein when all three proteins were ectopically expressed when compared with transfection of CBP and E2F1. Triple-transfected 293 cells grew more slowly, suggesting that there may have been cellular toxicity that influenced the efficiency of the coimmunoprecipitation and/or protein expression levels.

Immunoprecipitation with α-E2F1 (Fig. 4, panel B) demonstrates that C/EBPβ interacts with E2F1. Taken together, these findings suggest that CBP, E2F1, and C/EBPβ have the potential to form a complex in vivo. In addition, α-E2F1 specifically immunoprecipitated endogenous C/EBPβ protein in 40-h posthepatectomy lysates from wild type but not C/EBPβ−/− livers. The α-E2F1 antibody used in these studies (sc-193) can cross-react with E2F2 and E2F3 (5). Therefore, our data are consistent with the notion that either E2F1 or another activator E2F and C/EBPβ interact in vivo in the regenerating liver (panel C).

Although the Santa Cruz α-E2F1 antibody specifically detects ectopically expressed E2F1 protein in transfected HEK 293 cells (Fig. 4, A and B), we were unable to detect E2F1 protein in the immunoprecipitated complexes from regenerating liver lysates, suggesting that the level of endogenous E2F1 protein is below the antibody detection threshold under these conditions (not shown).

We next investigated whether C/EBPβ was necessary for E2F1 and/or E2F2 protein recruitment to mcm3, cdc6, msh2, or msh5 promoters. ChIP analysis demonstrated that E2F1 and E2F2 occupancy on mcm3, cdc6, msh2, and msh5 promoters was increased 40 h posthepatectomy relative to quiescent livers (Fig. 5). These results are consistent with previously published studies that have identified mcm3, cdc6, and msh2 as transcriptional targets of the proliferative E2Fs (28) and demonstrate for the first time that proliferative E2F occupancy increases on the msh5 promoter during cell cycle progression. However, E2F1 and E2F2 occupancy on mcm3, cdc6, msh2, and msh5 promoters was unchanged in C/EBPβ livers 40 h posthepatectomy relative to wild type livers, indicating that E2F1 and E2F2 protein recruitment to these promoters does not depend upon C/EBPβ and that recruitment of E2Fs to these promoters is not sufficient for maximal activation.

We investigated whether C/EBPβ activates the transcription of E2F target genes via recruitment of CBP and/or p300. We first established that the levels of CBP and p300 proteins were not reduced in C/EBPβ−/− relative to wild type livers (data not shown). We then performed quantitative ChIP assays in wild type and C/EBPβ−/− livers 40 h posthepatectomy to investigate whether maximal CBP and/or p300 occupancy of E2F target promoters was dependent on C/EBPβ. Both CBP and p300 occupancy were enriched to a similar extent on the mcm3 promoter in wild type and C/EBPβ−/− livers, suggesting that differential recruitment of other coactivators by C/EBPβ may contribute to expression of this gene posthepatectomy (Fig. 6B). However, CBP was significantly enriched on both the cdc6 and msh2 promoters, whereas p300 occupancy on both msh2 and msh5 was enhanced in wild type relative to C/EBPβ−/− livers (Fig. 6, A, C, and D). These data are consistent with previously published studies in which these coactivator proteins are recruited to E2F target gene promoters during transcriptional activation (47) and further demonstrate that CBP/p300 occupancy on these E2F-regulated genes is increased by C/EBPβ. These results indicate that C/EBPβ activates transcription of a subset of E2F-regulated target genes through enhanced recruitment of the coactivator proteins CBP and/or p300.

DISCUSSION

The transcription factor C/EBPβ is an important effector of growth signals in physiologic and neoplastic growth. However, the molecular mechanisms that mediate the pro-proliferative function of C/EBPβ have not been previously elucidated. The potential for C/EBPβ to directly regulate E2F target genes in the liver had not been appreciated thus far, in part because we and other investigators have been unable to demonstrate a positive effect of C/EBPβ in multiple cell culture systems. Here we have utilized the best currently available mammalian in vivo model of synchronous entry into the cell cycle (that is, recovery after partial hepatectomy) to define the events in chromatin that lead to activation of genes required for cell cycle progression. In this study we demonstrate that C/EBPβ occupancy on E2F binding sites in E2F target genes in vivo is required for maximal induction of E2F-regulated target genes involved in DNA synthesis and replication. Maximal C/EBPβ occupancy on cdc6 and mcm3 occurs at the peak of S-phase, coinciding with maximal histone H4 acetylation and gene transcription. C/EBPβ occupancy on two other E2F-regulated genes, msh2 and msh5, did not change between 0- and 40-h posthepatectomy, suggesting that mechanisms other than increased occupancy are likely to contribute to their regulation by C/EBPβ. Buck et al. (49) have shown that phosphorylation of C/EBPβ by transforming growth factor-α on threonine 217 is necessary for hepatocyte proliferation. Therefore, phosphorylation of this residue or other posttranslational modifications may be important for activation of Msh2 and Msh5 by C/EBPβ. An outstanding question in the E2F field is whether activator E2F proteins alone are
sufficient for proliferation. Lukas et al. (50) reported previously that mass restoration is not decreased in the livers of E2F1 knock-out mice. This observation most likely reflects functional compensation by other activator E2Fs in the setting of long-term loss of E2F1. In support of this notion, Kong et al. (51) recently showed that E2F3a protein levels are increased in mouse embryonic fibroblasts from E2F1−/− mice. Here we show that maximal induction of eight E2F-regulated genes is in part due to the action of C/EBPβ, strongly supporting an active role for C/EBPβ as a transcriptional activator of E2F target gene expression.

In contrast to the activation of E2F-regulated genes by C/EBPβ in the liver, Johnson and co-workers (52) provide evidence that C/EBPβ represses several E2F target genes in a fibroblast model of Ras-induced senescence. However, C/EBPβ was pro-proliferative in mouse embryonic fibroblasts (MEFs) in which all three pocket proteins were knocked out or in wild type MEFs expressing a dominant negative E2F1 mutant lacking the transactivation and pocket protein interaction domains. These observations suggest that activation or repression of E2F target gene activation by C/EBPβ may be dependent on the level of pocket protein occupancy on the promoters of these genes. Therefore, a reduction in pocket protein binding on E2F promoters in hepatocytes in late G1 and S phase may be necessary for C/EBPβ to activate E2F target gene expression in proliferating hepatocytes.

Recruitment of coactivators such as CBP and p300 has been thought to be important for the activation of E2F-dependent gene promoters (46, 53, 54). Morris et al. (46) proposed that p300 recruitment was mediated by E2F proteins themselves in response to cyclin E/cyclin-dependent kinase 2-induced phosphorylation of E2F5. C/EBPβ is known to recruit p300 and/or CBP to standard C/EBPβ target genes, thereby increasing their transcription rate (44, 45, 55, 56). Here we have shown that C/EBPβ can also enhance recruitment of CBP and/or p300 to E2F target promoters, providing a molecular explanation of how this coactivator can become part of the complex nucleated by E2F on its target genes. Although our data demonstrating C/EBPβ occupancy on these promoters and C/EBPβ-CBP/p300 protein-protein interactions suggest that increase CBP/p300 occupancy to E2F-regulated promoters is dependent upon physical interaction between C/EBPβ and CBP/p300, it is possible that C/EBPβ enhances CBP/p300 occupancy on E2F promoters indirectly. The small changes in CBP/p300 occupancy detected on E2F target promoters 40 h posthepatectomy between wild type and mutant livers most likely reflects the fact that only 60% of the liver cells are hepatocytes, and these are the only cells that have reached S phase at this time point. Therefore, we estimate that the true -fold difference in CBP/p300 occupancy on these promoters is more than 4-fold. C/EBPβ consensus sequences are present only a considerable distance (>350 bp) from the C/EBPβ contact site identified by ChIP. These observations in combination with our finding that C/EBPβ forms a complex with E2F1 in vivo suggest that activation of E2F target genes is dependent upon direct interactions between C/EBPβ and E2Fs on E2F binding sites. The observation that C/EBPα mutant proteins defective for repression of E2F target genes retain their ability to bind to C/EBP sites is consistent with the notion that C/EBPs regulate E2F target genes through interactions with E2F proteins rather than by binding to C/EBP sites on the promoters of these genes (13, 14). C/EBPε represses E2F target gene activation in granulocytes through interactions with E2F1 and pRB, suggesting that C/EBP-E2F interactions may be a general characteristic of this transcription factor family (57). The mechanism by which C/EBP-E2F interactions result in either E2F repression or activation is not known. However, it may depend on isoform-specific ability to enhance recruitment of either coactivator or corepressor proteins such as CBP/p300 by C/EBPβ or Brm by C/EBPα (15).

A model outlining the relationships between C/EBPβ, E2F, and CBP/p300 is shown in Fig. 6E. Although our data strongly support this model, we cannot exclude the possibility that C/EBPβ-E2F protein interactions are mediated through other mechanisms that bring the two proteins into direct contact such as chromosome looping, a mechanism of transcriptional synergy that has previously been demonstrated for C/EBPβ and myb (58). In summary, our findings demonstrate that C/EBPβ is a direct activator of E2F promoters and cell cycle progression in the mammalian cell cycle through a mechanism that involves recruitment of CBP and/or p300. The demonstration of a functional link between C/EBPβ and CBP/p300 for E2F target gene activation provides a potential mechanism for how coactivators such as CBP/p300 can enhance activation of E2F target genes in response to tissue-specific growth stimuli.

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REFERENCES

1. Frolow, M. V., and Dyson, N. J. (2004) J. Cell Sci. 117, 2173–2181
2. Cam, H., and Dynlacht, B. D. (2003) Cancer Cells 3, 311–316
3. Sherr, C., and Roberts, J. (1999) Genes Dev. 13, 1501–1512
4. Schlisio, S., Halperin, T., Vidal, M., and Nevins, J. R. (2002) EMBO J. 21, 5775–5786
5. Giangrande, P. H., Hallstrom, T. C., Tunyaplin, C., Calame, K., and Nevins, J. R. (2003) Mol. Cell. Biol. 23, 3707–3720
6. Giangrande, P. H., Zhu, W., Rempel, R. E., Laakso, N., and Nevins, J. R. (2004) EMBO J. 23, 1336–1347
7. Zhu, W., Giangrande, P. H., and Nevins, J. R. (2004) EMBO J. 23, 4615–4626
8. Blais, A., and Dynlacht, B. D. (2004) Curr. Opin. Genet. Dev. 14, 527–532
9. Fausto, N. (1988) in The Liver: Biology and Pathobiology (Arias, I. M., Jakoby, W. B., Popper, H., Schachter, D., and Shafritz, D. A., eds) pp. 53–68 Raven Press, New York
10. Taub, R. (2004) Nat. Rev. Mol. Cell Biol. 5, 837–847
11. Ramji, D. P., and Foka, P. (2002) Biochem. J. 365, 561–575
12. Wang, H., Iakova, P., Wilde, M., Welm, A., Goode, T., Roseler, W. J., and Timchenko, N. A. (2001) Mol. Cell 8, 817–828
13. Slomiany, B. A., D’Arigo, K. L., Kelly, M. M., and Kurtz, D. T. (2000) Mol. Cell. Biol. 20, 5986–5997
14. Porse, B. T., Pedersen, T. A., Xu, X., Lindberg, B., Weser, U. M., Friis-Hansen, L., and Nerlov, C. (2001) Cell 107, 247–258
15. Iakova, P., Awad, S. S., and Timchenko, N. A. (2003) Cell 113, 495–506
16. Greenbaum, L. E., Li, W., Cressman, D., Peng, Y., Ciliberto, G., Poli, V., and Taub, R. (1998) J. Clin. Investig. 102, 996–1007
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17. Wessels, J., Yakar, S., and Johnson, P. F. (2004) Mol. Cell. Biol. 24, 3238–3250
18. Iyer, V. V., Kadakia, T. B., McCabe, L. R., and Schwartz, R. C. (2004) Exp. Cell Res. 295, 128–137
19. Buck, M., and Chojkier, M. (2003) Hepatology 37, 731–738
20. Bundy, L. M., and Sealy, L. (2003) Oncogene 22, 869–883
21. Shuman, J. D., Sebastian, T., Kaldis, P., Copeland, T. D., Zhu, S., Smart, R. C., and Johnson, P. F. (2004) Mol. Cell. Biol. 24, 7380–7391
22. Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F., and Smart, R. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 207–212
23. Glisin, V., Crkvenjakov, R., and Byus, C. (1974) Biochemistry 13, 2633–2637
24. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakov, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A., Podkolodny, N. L., and Kolchanov, N. A. (1998) Nucleic Acids Res. 26, 364–370
25. Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., and Haussler, D. (2002) Genome Res. 12, 996–1006
26. Friedman, J. R., Larris, B., Le, P. P., Peiris, T. H., Arsenlis, A., Schug, J., Tobias, J. W., Kaestner, K. H., and Greenbaum, L. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12986–12991
27. Wells, J., Graveel, C. R., Bartley, S. M., Madore, S. J., and Farnham, P. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3890–3895
28. Iwanaga, R., Komori, H., and Ohtani, K. (2004) J. Neurosci. Res. 77, 4139–4145
29. Cam, H., Balciunaite, E., Blais, A., Spektor, A., Scarpulla, R. C., Young, R., Tobias, J. W., Kaestner, K. H., and Greenbaum, L. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12986–12991
30. Yoshida, K., and Inoue, I. (2004) Oncogene 23, 6250–6260
31. Yoshida, K., and Inoue, I. (2004) Oncogene 23, 3802–3812
32. Barker, P. A., and Salehi, A. (2002) J. Neurosci. Res. 67, 705–712
33. Karakaides, P., Taraviras, S., Vassiliou, L. V., Zacharatos, P., Kastrinakis, N. G., Kougiou, D., Kouloukoussa, M., Nishitani, H., Papavassiliou, A. G., Lygerou, Z., and Gorgoulis, V. G. (2004) Am. J. Pathol. 165, 1351–1365
34. Williams, G. H., Romanowski, P., Morris, L., Madine, M., Mills, A. D., Stoeber, K., Marr, J., Laskey, R. A., and Coleman, N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14932–14937
35. Pelizon, C. (2003) Trends Cell Biol. 13, 110–113
36. Chong, J. P., Thommes, P., and Blow, J. J. (1996) Trends Biochem. Sci. 21, 102–106
37. Glisin, V., Crkvenjakov, R., and Byus, C. (1974) Biochemistry 13, 2633–2637
38. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakov, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A., Podkolodny, N. L., and Kolchanov, N. A. (1998) Nucleic Acids Res. 26, 364–370
39. Oehlmann, M., Score, A. J., and Blow, J. J. (2004) J. Biol. Chem. 165, 181–190
40. Mailand, N., and Diffley, J. F. (2005) Cell 122, 915–926
41. Taubert, S., Gorrini, C., Frank, S. R., Parisi, T., Fuchs, M., Chan, H.-M., Livingston, D. M., and Amati, B. (2004) Mol. Cell. Biol. 24, 4546–4556
42. Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W. (1990) Mol. Cell. Biol. 10, 6264–6272
43. Chan, H. M., and La Thangue, N. B. (2001) J. Cell Sci. 114, 2363–2373
44. Kovacs, K. A., Steinmann, M., Magistretti, P. J., Halfan, O., and Cardinaux, J. R. (2003) J. Biol. Chem. 278, 36959–36965
45. Schwartz, C., Beck, K., Mink, S., Schmolke, M., Budde, B., Wenning, D., and Kelpnauer, K. H. (2003) EMBO J. 22, 882–892
46. Morris, L., Allen, K. E., and La Thangue, N. B. (2000) Nat. Cell Biol. 2, 232–239
47. Caretti, G., Salci, V., Vecchi, C., Imbrini, C., and Mantovani, R. (2003) J. Biol. Chem. 278, 30435–30440
48. Lill, N. L., Grossman, D., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997) Nature 387, 823–827
49. Buck, M., Poli, V., van der Geer, P., Chojkier, M., and Hunter, T. (1999) Mol. Cell. Biol. 19, 1087–1092
50. Lukas, E. R., Bartley, S. M., Graveel, C. R., Diaz, Z. M., Dyson, N., Harlow, E., Yamasaki, L., and Farnham, P. J. (1999) Mol. Carcinog. 25, 295–303
51. Kong, L.-J., Chang, J. T., Bild, A. H., and Nevins, J. R. (2007) Oncogene 26, 321–327
52. Sebastian, T., Malik, R., Thomas, S., Sage, J., and Johnson, P. F. (2005) EMBO J. 24, 3301–3312
53. Trouche, D., Cook, A., and Kouzarides, T. (1996) Nucleic Acids Res. 24, 4139–4145
54. Lee, C. W., Sorensen, T. S., Shkama, N., and La Thangue, N. B. (1998) Oncogene 16, 2695–2710
55. Oelgeschlager, M., Janknecht, R., Krieg, J., Schreek, S., and Luscher, B. (1996) EMBO J. 15, 2771–2780
56. Mink, S., Haenig, B., and Klempnauer, K. H. (1997) Mol. Cell. Biol. 17, 6609–6617
57. Gery, S., Gombart, A. F., Fung, Y. K., and Koeffler, H. P. (2004) Blood 103, 828–835
58. Tahirov, T. H., Sato, K., Ichikawa-Iwata, E., Sasaki, M., Inoue-Bungo, T., Shiina, M., Kimura, K., Takata, S., Fujikawa, A., Morii, H., Kumasaka, T., Yamamoto, M., Ishii, S., and Ogata, K. (2002) Cell 108, 57–70