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Endogenous CCAAT/Enhancer Binding Protein β and p300 Are Both Regulated by Growth Hormone to Mediate Transcriptional Activation

Tracy Xiao Cui, Graciela Piwien-Pilipuk, Jeffrey S. Huo, Julianne Kaplani, Roland Kwok, and Jessica Schwartz

Department of Molecular & Integrative Physiology (T.X.C., G.P.-P., J.K., J.S.), Program in Cellular and Molecular Biology (J.S.H., J.S.), Departments of Biological Chemistry and Obstetrics & Gynecology (R.K.), University of Michigan, Ann Arbor, Michigan 48109

The regulation of c-*fos* transcription by GH involves multiple factors, including CCAAT/enhancer binding protein (C/EBP) β. Knockdown of C/EBPβ by RNA interference prevents stimulation of endogenous c-*fos* mRNA by GH, indicating a key role for C/EBPβ in GH-stimulated c-*fos* transcription. GH rapidly increases the occupancy of both endogenous C/EBPβ and p300 on the c-*fos* promoter in 3T3-F442A preadipocytes as indicated by chromatin immunoprecipitation. The transient occupancy of p300 on c-*fos* and the presence of p300 in the anti-C/EBPβ immunoprecipitate coincide with the transient increase in c-*fos* transcription with GH, suggesting that a nuclear complex containing both p300 and C/EBPβ occupies the c-*fos* promoter in response to GH. Expression of p300 with C/EBPβ markedly increases c-*fos* promoter activity when neither alone is effective, indicating that p300 coactivates C/EBPβ-mediated c-*fos* promoter activation. Such coactivation can determine a baseline for c-*fos* activation by GH. Furthermore, the occupancy of phosphorylated murine C/EBPβ (T188) on c-*fos* upon GH treatment is simultaneous with increased occupancy by p300, suggesting that phospho-C/EBPβ recruits p300 in response to GH. Thus, endogenous C/EBPβ and p300 on c-*fos* are dynamically regulated by GH to determine transcriptional activation. Phosphorylated C/EBPβ and p300 appear to function as part of a regulated complex that mediates GH-stimulated transcription. (*Molecular Endocrinology* 19: 2175–2186, 2005)

EUKARYOTIC TRANSCRIPTION involves coordination of multicomponent complexes that include transcription factors, coactivators, corepressors, and other nuclear proteins, which in combination enhance or repress transcription (1–5). The coactivators p300 and the homologous cAMP response element binding protein-binding protein (CBP) have been documented to enhance transcription through interactions with a variety of DNA-bound transcription factors, facilitating activation of the basal transcription machinery (6, 7). The formation and components of such complexes provide potential targets for regulation of transcription, in some instances by recruitment of factors to complexes, or by modification of components of the complexes, which leads to changes in their function. For example, estrogen modulates cyclical recruitment to the estrogen receptor of coactivators, chromatin remodeling factors, and components of the mediator complex on the cathepsin D and pS2 promoters (8, 9). In addition, modifications such as phosphorylation, acetylation, and sumoylation can alter the function of transcription factors, coregulators, and other components of nucleoprotein complexes (10–12). Some of the effects of coactivators on transcription are related to their ability to function as acetyltransferases that acetylate histones or other proteins, leading to changes such as chromatin remodeling (13–16).

GH regulates normal growth by modulating gene expression. Among GH-responsive genes, c-*fos* is rapidly and transiently stimulated in various cell types (17, 18), including 3T3-F442A preadipocytes which require GH to differentiate to adipocytes (19). The activation of c-*fos* by GH is mediated by an enhancer/promoter region, which contains multiple sequences that are regulated by transcription factors in a GH-dependent manner, based on *in vitro* studies. Among these GH-regulated factors are CCAAT/enhancer binding protein (C/EBP) β and C/EBPδ, which bind to the C/EBP site (20–22); serum response factor (SRF) and Elk-1, which bind to the serum response element (SRE) (23–25); and signal transducers and activators of

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Abbreviations: Ac-H4, Acetylated histone 4; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; CBP, cAMP response element binding protein-binding protein; CHO, Chinese hamster ovary; CMV, cytomegalovirus; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethylsulfonylfluoride; QT-PCR, quantitative real-time PCR; P-Pol II, phosphorylated RNA polymerase II; RLU, relative luciferase units; SDS, sodium dodecyl sulfate; siC/EBPβ, a hairpin short interfering RNA targeting C/EBPβ; SRF, serum response factor; Stat, signal transducers and activators of transcription.

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transcription (Stats) 1 and 3, which bind to the Sis-inducible element (26–28). Although each of these factors can mediate transcriptional activation in response to GH, whether and how they are coordinated to regulate c-*fos* expression is not known. The possibility that p300 plays a coordinating role is suggested by observations that each of these transcription factors can interact individually with the coactivators p300/CBP (29–34). Therefore, determining whether p300 is involved in GH-regulated events in c-*fos* promoter activation can provide insight into whether a nucleoprotein complex contributes to the coordinated regulation of c-*fos* by GH.

Among the GH-regulated transcription factors binding to the c-*fos* promoter, C/EBPβ has been well studied. GH stimulates the binding of murine C/EBPβ to the c-*fos* C/EBP site and stimulates its phosphorylation via MAPK at Thr188 to determine its transcriptional activation (20, 35, 36). This study establishes a critical role for C/EBPβ in GH-stimulated expression of endogenous c-*fos* mRNA, and in c-*fos* promoter activation because these responses to GH are blocked when C/EBPβ is knocked down by RNA interference. Endogenous C/EBPβ and endogenous p300 are shown to occupy the c-*fos* promoter in response to GH. Not only does GH dynamically induce the rapid and transient occupancy of endogenous C/EBPβ and p300 on c-*fos* in a manner that corresponds with GH-stimulated transcription, but p300 coactivates C/EBPβ on c-*fos*, establishing a transcriptional baseline that determines the extent of GH-stimulated c-*fos* expression. The presence of phosphorylated C/EBPβ on c-*fos* in response to GH suggests that phospho-C/EBPβ and p300 function as part of a regulated nuclear complex that mediates GH-stimulated transcription.

RESULTS

GH-Stimulated c-*fos* Expression Is Dependent on C/EBPβ

The transcription of c-*fos* is stimulated by GH rapidly and transiently in various cell types, including 3T3-F442A preadipocytes (17, 18, 37) and Chinese hamster ovary (CHO) cell lines stably expressing GH receptors (CHO-GHR) (38). Quantitative real-time PCR (QT-PCR) confirms that c-*fos* mRNA expression peaks 30 min after GH treatment in 3T3-F442A and CHO-GHR cells, then subsides within 1 h and is almost undetectable at later times (Fig. 1A), consistent with previous Northern analysis (17, 37, 38). The pattern of

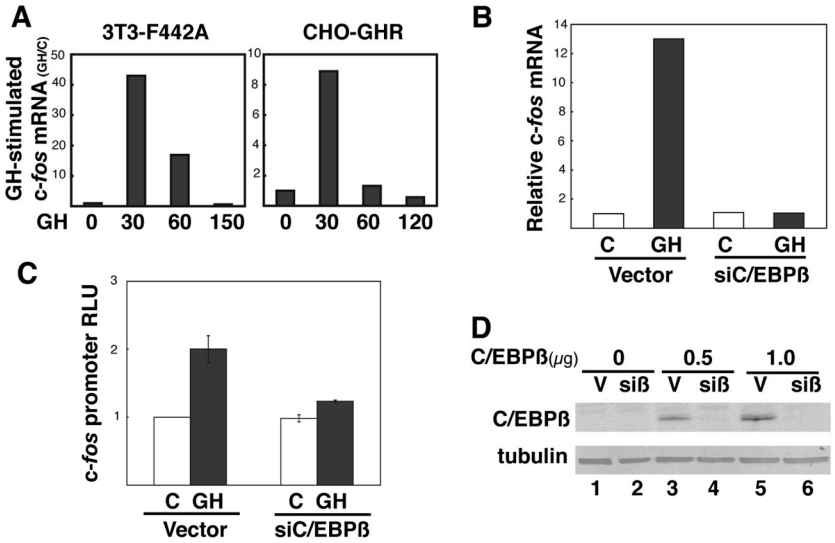

Fig. 1. GH-Induced c-*fos* Expression Depends on C/EBPβ

A, 3T3-F442A or CHO-GHR cells were treated with GH for various times (min). Total RNA was prepared and used for QT-PCR with c-*fos* primers. c-*fos* Expression was analyzed for each sample and normalized to GAPDH. The increase in c-*fos* mRNA expression due to GH is presented as the ratio of GH to control (GH/C) for each time point. Similar results were obtained in three independent experiments. B, Plasmid siC/EBPβ or vector mU6pro was expressed in CHO-GHR cells. Cells were treated 48 h later with or without GH for 30 min, and RNA was prepared for QT-PCR with c-*fos* primers. Expression of c-*fos* mRNA with GH or siC/EBPβ is presented relative to vector in untreated cells. Similar results were obtained in three independent experiments. C, CHO-GHR cells were transfected with the plasmid siC/EBPβ or vector (200 ng each) in the presence of fos-Luc (0.4 μg) and CMV-βgal (0.1 μg). Cells were treated with (*black bars*) or without (*open bars*) GH and analyzed for luciferase activity as described. c-*fos* Promoter activation is expressed as RLU compared with vector control = 1. Data are expressed as mean ± SE in this and subsequent figures (n = 3 independent experiments). The increase due to GH is significant ($P < 0.05$) in cells transfected with vector, but not in cells transfected with siC/EBPβ. D, Plasmid siC/EBPβ (siβ) or vector (V, 5 μg each) was coexpressed with or without CMV-C/EBPβ in indicated amounts (μg) in CHO-GHR cells. Nuclear extracts were analyzed by immunoblotting using anti-C/EBPβ (1:1000, *upper panel*). Antibody against α-tubulin (1:1000) was used to determine loading (*lower panel*).

the response to GH is comparable in both cell types, although the magnitude of the response to GH is about five times greater in highly responsive 3T3-F442A preadipocytes than in CHO-GHR cells.

The GH-regulated transcription factor C/EBPβ is expressed in both 3T3-F442A (20, 39, 40) and CHO-GHR cells (data not shown). The dependence of GH-stimulated endogenous c-*fos* expression on cellular C/EBPβ is demonstrated by blockade of the response to GH in the absence of C/EBPβ. RNA interference against C/EBPβ using a hairpin short interfering RNA (siC/EBPβ) inhibits GH-induced expression of endogenous c-*fos* mRNA in CHO-GHR cells detected by QT-PCR (Fig. 1B). Consistent with its inhibition of endogenous c-*fos* mRNA expression, siC/EBPβ also inhibits the ability of GH to stimulate c-*fos* promoter activation when a luciferase reporter gene driven by a c-*fos* enhancer sequence (−379 to +1, fos-Luc) is coexpressed with siC/EBPβ in GH-treated CHO-GHR cells (Fig. 1C). Interestingly, the basal c-*fos* expression is not altered by siC/EBPβ (Fig. 1, B and C). In the same experimental setting, neutralization of cellular C/EBPβ protein is demonstrated by the fact that when increasing amounts of C/EBPβ are expressed (Fig. 1D, lanes 3–6), siC/EBPβ completely reduces C/EBPβ even when the endogenous protein levels of C/EBPβ are too low to be detected under these conditions (Fig. 1D, lanes 1–2). Taken together, these findings demonstrate that endogenous C/EBPβ plays a key role in GH-stimulated c-*fos* expression.

GH Increases the Occupancy of Endogenous C/EBPβ on the c-*fos* Promoter

To examine *in vivo* how GH promotes c-*fos* activation via C/EBPβ, the occupancy of endogenous C/EBPβ on the c-*fos* promoter was evaluated using chromatin immunoprecipitation (ChIP). Chromatin-bound proteins were immunoprecipitated with anti-C/EBPβ from nuclei of 3T3-F442A cells treated with or without GH for various times. DNA fragments associated with immunoprecipitated proteins were amplified by PCR using primers (p5 and p3), which generate a 330-bp fragment of the c-*fos* promoter (Fig. 2; and supplemental Fig. 8A published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Endogenous C/EBPβ immunoprecipitates with c-*fos* promoter DNA in untreated cells (Fig. 2, t = 0), consistent with previous observations by EMSA of constitutive binding of C/EBPβ (20, 35). Nevertheless, within 15 min of GH treatment, the amount of endogenous C/EBPβ which occupies the c-*fos* promoter *in vivo* is consistently greater in GH-treated cells than in controls. The occupancy of C/EBPβ on the c-*fos* promoter peaks at 30 min and subsides by 60 min but remains elevated relative to untreated cells. Immunoblotting shows that the amount of endogenous C/EBPβ in the immunoprecipitates used for the ChIP assay does not change with GH treatment (supplemental Fig. 9), indicating that it is the amount of

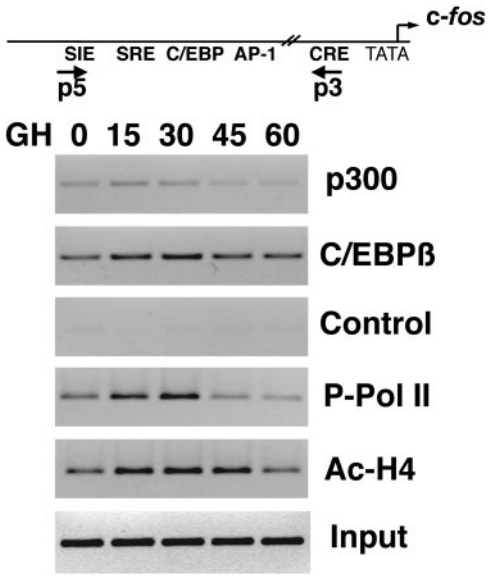

Fig. 2. GH Increases Occupancy of Endogenous C/EBPβ and p300 on the c-*fos* Promoter

Schematic at top shows the c-*fos* promoter (−379 to +1) containing multiple GH-regulated sites including a C/EBP site (not to scale). *Arrows* indicate the locations of primers (p5 and p3) used for ChIP PCR. For ChIP (*lower panels*), 3T3-F442A cells were treated with GH for the indicated times (min). ChIP was performed using anti-C/EBPβ (4 μg), anti-p300 (6 μg), anti-P-Pol II (4 μg), anti-Ac-H4 (3 μg), or no antibody (control). Proteins recognized by IP are indicated on right. 1% Input is shown in *bottom panel*. Purified DNA was used for PCR (33 cycles). Similar data were obtained in four other experiments.

C/EBPβ occupying the c-*fos* promoter that increases. By ChIP, the bands representing c-*fos* promoter DNA identified with anti-C/EBPβ contrast with the complete lack of signal when no antibody (Fig. 2; control) or normal rabbit IgG (data not shown) is used in place of anti-C/EBPβ. The amounts of phosphorylated RNA polymerase II (P-Pol II) and acetylated histone 4 (Ac-H4) associated with the c-*fos* promoter also increase 15 and 30 min after GH treatment and subside by 60 min. This timing for activation of Pol II by phosphorylation and for acetylation of H4 associated with the c-*fos* promoter parallels the timing of the activation of c-*fos* transcription by GH (17). Taken together, these findings indicate that GH increases the occupancy of endogenous C/EBPβ on c-*fos*, most likely by recruiting additional C/EBPβ to the c-*fos* promoter as it initiates transcription.

GH Induces the Transient Occupancy of Endogenous p300 on the c-*fos* Promoter

Transcription complexes are thought to assemble on promoters by association of nuclear proteins, including coactivators such as p300, with the DNA-bound transcription factors such as C/EBPβ. In fact, p300 can associate with C/EBPβ (data not shown; and Refs. 29 and 30). The occupancy of p300 with the c-*fos*

promoter in response to GH was therefore examined for insight into whether GH regulates the appearance of a coactivator on c-*fos,* possibly in conjunction with C/EBPβ and activation of transcription. GH was found to increase the occupancy of endogenous p300 on the c-*fos* promoter within 15 min (Fig. 2; p300). Not only does the GH-stimulated increase in p300 coincide with the rapid increase in transcription of c-*fos* in response to GH, but the association of p300 with the promoter subsides to control levels by 60 min, as c-*fos* transcription does. The same time course for p300 was detected with two different antibodies against p300 (data not shown), suggesting that the changes in p300 are unlikely to reflect factors such as epitope masking during hormone treatment and cross-linking (41). p300 Appears to be slightly detectable on the c-*fos* promoter in untreated cells. The protein levels of p300 in the lysates for ChIP are not altered by GH treatment (data not shown). These findings indicate that GH increases the occupancy of p300 on the promoter at the same time (15–30 min) that it increases the occupancy of C/EBPβ on c-*fos*, opening the possibility that GH promotes rapid assembly of C/EBPβ and p300 on c-*fos* as part of a transcription complex.

The ability of GH to increase the occupancy of p300 on the c-*fos* promoter suggests that p300 may be involved in GH-stimulated c-*fos* transcription. Consistent with the latter, expression of the adenoviral E1A oncoprotein, which interacts with p300 and can repress its coactivator functions (42, 43), inhibits the GH-induced expression of endogenous c-*fos* mRNA in CHO-GHR cells (Fig. 3A). The stimulation by GH of c-*fos* promoter activation was also blocked by coexpression of E1A with fos-Luc in CHO-GHR cells (Fig. 3B). Thus, inhibition of GH stimulation by E1A is suggestive that p300 contributes to GH-stimulated c-*fos* expression. The transient nature of GH-stimulated c-*fos* transcription may therefore be related to the transient increase in the occupancy of p300 observed on the c-*fos* promoter in response to GH.

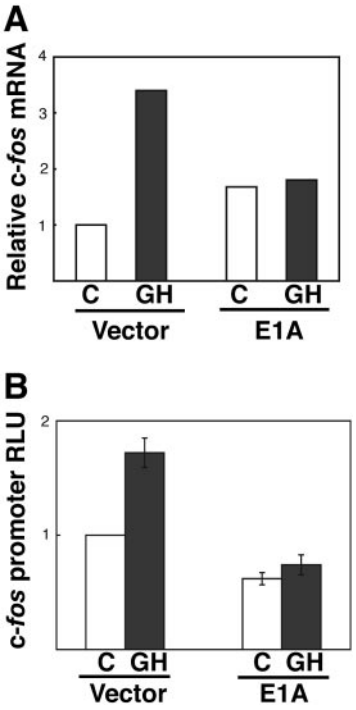

Fig. 3. E1A Interferes with GH-Stimulated c-*fos* Expression

A, Plasmid E1A or vector pRc/RSV (5 μg each) was expressed in CHO-GHR cells. Cells were treated without (*open bars*) or with (*black bars*) GH for 30 min, and RNA was prepared for QT-PCR with c-*fos* and GAPDH primers. Expression of endogenous c-*fos* mRNA is presented relative to vector-transfected cells without GH treatment (C = 1). Similar data were obtained in three independent experiments. B, The plasmid fos-Luc was expressed with a plasmid for E1A or vector (0.5 μg each) in CHO-GHR cells. Cells were treated without (C, *open bars*) or with GH (*black bars*) and luciferase measured (n = 3 experiments). The increase due to GH ($P < 0.05$) in controls with vector is significantly inhibited ($P < 0.05$) in the presence of E1A.

A Complex Containing Endogenous C/EBPβ and p300 Occupies the c-*fos* Promoter upon GH Treatment

Because C/EBPβ binds directly to the c-*fos* promoter, and because C/EBPβ and p300 can form a complex, it is likely that the association of p300 with c-*fos* DNA is mediated by a DNA-bound factor, such as C/EBPβ. To assess whether C/EBPβ and p300 occupy the same c-*fos* promoter DNA, a Re-ChIP assay was employed. After GH treatment, cross-linked nuclear lysates were first immunoprecipitated with anti-C/EBPβ. Then the immunoprecipitate was washed and the DNA with the associated protein complex was eluted from the beads and subjected to a second immunoprecipitation with anti-p300 before processing for PCR. The first IP confirms that GH increases the occupancy of C/EBPβ on c-*fos* promoter DNA within 15 min (Fig. 4; 1st IP). The second IP identifies p300 in the C/EBPβ immunoprecipitate (Fig. 4; 2nd IP, *bottom*), indicating that a complex containing both p300 and C/EBPβ occupies the same c-*fos* promoter DNA. Conversely, when the first anti-p300 immunoprecipitate was similarly reimmunoprecipitated with anti-C/EBPβ, C/EBPβ was also identified in the p300 complex associated with c-*fos* DNA (Fig. 4; 2nd IP, *top*). These findings indicate that in response to 15 min GH treatment, a complex containing both p300 and C/EBPβ occupies c-*fos* promoter DNA.

p300 Coactivates C/EBPβ-Mediated c-*fos* Promoter Activation in the Absence and Presence of GH

Because endogenous C/EBPβ and p300 occupy the same c-*fos* promoter after GH treatment, it was of interest to examine whether p300 modulates C/EBPβ-mediated c-*fos* promoter activation. Expression of C/EBPβ alone, or of increasing amounts of p300 alone, slightly increases c-*fos* promoter activity com-

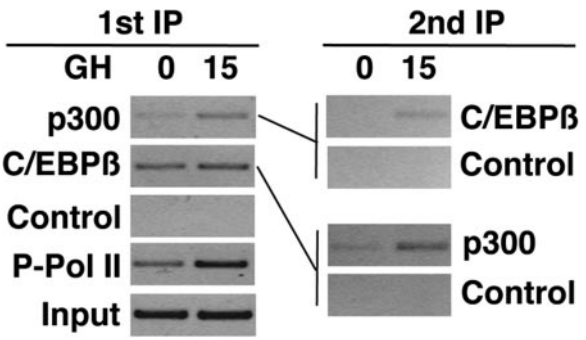

Fig. 4. GH Promotes the Occupancy of a Complex Containing both C/EBPβ and p300 on the c-*fos* Promoter *in Vivo*

3T3-F442A cells were treated with GH for 0 or 15 min. Primary ChIP was performed using anti-C/EBPβ (4 μg), anti-p300 (4 μg) or anti-P-Pol II (4 μg) as described for Fig. 2. The beads from the first IP were washed and eluted for the second IP as described. The eluate from the first IP with anti-C/EBPβ was used for the second IP with anti-p300 (4 μg) or no antibody as control (2nd IP, *bottom*). The eluate from the first IP with anti-p300 was used for the second IP with anti-C/EBPβ (4 μg) or no antibody as control (2nd IP, *top*). Proteins recognized by IP are indicated on right. 1% Input is also shown. Results of PCR (35 cycles) are shown. *Brackets* indicate which of the first IP was used for second IP. Similar data were obtained in another experiment.

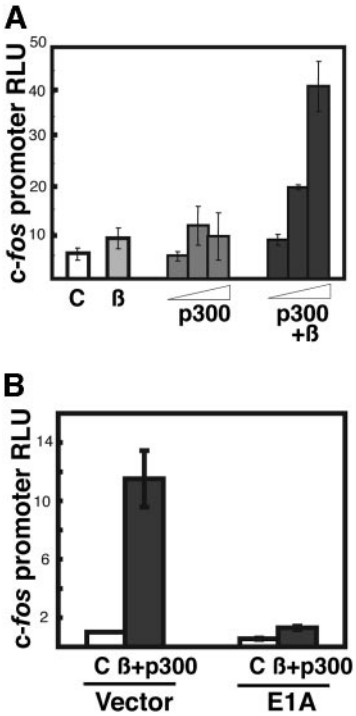

Fig. 5. p300 Coactivates C/EBPβ-Mediated c-*fos* Promoter Activity

A, Plasmids for C/EBPβ (β, *light gray bar,* 5 ng) and p300 (*gray bars)* were transfected at 0.1, 0.5, or 1 μg/well, alone or in combination (*black bars*), together with fos-Luc (*open bar*) in CHO-GHR cells. Luciferase activity 48 h after transfection is shown. The increase in c-*fos* promoter activation over control is significant only with the combination of C/EBPβ and p300, when p300 is used at 0.5 μg ($P < 0.05$) and 1.0 μg ($P < 0.01$). B, The plasmid fos-Luc was expressed with plasmids for C/EBPβ (β, 5 ng) plus p300 (1 μg) (β + p300), or control DNA, in CHO-HGR cells. Additionally, E1A or its vector (0.5 μg each) was coexpressed. Control RLU without E1A is set at 1.0 (n = 3 experiments). The coactivation in the presence of C/EBPβ plus p300 is significantly ($P < 0.01$) inhibited by E1A.

pared with control (Fig. 5A). In contrast, when the same amounts of p300 are coexpressed in combination with C/EBPβ, c-*fos* promoter activity is markedly increased (Fig. 5A, *black bars*). The enhanced activation of transcription with p300 increases as the amount of p300 is increased. The increase in transcription with the combined expression of C/EBPβ and p300 was much greater than the modest increase observed with expression of either alone. Immunoblotting shows that the protein level of C/EBPβ is not increased by p300 (supplemental Fig. 10). Overall, these results indicate that p300 coactivates C/EBPβ-mediated c-*fos* promoter activation. The role of p300 is substantiated by reversal of coactivation when E1A is coexpressed with C/EBPβ and p300 (Fig. 5B). Taken together, these findings indicate that p300 can interact with C/EBPβ and coactivate transcription on the c-*fos* promoter.

To examine whether the coactivation of C/EBPβ by p300 contributes to GH-stimulated c-*fos* promoter activation, cells were additionally treated with GH. GH typically elicits a significant and reproducible doubling of the activation of the c-*fos* promoter compared with untreated cells (Fig. 6). Stimulation by GH (Fig. 6, *black bars*) also occurs when C/EBPβ or p300 alone is expressed. Even when coactivation in the combined presence of C/EBPβ and p300 raises the level of c-*fos* promoter activity, GH significantly increases transcription above the elevated level (Fig. 6, *rightmost pair of bars*). It appears that C/EBPβ and p300 modulate transcription via the c-*fos* promoter in untreated cells, resetting the basal levels, which are highest when they are expressed in combination. GH appears to activate transcription above whatever basal level prevails. These findings raise the possibility that beyond coactivation, an additional GH-dependent event occurs upon occupancy of C/EBPβ and p300 on the c-*fos* promoter.

GH Promotes Transient Occupancy of Phosphorylated C/EBPβ on the c-*fos* Promoter *in Vivo*

GH transiently increases MAPK-dependent phosphorylation of mouse C/EBPβ on Thr188 (P-C/EBPβ, corresponding to Thr235 of human C/EBPβ), a modification which is required for c-*fos* promoter activation by C/EBPβ in response to GH (36). To determine whether GH regulates the occupancy of endogenous P-C/EBPβ on the c-*fos* promoter *in vivo*, ChIP was performed with an antibody specific for P-C/EBPβ. GH was found to increase the presence of endogenous

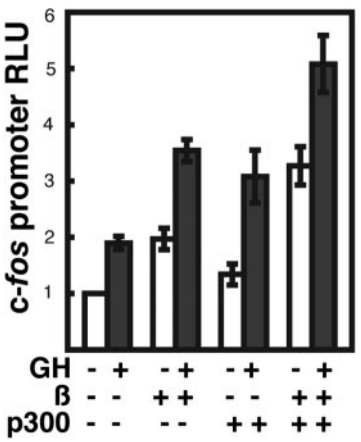

Fig. 6. GH Increases c-*fos* Promoter Activation in the Presence of C/EBPβ and p300

The plasmid fos-Luc was coexpressed with C/EBPβ (5 ng) or p300 (0.5 μg) alone or in combination in CHO-GHR cells. Cells were treated 48 h later without (*open bars*) or with GH (*black bars*) and luciferase was measured (n = 3). The increase due to GH is statistically significant ($P < 0.04$) for each pair.

P-C/EBPβ on the c-*fos* promoter within 15 min of treatment, as detected by ChIP (Fig. 7). The presence of P-C/EBPβ was barely evident without GH treatment and was almost undetectable 60 min after GH. The timing of the transient occupancy by P-C/EBPβ *in vivo* is consistent with previous findings that GH transiently increases phosphorylation of C/EBPβ at Thr188 in 15 min, and that P-C/EBPβ transiently binds to the c-*fos* C/EBP site *in vitro* (36). The pattern of occupancy of P-C/EBPβ on the c-*fos* promoter *in vivo* coincides with the transient pattern of occupancy of p300 on the same region of the c-*fos* promoter DNA (Figs. 2, 4, and 7), and coincides with the timing of GH-induced c-*fos* transcription. These findings suggest that a posttranslational modification such as phosphorylation, stimulated by GH on C/EBPβ, might play a role in GH-stimulated c-*fos* gene expression *in vivo*, possibly by recruiting factors such as p300 into a complex on c-*fos* promoter DNA.

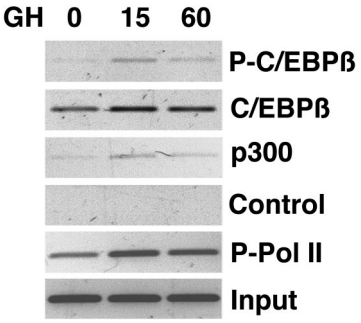

Fig. 7. GH Induces the Occupancy of Phosphorylated C/EBPβ on the c-*fos* Promoter *in Vivo*

3T3-F442A cells were treated with GH for 0, 15, or 60 min. ChIP was performed using anti-P-C/EBPβ (4 μg), anti-C/EBPβ (4 μg), anti-p300 (6 μg), anti-P-Pol II (4 μg), or no antibody (control). Proteins recognized by IP are indicated on right. 1% Input is also shown. Purified DNA was used for PCR (33 cycles). Similar data were obtained in two other experiments.

DISCUSSION

Endogenous C/EBPβ Is Essential for GH-Stimulated c-*fos* Transcription

These studies establish the physiological relevance of endogenous C/EBPβ for the stimulation of c-*fos* transcription by GH. Neutralization of C/EBPβ by RNA interference blocks both the ability of GH to stimulate endogenous c-*fos* mRNA expression, and to activate the c-*fos* promoter. The requirement for C/EBPβ *in vivo* is consistent with reduction of GH-stimulated c-*fos* promoter activation when the C/EBP site is mutated to prevent the binding of C/EBPβ (data not shown). Interestingly, neutralization of C/EBPβ by siC/EBPβ does not alter basal c-*fos* mRNA expression or promoter activation, suggesting that C/EBPβ is necessary to mediate regulated, but not basal c-*fos* transcription. The present studies suggest that phosphorylation of endogenous C/EBPβ *in vivo* is one of the events involved in the regulation of c-*fos* transcription by GH.

The importance of C/EBPβ for GH-stimulated c-*fos* expression is consistent with another novel finding in this study, that GH regulates the occupancy of endogenous C/EBPβ on c-*fos* promoter DNA *in vivo*. Upon addition of GH to 3T3-F442A cells, the occupancy of C/EBPβ on the c-*fos* promoter increases within 15 min, coincident with the timing of activation of c-*fos* transcription by GH (17). The C/EBPβ associated with c-*fos* increases even though the total amount of C/EBPβ present in GH-treated cells is unaltered. This implies that C/EBPβ is redistributed within the nucleus in response to GH, and may be related to our previous observations of rapid (5–15 min) relocalization of C/EBPβ in nuclei of 3T3-F442A cells treated with GH (44). C/EBPβ remains associated with c-*fos* DNA 60 min after GH, even though GH-induced transcription has subsided by this time. This difference in timing implicates regulatory events in addition to enhanced binding to DNA in modulating the dynamics of C/EBPβ in the transcriptional response to GH.

In addition to C/EBPβ, GH also regulates other transcription factors that can mediate activation of c-*fos* transcription on the same c-*fos* promoter sequence (−379 bp to +1 bp), including Elk-1 (25), SRF (23, 24), Stats 1 and 3 (26, 27, 45, 46), and C/EBPδ (20), which is not neutralized by siC/EBPβ (Kaplani, J., and J. Schwartz, data not shown). C/EBPβ may work in coordination with these or other transcription factors on c-*fos* in responding to GH because interactions of C/EBPβ with SRF and Elk-1 have been reported (47, 48). Neutralization of C/EBPβ by siC/EBPβ may dis-

rupt its ability to form a complex with other proteins associated with c-*fos*, thereby interfering with transcription of the gene. The substantial reduction in c-*fos* expression when C/EBPβ function is impaired reinforces that C/EBPβ has a central role in stimulation of c-*fos* transcription by GH, and that C/EBPβ makes a major contribution in this regard in an endogenous setting. C/EBPβ may play a similar role in the regulation by GH of other genes known to bind C/EBPβ, such as genes for Spi2.1 (49), IGF-I (50), or alcohol dehydrogenase (51).

GH Promotes the Recruitment of Endogenous p300 to the c-*fos* Promoter

GH was found to increase the occupancy of p300 on the c-*fos* promoter *in vivo*. The timing of the response to GH is notable both because p300 increases within 15 min of GH treatment and because p300 is no longer evident 60 min after GH. The rapid and transient appearance of p300 on c-*fos* coincides with the timing of GH-stimulated transcription of c-*fos* (17), making it tempting to speculate that the presence of p300 is a feature that determines the pattern of c-*fos* transcription.

The appearance of p300 and C/EBPβ on the c-*fos* promoter in response to GH is accompanied by a simultaneous increase in the presence of Ac-H4 and phosphorylated RNA Pol II on c-*fos*. Acetylation of lysines on the tails of H4 at the nucleosome core (52) is believed to mediate c-*fos* activation by facilitating the unwinding of DNA in chromatin, allowing regulated transcription factors, such as C/EBPβ, to associate with the promoter. The parallel timing of the increase in P-Pol II, an indicator of transcription initiation, on the c-*fos* promoter in response to GH suggests that the simultaneous occupancy of these proteins on c-*fos* is part of a coordinated set of changes in proteins associated with the DNA that leads to the rapid and transient increase in transcription with GH treatment.

These studies also show that p300 and C/EBPβ occupy the same c-*fos* promoter DNA in response to GH, suggesting that they are part of a complex. Furthermore, GH simultaneously increases the occupancy of p300 and C/EBPβ. It is not clear whether C/EBPβ and p300 form a complex upon the binding of C/EBPβ to the DNA, or whether a preformed complex of endogenous C/EBPβ and p300 associates with the DNA in response to GH. Because GH regulates the phosphorylation of C/EBPβ, this modification may be a factor in its ability to recruit p300 in response to GH, as it appears to be for association of C/EBPβ with SRF (53) and components of the mediator complex (54) in Ras-expressing cells. It is also possible that the association of p300 with other proteins bound to c-*fos* is involved in the formation of complexes containing p300 and enhances c-*fos* transcription because p300 serves widely as a scaffold for nucleoprotein complexes (55, 56). The pattern of transient recruitment of p300 in response to GH follows a kinetic profile comparable to that reported for c-*fos* after T cell activation. This transient pattern is distinct from a sustained recruitment of p300 to the promoters for p21 and other genes (57). An alternative pattern is evident on the phosphoenolpyruvate carboxykinase promoter, where occupancy by CBP is reduced by insulin, in part by displacement of the activating liver-enriched activating protein form of C/EBPβ with the inhibitory liver-enriched transcriptional inhibitory protein form (41). These observations indicate the importance of the distinct pattern of occupancy by p300 and simultaneous occupancy by phosphorylated C/EBPβ in response to GH for determining c-*fos* transcription.

p300 Coactivates C/EBPβ on the c-*fos* Promoter

The interaction of C/EBPβ and p300 on the native c-*fos* promoter can result in coactivation. Others have reported coactivation of C/EBPβ by p300 on other promoters (29, 30). Here, coactivation may be related to the simultaneous recruitment of p300 and C/EBPβ to the c-*fos* promoter. These studies suggest that the formation of a complex containing C/EBPβ and p300 on the c-*fos* promoter determines transcription in untreated cells expressing the proteins. When C/EBPβ is overexpressed in 293T cells, it is constitutively phosphorylated at the MAPK site as well as other sites (36). Phosphorylation of at least some of the expressed C/EBPβ at T188 was also observed in CHO-GHR cells (data not shown). Whether constitutive phosphorylation of C/EBPβ expressed in CHO-GHR cells in these experiments is required for the coactivation remains to be determined. Conversely, the association of C/EBPβ with p300 or CBP promotes avid phosphorylation of these coactivators, which contributes to their ability to coactivate C/EBPβ-dependent transcription (30, 58). Other modifications, such as acetylation of either C/EBPβ [Cesena, T. I., and J. Schwartz, unpublished observations (59)] or p300/CBP (60–62), possibly mediated by the acetyltransferase activity associated with p300/CBP, which can contribute to c-*fos* activation (63), may also contribute to recruitment and/or coactivation.

C/EBPβ Phosphorylation May Mediate Recruitment of p300 for GH-Induced c-*fos* Promoter Activation

In cells expressing C/EBPβ or p300 alone or in combination, GH stimulates c-*fos* promoter activation. The ability of GH to increase c-*fos* transcription two to three times basal values is consistently observed and depends on C/EBPβ. The actual level of transcription achieved in the presence of GH varies depending on the prevailing basal level of transcription in the cell, which in turn is determined by the expression of C/EBPβ and p300 in these experiments. The changing baseline affords great flexibility and range to the re-

sponsiveness of the c-*fos* promoter to a regulator such as GH. Presumably, when overexpressed, C/EBPβ and p300 associate with c-*fos* either alone or in combination, and determine transcription in the absence of GH. In response to GH, the additional C/EBPβ and p300 that rapidly occupy the c-*fos* promoter appear to mediate the increase in promoter activation above basal levels.

In addition to recruitment, the GH-dependent event that may trigger the increase in transcription above basal levels is likely to be related to the phosphorylation of C/EBPβ. Its transient phosphorylation at T188 in response to GH, mediated by ERKs 1 and 2, has been found to determine its binding and is required for GH-stimulated c-*fos* transcription (36). It has been proposed that phosphorylation of C/EBPβ at this site is associated with activation of transcription by derepression (64, 65). The present studies show that the GH-stimulated increase in occupancy by phosphorylated C/EBPβ on c-*fos* is simultaneous with the ability of GH to increase the occupancy of p300 on the promoter. Furthermore, C/EBPβ and p300 occupy the same promoter DNA in GH-treated cells. Thus, the transient time course for GH-stimulated phosphorylation that parallels p300 occupancy also parallels the time course of GH-stimulated transcription of c-*fos*. Taken together, these observations suggest that phosphorylation of C/EBPβ may determine the ability of GH to recruit p300 and to increase transcription. Thus, phosphorylation of C/EBPβ at T188 may contribute to formation of an active regulatory complex on c-*fos* promoter DNA, leading to the transient stimulation of c-*fos* expression in response to GH. Different proteins may be recruited to a complex when C/EBPβ is phosphorylated. In this context, it is of interest that dephosphorylation of C/EBPβ, likely at a GSK3 site, is observed 60 min after GH treatment (supplemental Fig. 9, lane 3; and Ref. 35), and might contribute to reducing c-*fos* expression by altering the complex on the promoter.

GH may also induce additional phosphorylation at other sites on C/EBPβ, or other modifications of C/EBPβ and/or p300, as well as participation of other proteins in a complex with them. A newly assembled complex may enable GH to stimulate transcription beyond the prevailing baseline level. These events may mediate integration of the function of C/EBPβ with other transcription factors or nuclear proteins that are regulated in diverse ways upon GH treatment (22). Such events may involve formation of an enhanceosome (3, 66, 67) containing additional nuclear proteins, including other coregulators (66), associated deacetylases (59) or other enzymatic activities, and/or architectural proteins such as high-mobility group protein I (HMG I/Y) (68). The present findings that GH elicits a simultaneous increase in the occupancy of both endogenous P-C/EBPβ and p300 on c-*fos*, and that C/EBPβ and p300 on c-*fos* determine a baseline for promoter activation in response to GH, are likely to be components among multiple events which are integrated in the nucleus to contribute to GH-regulated transcription.

MATERIALS AND METHODS

Materials

Murine 3T3-F442A preadipocytes were provided by H. Green (Harvard University, Cambridge, MA) and M. Sonenberg (Sloan-Kettering, New York, NY). CHO cells stably expressing a truncated GH receptor (CHO-GHR, GHR1–454) were provided by G. Norstedt (Karolinska Institute, Stockholm, Sweden) and N. Billestrup (Novo Nordisk, Gentofte, Denmark) (69) and used as described (35, 38). Human GH was generously provided by Eli Lilly Inc. (Indianapolis, IN). Culture media, calf serum, fetal calf serum, L-glutamine, and antibiotic-antimycotic were purchased from Invitrogen (Carlsbad, CA). BSA (CRG7) was from Serologicals Corp. (Norcross, GA). The protease inhibitors leupeptin and aprotinin were purchased from Roche Molecular Biochemicals (Indianapolis, IN), phenylmethylsulfonylfluoride (PMSF) from Mallinckrodt, and sodium orthovanadate from Sigma (St. Louis, MO). RNA STAT60 was purchased from Tel-Test B, Inc. (Friendswood, TX), and Taqman Reverse Transcription Kit and SYBR green I from Applied Biosystems (Foster City, CA). The Ac-H4 ChIP assay kit was purchased from Upstate (Lake Placid, NY). Formaldehyde was purchased from Sigma. Immobilized Protein A was purchased from Repligen, sonicated salmon sperm DNA from Stratagene (La Jolla, CA), and the PCR Purification Kit from QIAGEN (Valencia, CA). Luciferin was purchased from Promega (Madison, WI), β-galactosidase chemiluminescence reagents from Tropix (Bedford, MA), and the enhanced chemiluminescence detection system from Amersham Biosciences (Arlington Heights, IL). Protein molecular weight standards were from Invitrogen.

Cell Culture and GH Treatment

3T3-F442A cells were grown in DMEM containing 4.5 g/liter glucose and 8% calf serum in an atmosphere of 10% CO_2/95% air at 37 C. CHO-GHR cells were grown in Ham's F-12 medium containing 10% fetal calf serum and 0.5 mg/ml G418 in an atmosphere of 5% CO_2/95% air at 37 C. All media were supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. Before GH treatment, cells were deprived of serum for 18–20 h in the appropriate medium containing 1% BSA instead of serum, and then treated with GH (500 ng/ml, 23 nM) for the times indicated.

Plasmids and Antibodies

The plasmid for rat C/EBPβ encoding liver-enriched activating protein driven by the cytomegalovirus (CMV) promoter (referred to as CMV-C/EBPβ) was a gift from U. Schibler (University of Geneva, Geneva, Switzerland) and L. Sealy (Vanderbilt University, Nashville, TN). The expression plasmid for hemagglutinin-tagged p300 (CMV-p300) was provided by D. Livingston (Harvard Medical School, Boston, MA), courtesy of O. MacDougald (University of Michigan). To reduce the expression of C/EBPβ, a hairpin short interfering RNA sequence (siC/EBPβ, 5′GAGCGACGAGTACAAGATG3′), which is present in mouse, rat, and human C/EBPβ, was inserted into *Bbs*I and *Xba*I sites in the mU6pro vector. Sequences were confirmed by the University of Michigan Sequencing Core. The mU6pro vector containing the mouse U6 promoter for RNA polymerase III was kindly provided by D. Turner (University of Michigan) (70). The plasmids encoding

$E1A_{289}$ and its backbone vector pRc/RSV were gifts from J. Lundblad (Oregon Health Sciences University, Portland, OR) (71). The reporter plasmid fos-Luc containing the mouse c-*fos* enhancer (−379 to +1, referred to as "promoter" throughout), upstream of the luciferase gene was provided by W. Wharton (University of Southern Florida, Gainesville, FL) and B. Cochran (Tufts University, Boston, MA) (72). pBR322 DNA, provided by M. Lomax (University of Michigan) was used to normalize amounts of transfected DNA. The plasmid CMV-β galactosidase (CMV-β gal) was provided by M. Uhler (University of Michigan).

Rabbit antibody against the C terminus of C/EBPβ, against the N terminus of p300, and against the C-terminal sequence (YSPT[PS]PS) of phosphorylated RNA polymerase II (P-Pol II), as well as normal rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against Ac-H4 and against the C terminus of p300 were from Upstate. Antibody against phospho-Thr188 of mouse C/EBPβ (equivalent to Thr235 of human C/EBPβ) was from Cell Signaling Technology, Inc. (Beverly, MA) (36). Antibodies against porcine α tubulin (residues 1–451), and horseradish peroxidase-conjugated antirabbit IgG were from Santa Cruz. IRDye800-conjugated antirabbit and antimouse IgG were obtained from Rockland Inc. (Gilbertsville, PA).

RNA Interference

To establish that siC/EBPβ reduces expression of C/EBPβ, plasmids for siC/EBPβ or mU6pro vector (5 μg each) were coexpressed with CMV-C/EBPβ (0, 0.5, or 1.0 μg) in CHO-GHR cells (10-cm dish) using calcium phosphate precipitation. Forty-eight hours after transfection cells were scraped in ice cold PBS containing inhibitors [1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin, and 1 mM dithiothreitol (DTT)]. After centrifugation, cell pellets were resuspended in hypotonic buffer [20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, containing inhibitors as above] and centrifuged (10,000 × *g*, 30 sec) to obtain a nuclear pellet. The nuclear pellet was dissolved in lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 6 mM EGTA, 150 mM NaCl, 0.1% Nonidet P-40 containing inhibitors as above] and subjected to immunoblot analysis. Alternatively, RNA was prepared from CHO-GHR cells for c-*fos* QT-PCR as described in *QT-PCR*. For functional measurements, siC/EBPβ or mU6pro vector (200 ng each) were cotransfected with the reporter plasmid fos-luc (0.4 μg) in CHO-GHR cells. Forty-eight hours after transfection cells were treated with or without GH for 4 h, lysed, and luciferase activity measured (35).

QT-PCR

Total RNA was isolated from 3T3-F442A or CHO-GHR cells with RNA STAT60 and reverse transcribed with the Taqman Reverse Transcription Kit. The resulting cDNAs were used to perform QT-PCR in duplicate with the iCycler system (Bio-Rad Laboratories, Hercules, CA) using SYBR green I. The primer pair to amplify mouse c-*fos* was 5′-TTCCTGGCAATAGCGTGTTC-3′ (forward) and 5′-TTCAGACCACCTCGACAATG-3′ (reverse) (73), and to amplify control murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (not responsive to GH, data not shown) was 5′-ATGTTCCAGTATGACTCCACTCACG-3′ (forward) and 5′-GAAGACACCAGTAGACTCCACGACA-3′ (reverse) (74). Results were analyzed using iCycler iQ real-time detection system software (Bio-Rad Laboratories). All c-*fos* values were normalized to GAPDH. When GH was administered for various times, results are expressed as the ratio of GH to control (GH/C) at each time point. In some experiments, plasmids for siC/EBPβ or mU6pro vector (5 μg each), or for E1A or Rc/Rsv vector (5 μg each) were transfected in CHO-GHR cells as described (35). Forty-eight hours later, serum-deprived CHO-GHR cells were treated with or without GH for 30 min before RNA preparation. When siC/EBPβ or E1A were expressed, mRNA expression for each condition, normalized to GAPDH, is presented.

ChIP

After GH treatment, 3T3-F442A preadipocytes were rinsed with cold PBS and cross-linked with 1% formaldehyde in PBS for 10 min. The cells were scraped in ice cold PBS containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin, and 1 mM DTT. After centrifugation, cell pellets were resuspended in hypotonic buffer [20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, containing inhibitors 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin, and 1 mM DTT] and centrifuged (10,000 × *g*, 30 sec) to obtain a nuclear pellet. The ChIP assay was performed following the instructions for the Ac-H4 ChIP Assay kit. The nuclear pellet was dissolved in ChIP sodium dodecyl sulfate (SDS) lysis buffer [50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% SDS, with 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin, and 1 mM DTT] and nuclear extracts were sheared to generate DNA fragments of 500–800 bp (15 sec, seven times, at 4.5 output of Hert Systems sonicator) (supplemental Fig. 8B). Samples were diluted 1:10 with ChIP dilution buffer [16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin] and precleared with 10 μg of salmon sperm DNA (41) and 80 μl of packed Protein A-agarose beads per ml ChIP dilution buffer. For immunoprecipitation, samples containing 100 μg of nuclear protein were incubated overnight at 4 C with the following antibodies individually: anti-C/EBPβ, anti-P-C/EBPβ, anti-p300, anti-Ac-H4, or antiphosphorylated Pol II (P-Pol II). Normal rabbit IgG and samples with no antibody served as negative controls. Then, each immunoprecipitate was incubated for 1 h with 10 μg of salmon sperm DNA (41) and 40 μl of protein A agarose beads. The beads were washed, and eluted, and DNA purified with a PCR purification kit. A single 330-bp fragment (−364 bp to −34 bp) of the mouse c-*fos* promoter (supplemental Fig. 8A) was amplified with 31–35 cycles of PCR (94 C for 20 sec, 60 C for 20 sec, and 72 C for 30 sec) using ChIP primers p5 (5′ GGCTGCAGCCGGCGAGCTG 3′) and p3 (5′ AGAAGCGCTGTGAATGGATG 3′). In each experiment, all of the immunoprecipitated samples were analyzed with the same PCR conditions, for insight into relative amounts of each protein associated with the promoter. Samples were separated on 2% agarose gels and stained with ethidium bromide. Images were visualized and band density calculated using a BioImaging Systems (Ultra-Violet Products, Ltd., Cambridge, UK). When proteins were analyzed in the immunoprecipitates, half of the beads were washed and eluted for immunoblotting analysis under the conditions used for ChIP.

Re-ChIP was performed as described (8) with the following modifications: after the primary immunoprecipitation (1st IP), the beads were washed and incubated with 20 mM DTT at 37 C for 30 min and diluted 1:50 with re-ChIP dilution buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin]. The supernatants were precleared with 10 μg salmon sperm DNA and 80 μl of protein A beads/ml sample. Then the reimmunoprecipitation (2nd IP) with the second antibodies (or control with no antibody) followed the procedures described for the primary immunoprecipitations. After purification of the second IP samples with a PCR purification kit, 35 cycles of PCR were performed with same conditions and primers described for the primary ChIP.

Immunoblotting Analysis

Nuclear extracts or IP eluates were separated by SDS-PAGE (12%), transferred to polyvinylidene difluoride membrane, and incubated with the indicated antibodies overnight at 4 C, as described previously (20). The immunoprecipitated proteins were visualized using enhanced chemiluminescence (supplemental Fig. 9) or with IRDye 800-coupled antirabbit IgG (1:12000) or antimouse IgG (1:12,000) on an Odyssey infrared scanning system (LI-COR, Inc., Lincoln, NE). Molecular weight was estimated using protein molecular weight standards from Invitrogen.

Luciferase Assay

CHO-GHR cells (2×10^5 cells/well) were transiently transfected as described (35, 75) with the fos-Luc reporter plasmids (0.5 μg/well), and plasmids for C/EBPβ, p300, siC/EBPβ, E1A or their respective control vectors, as indicated. Cotransfection with CMV-β-gal (10 ng) was used to normalize for transfection efficiency in all experiments. Approximately 24 h after transfection, cells were deprived of serum by incubation in the appropriate medium containing 1% BSA for 18 h. When indicated, cells were treated with GH for 4 h, then, were lysed for measurement of luciferase and β-galactosidase using an Opticomp Luminometer as described previously (20, 35). Lysates were also used for immunoblotting. Results of luciferase assays, normalized for β-gal, are shown as RLU (relative luciferase units). Controls are set to 1. Luciferase activity is presented as mean ± SE for at least three independent experiments, each performed in triplicate. Statistical analysis of the increment due to GH, or in the presence of other plasmids, was performed using *t* test or two-way ANOVA (Prism version 3; www.GraphPad.com).

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Address all correspondence and requests for reprints to: Dr. Jessica Schwartz, Department of Molecular & Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0622. E-mail: jeschwar@umich.edu.

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Current address for G.P.-P.: Instituto Investigaciones Bioquimicas Fundación Instituto Leloir, Universidad Buenos Aires and Consejo Nacional de Investigaciones Cientifícas y Tećnicas.

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