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CCAAT/Enhancer-binding Protein β (C/EBPβ) and C/EBPδ Contribute to Growth Hormone-regulated Transcription of c-*fos**

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Using the c-*fos* enhancer as a model to analyze growth hormone (GH)-promoted gene expression, the roles of CCAAT/enhancer-binding proteins (C/EBPs) in GH-regulated transcription were investigated. In 3T3-F442A fibroblasts stably expressing the c-*fos* promoter mutated at the C/EBP binding site upstream of luciferase, c-*fos* promoter activity is stimulated by GH 6–7-fold; wild type c-*fos* promoter shows only a 2-fold induction by GH. This suggests that C/EBP restrains GH-stimulated expression of c-*fos*. Electrophoretic mobility shift assays with nuclear extracts from 3T3-F442A cells indicate that GH rapidly (2–5 min) increases binding of C/EBPβ and C/EBPδ, to the c-*fos* C/EBP binding site. Both liver activating protein (LAP) and liver inhibitory protein (LIP), forms of C/EBPβ, are detected in 3T3-F442A cells by immunoblotting. GH increases the binding of LAP/LAP and LAP/LIP dimers. Overexpression of LIP interferes with GH-promoted reporter expression in CHO cells expressing GH receptors, consistent with the possibility that LIP restrains GH-stimulated c-*fos* expression. Overexpression of LAP elevates basal luciferase activity but does not influence promoter activation by GH, while overexpressed C/EBPδ elevates basal promoter activity and enhances the stimulation by GH. GH stimulates the expression of mRNA for C/EBPβ and -δ and increases levels of C/EBPδ. Although C/EBPβ is not detectably altered, GH induces a shift to more rapidly migrating forms of LIP and LAP upon immunoblotting. Treatment of extracts from GH-treated cells with alkaline phosphatase causes a shift of the slower migrating form to the rapidly migrating form, consistent with GH promoting dephosphorylation of LIP and LAP. These studies implicate C/EBPβ and -δ in GH-regulated gene expression. They also indicate that GH stimulates the binding of C/EBPβ and -δ to the c-*fos* promoter and promotes the dephosphorylation of LIP and LAP. These events may contribute to the ability of C/EBPβ and -δ to regulate GH-stimulated expression of c-*fos*.

For insight into mechanisms by which growth hormone (GH)[1] regulates gene expression, analysis of transcriptional regulation of the proto-oncogene c-*fos* provides an excellent model. The c-*fos* gene product is believed to participate in cell growth and differentiation (1), processes associated with the normal growth regulated by GH. Further, the upstream regulatory sequences of c-*fos* contain several response elements now known to be regulated by GH (2–5). Among these, the Sis-inducible element of c-*fos* binds activated signal transducers and activators of transcription (STATs) 1 and 3 in response to GH (3, 5, 6) and can mediate reporter expression in response to GH when STAT 3 and GH receptor are overexpressed (7). Tyrosyl phosphorylation of STATs 1 and 3 in response to GH is a prerequisite for GH-promoted binding and function of STATs (3, 5, 6). A highly GH-responsive sequence, the c-*fos* serum response element (SRE) mediates transcriptional activation in response to GH (2, 8). Such stimulation by GH requires the transcription factor serum response factor (SRF) and a ternary complex factor family member such as Elk-1 (8). GH stimulates the serine phosphorylation of Elk-1 in conjunction with stimulating transcriptional activation mediated by Elk-1 (8, 9). Thus, multiple regulatory sequences and multiple types of post-translational modifications of transcription factors contribute to GH-regulated c-*fos* expression.

Numerous proteins in addition to STATs 1 and 3, SRF, and Elk-1, representing a variety of transcription factor families, also associate with regulatory sequences in c-*fos* (10) and might therefore be regulated by GH. In fact, in our previous studies of the SRE, a prominent unidentified complex which appeared to be regulated by GH was usually observed in electrophoretic mobility shift assays (8). This complex is reported here to reflect binding to a CCAAT/enhancer-binding protein (C/EBP) site, which lies immediately downstream of the SRE.

The C/EBPs belong to the basic region-leucine zipper family of transcription factors, which includes C/EBPα, -β, and -δ (11). C/EBPβ expression increases with hormone stimulation of preadipocyte differentiation and then gradually decreases as differentiation proceeds (12, 13). C/EBPβ occurs as alternate translation products in both an activating form known as liver activating protein (LAP) and an inhibitory form known as liver inhibitory protein (LIP), which lacks the N-terminal transcriptional activation domain found in LAP (14). In the c-*fos* enhancer, C/EBPβ (also known as NF-IL6) binds to a sequence at −303 to −295, just downstream of the SRE (13, 15–17). It was

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[1] The abbreviations used are: GH, growth hormone; STAT, signal transducer and activator of transcription; SRE, serum response element; SRF, serum response factor; C/EBP, CCAAT/enhancer-binding protein; LAP, liver activating protein; LIP, liver inhibitory protein; CHO, Chinese hamster ovary; GHR, GH receptor; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay.

recently suggested that C/EBPβ may play a role in conjunction with SRF in ternary complex factor-independent signaling for SRE activation of c-*fos* in response to serum (17). The present study implicates C/EBPβ and -δ as GH-responsive transcription factors that appear to contribute to the regulation of c-*fos* by GH.

The above observations, particularly the proximity of the C/EBP site to the SRE and the presence of an unidentified GH-induced complex associated with the SRE, led us to investigate whether C/EBPs might contribute to GH-regulated gene expression and might be regulated by GH. Evidence implicating C/EBPβ and δ in GH-regulated transcription is provided here by the observation that mutation of the C/EBP site in the c-*fos* promoter enhances responsiveness of this promoter to GH, suggesting that C/EBPs restrain the induction of c-*fos* by GH. Overexpression of LIP interferes with GH-stimulated gene expression. In contrast, overexpression of LAP does not alter the response to GH, and C/EBPδ enhances it. GH was found to regulate C/EBPβ and -δ in at least three ways. First, GH increases C/EBPβ and δ mRNA and C/EBPδ protein levels. Second, treatment with GH is associated with a rapid increase in binding of LAP/LAP, LAP/LIP, and C/EBPδ to the c-*fos* promoter. Third, GH produces a transient dephosphorylation of both forms of C/EBPβ. These findings are consistent with an interplay of effects of GH on LIP, LAP, and C/EBPδ contributing to determining responsiveness of the c-*fos* promoter to GH.

EXPERIMENTAL PROCEDURES

Materials—3T3-F442A cells were provided by Dr. H. Green (Harvard University) and Dr. M. Sonenberg (Sloan-Kettering). Chinese hamster ovary (CHO) cells expressing rat GH receptor containing the N-terminal half of the cytoplasmic domain (GHR-(1–454)) (18) were provided by Dr. Gunnar Norstedt (Karolinska Institute). 293T cells were provided by Dr. M. Lazar (University of Pennsylvania). Recombinant human GH was provided by Lilly. Culture media were purchased from Irvine Scientific, and sera, G418, and LipofectAMINE were from Life Technologies, Inc. Luciferin was purchased from Promega, and β-galactosidase chemiluminescence reagents from Tropix. Leupeptin, aprotinin, pepstatin, and alkaline phosphatase were purchased from Roche Molecular Biochemicals, vanadate from Sigma, bovine serum albumin (BSA, CRG7) from Intergen, and radioisotopes from NEN Life Science Products. Expresshyb® was purchased from CLONTECH, and the enhanced chemiluminescence (ECL) detection system and Rediprime® labeling kit were purchased from Amersham Pharmacia Biotech.

Cell Culture and Hormone Treatment—3T3-F442A preadipocytes were grown to confluence in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 8% calf serum in an atmosphere of 10% CO_2, 90% air at 37 °C. In CHO cells expressing full-length GHR or GHR-(1–454), GH induces c-*fos* mRNA and stimulates transcriptional activation via the SRE to comparable extents (19), so these cells were used interchangeably. CHO cells expressing GHR were grown in Ham's F-12 medium containing 0.5 mg/ml G418 and 10% fetal calf serum (FCS) in an atmosphere of 5% CO_2, 95% air at 37 °C. All media were supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. Prior to treatment, cells were deprived of serum overnight in the appropriate medium containing 1% BSA instead of serum unless indicated otherwise. Cells were then incubated with GH at 500 ng/ml (22 nM) or as indicated. 293T cells were maintained as described previously (20).

Plasmids, Probes, and Antibodies—The plasmid wtFos-Luc contains 379 base pairs of the mouse *c-fos* promoter immediately 5′ of the transcription start site, directly upstream of the luciferase gene. mC/EBP-Luc (mC/EFos-Luc) is identical to wtFos-luc except that the C/EBP site has been mutated (sequence below). Both plasmids were provided by Dr. W. Wharton (University of South Florida) (21). Plasmids encoding LAP or LIP driven by the CMV promoter (CMV-LAP and CMV-LIP) were gifts from Dr. U. Schibler (University of Geneva). The plasmid CMV-C/EBPδ was provided by Dr. S. McKnight (University of Texas Southwestern). The reporter plasmid TK-Luc was provided by Dr. J. Pessin (University of Iowa). The plasmid RSV-β-galactosidase was provided by Dr. M. Uhler (University of Michigan), and RSV-neo was provided by Dr. Nils Billestrup (Hagedorn Lab, Gentofte, Denmark). Oligonucleotides contained the following sequences (changes from wild type are underlined): wild type *c-fos* C/EBP site and flanking SRE (wtC/EBP-SRE, previously designated SREw (8)), 5′-gatcGGATGTCCATATTAGGACATC-3′; wtC/EBP-SRE mutated in the C/EBP binding site (mC/EBP), 5′-gatcGGATGTCCATATTAGGAGTTC-3′; the C/EBP binding site from the *422/aP2* gene, gatcCAAAGTTGAGAAATTTCTATTAAAAA (−150 to −125) (22).

Specific rabbit polyclonal antibodies against peptides corresponding to amino acids 278–295 at the C terminus of C/EBPβ (12), amino acids 115–130 of C/EBPδ (12), and an internal amino acid sequence of C/EBPα (23) were prepared as described previously.

Transfection—3T3-F442A cells were plated at 10^4 cells/cm^2 on 100-mm plates, and the next day they were transfected using LipofectAMINE with the plasmids RSV-neo (2 μg of DNA/plate) and either wtFos-Luc or mC/EBP-Luc (8 μg DNA/plate). Pooled clones were maintained in the presence of 0.6 mg/ml G418 and used for experiments. CHO cells expressing GHR-(1–454) were transiently transfected by the calcium phosphate coprecipitation procedure (24) with 0.4 μg of wtFos/Luc plasmid, in the presence of CMV-LAP (1 ng), CMV-LIP (0.15 μg), CMV-C/EBPδ (0.1 μg), or corresponding amounts of pcDNA3 vector plasmid per 35-mm well. After 24 h, the cells were deprived of serum by incubation in medium containing 1% BSA for 18–24 h prior to treatment as indicated. 293T cells were transfected using calcium phosphate, as described (20) with plasmids CMV-LAP (1 μg) or CMV-LIP (1 μg). 24 h later, cell lysates were prepared using high salt buffer (8) and were used for analysis.

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed as described previously (8). Briefly, confluent cells were deprived of serum overnight and incubated at 37 °C for the indicated times with hormone, serum, or vehicle. Nuclear extracts were prepared and analyzed as described (3, 8). Binding reactions proceeded for 30 min at 30 °C and were analyzed by nondenaturing polyacrylamide gel electrophoresis followed by autoradiography. Where indicated, nuclear extracts were preincubated for 20 min at room temperature with 1 μl of antisera against C/EBPα, C/EBPβ, or C/EBPδ, each at 1:10, or combinations of these antibodies. In the experiment in Fig. 3*B*, EMSA was performed as described previously (17). Data were analyzed using Bio-Rad Multi-Analyst, version 1.0.2.

Luciferase Assay—Cell lysates were prepared in reporter lysis buffer (100 mM potassium phosphate, 0.2% Triton X-100, 1 mM DTT), and luciferase or β-galactosidase activity was measured using an Autolumat or Opticomp Luminometer. The luciferase values were normalized to β-galactosidase activity. Each condition was tested in triplicate in each experiment. Analysis of variance with factorial Scheffe F-test was used to analyze data from five or six individual experiments. Data are presented as percentage or -fold stimulation relative to a control = 1.

Analysis of RNA—Total RNA was isolated from confluent 3T3-F442A preadipocytes as described (9, 19). C/EBPβ and C/EBPδ mRNA were assessed by Northern blot analysis (9). The DNA fragments used as probes for C/EBPβ and C/EBPδ mRNA have been described previously (12).

Immunoblotting—3T3-F442A cells in 100-mm plates were washed with PBS and scraped into 0.5 ml of SDS lysis buffer (60 mM Tris-HCl, pH 6.8, 1% SDS). Lysates were boiled for 3 min, vortexed, and then boiled for an additional 7 min prior to storing at −80 °C (12). Some experiments used hypotonic buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 0.2% Nonidet P-40, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml each aprotinin, leupeptin, and pepstatin) to lyse the cells prior to lysing nuclei with SDS lysis buffer. Whole cell lysates (35–50 μg) or nuclear extracts (20 μg) were analyzed by immunoblotting as described (12) using anti-C/EBPβ (1:1000) or anti-C/EBPδ (1:1000). In some experiments, whole cell lysates from cells treated with GH for 1 h were incubated with 40 units of alkaline phosphatase, in the presence or absence of vanadate (10 mM), for 1 h at 37 °C prior to immunoblotting (25). The apparent M_r values indicated in the figures are based on prestained molecular weight standards (Life Technologies).

RESULTS

Mutation of the C/EBP Site Enhances Responsiveness of the c-fos Promoter to GH—To determine whether the C/EBP site in the upstream regulatory sequence of c-*fos* is responsive to GH, the influence of mutating the C/EBP binding site on the ability of GH to regulate c-*fos* promoter activity was examined. GH was added to pools of 3T3-F442A fibroblasts stably expressing the wild type c-*fos* promoter (from −379 to +1) immediately upstream of the luciferase gene (wtFos-Luc) and to pools of

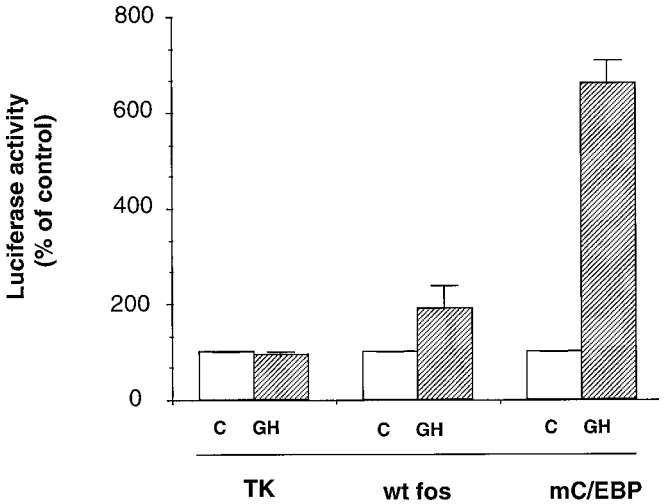

FIG. 1. **Mutation of the C/EBP site enhances responsiveness of the c-*fos* promoter to GH.** 3T3-F442A cells stably expressing wtFos-Luc (*wt fos*), mC/EBP-Luc (*mC/EBP*), or TK-Luc (*TK*) were incubated without (*open bars*) or with 500 ng/ml GH (*hatched bars*) for 4 h. Cell extracts were analyzed for luciferase activity. Basal luciferase values were as follows: wild-type Fos-luc, 262 relative luciferase units; mC/EBP-luc, 91,370 relative luciferase units; TK-Luc, 162,000 relative luciferase units. Each *bar* represents the mean ± S.E. luciferase activity expressed as a percentage of control (set at 100%) for triplicate observations averaged for five independent experiments.

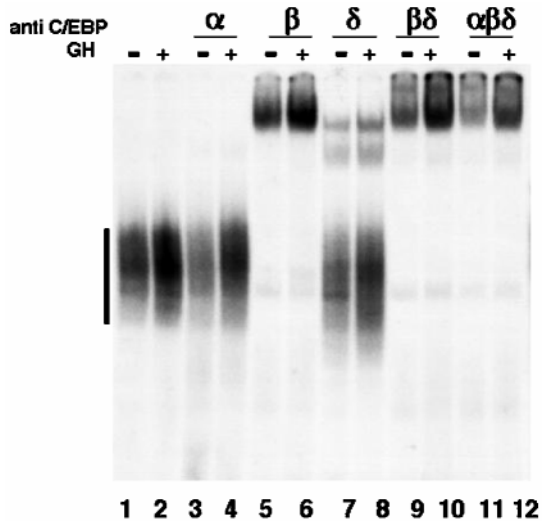

FIG. 2. **GH induces binding of C/EBPβ and C/EBPδ to a C/EBP binding site.** 3T3-F442A fibroblasts were incubated without (−) or with GH (+) for 5 min, as indicated. Nuclear extracts were analyzed by EMSA using the C/EBP site from the *422/aP2* gene as probe. The *bar* on the *left* indicates the C/EBP-containing complex. The indicated antisera specific for C/EBPα, -β, or -δ were added to nuclear extracts alone or in combination, as described under "Experimental Procedures." Similar results were obtained in two different experiments.

cells expressing the c-*fos* promoter mutated in the C/EBP binding site (mC/EBP-Luc). Treatment with GH causes a 2-fold increase in luciferase expression mediated by the wtFos promoter (Fig. 1). However, when the C/EBP site is mutated in the context of the c-*fos* promoter, the response to GH rises to more than 6 times control values (Fig. 1). A luciferase gene without c-*fos* promoter sequences (*TK*) fails to respond to GH. These findings indicate that the C/EBP site in the c-*fos* promoter is responsive to GH and suggest that proteins bound to the C/EBP site may play a restraining role in GH-promoted c-*fos* expression.

GH Increases the Binding of C/EBPβ and C/EBPδ—To determine whether GH can regulate proteins that bind to a well characterized C/EBP site, nuclear extracts were analyzed by EMSA using a probe based on the C/EBP site from the *422/aP2* gene (22), which recognizes C/EBPα, -β, and -δ. Nuclear proteins from 3T3-F442A cells bind to the C/EBP site as a diffuse band in EMSA (Fig. 2, *lane 1*). This band is not evident in the presence of a 100-fold excess of unlabeled homologous probe (data not shown). Treatment of cells with GH for 5 min increases the intensity of the complex bound to the C/EBP site (*lane 2*). Differences in binding cannot be explained by differences in protein levels as assessed by immunoblots. The addition of antisera specific for C/EBPβ causes almost complete disappearance of the complex and causes the appearance of a slower migrating, supershifted band in extracts from both control and GH-treated cells (*lanes 5* and *6*), indicating that C/EBPβ is a major component of the complex. Antibodies against C/EBPδ reduce the amount of binding slightly and induce a supershift in extracts from control or GH-treated cells (*lanes 7* and *8*), indicating the presence of some C/EBPδ in the complex. The addition of antibodies to C/EBPβ and -δ in combination results in a pattern of binding similar to that with anti-C/EBPβ alone (*lanes 9* and *10*). In contrast, antibodies against C/EBPα have no effect on binding to the C/EBP site (*lanes 3*, *4*, *11*, and *12*). These observations indicate that GH can rapidly increase the binding of C/EBPβ and δ to a well characterized C/EBP site.

C/EBPβ and C/EBPδ Are Present in a GH-stimulated Complex Bound to c-fos—To determine whether GH similarly regulates proteins bound to the C/EBP site in c-*fos*, nuclear extracts were analyzed with a probe based on the sequence of the wild type c-*fos* C/EBP site and adjacent (5′) SRE (wtC/EBP-SRE). Three complexes are associated with the wtC/EBP-SRE probe (Fig. 3*A*, *lane 1*), each of which is increased by treatment with GH for 5 min (*lane 2*). One of these complexes (SRF) consists of SRF bound to the probe; a slower-migrating band contains Elk-1 as well as SRF in a ternary complex, as identified previously by supershifting with antibodies specific for SRF or Elk-1 (8). The fastest migrating complex consists of a broad diffuse band. All three complexes are absent in the presence of an excess of unlabeled probe but are unaffected by an unrelated (SIE) probe (data not shown).

Antibodies specific for C/EBPβ cause disappearance of the broad diffuse band and cause appearance of a supershifted band that migrates more slowly than any of the other complexes (Fig. 3*A*, *lanes 5* and *6*). This indicates that C/EBPβ or an immunologically related protein is a major component of this diffuse complex (hereafter labeled "C/EBP") and is increased in GH-treated cells (*lane 6*). Antibodies against C/EBPδ also cause a supershift and reduce the intensity of the C/EBP complex in the presence and absence of GH (Fig. 3*A*, *lanes 7* and *8*), indicating that C/EBPδ is present in the complex in control and GH-treated cells, but at reduced levels compared with C/EBPβ. The C/EBP complex is not altered when antibodies to C/EBPα are added (Fig. 3*A*, *lanes 3* and *4*), indicating that C/EBPα is not present in this complex. The addition of antibodies specific for C/EBPβ, δ, or α in combination (*lanes 9–16*) do not differ from those using anti-C/EBPβ and δ alone; however, the supershift appears greater when anti-C/EBPβ and -δ are added in combination (*lanes 13–16*) rather than individually. The diffuse C/EBP complex appears to be superimposed on three constitutive bands that remain despite the presence of anti-C/EBPβ and are not altered by GH. Thus, the GH-regulated C/EBP complex bound to c-*fos* contains C/EBPβ as a major constituent and C/EBPδ to a lesser extent. When the C/EBP site mutation is introduced into the oligonucleotide probe (mC/EBP), the diffuse C/EBP complex disappears from the EMSA (not shown). Taken together, these observations

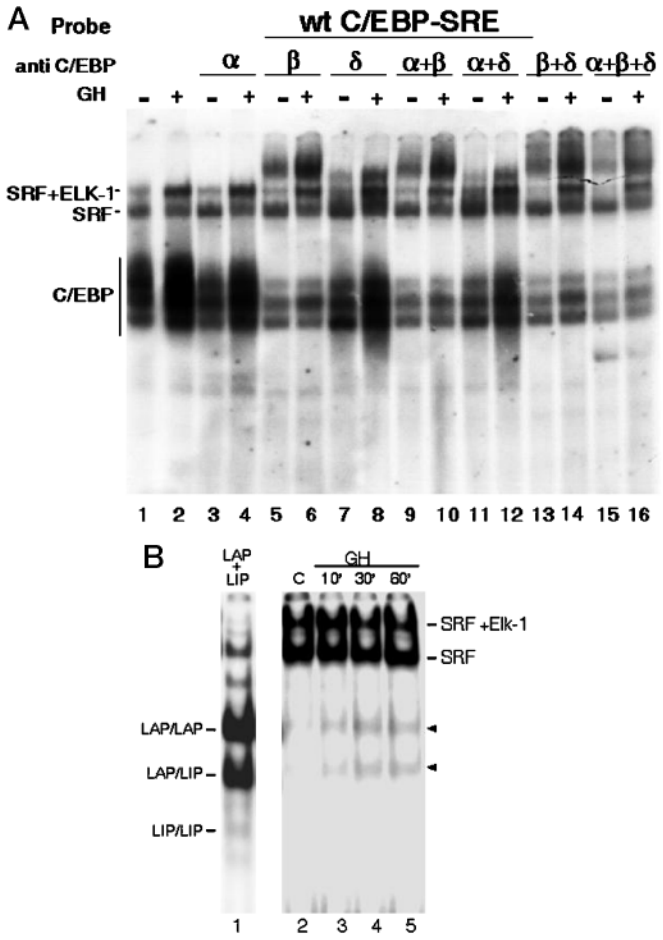

FIG. 3. **C/EBPβ is present in a GH-stimulated complex bound to the c-*fos* promoter.** *A*, nuclear extracts were analyzed by EMSA using the wtC/EBP-SRE probe containing the wild type C/EBP site and flanking SRE. Antisera were added as for Fig. 2. *B*, extracts from 293T cells overexpressing LAP or LIP were combined and analyzed by EMSA (*lane 1*). When extracts containing LAP or LIP alone were analyzed, only the appropriate homodimer, and no heterodimer, was observed. Nuclear extracts from 3T3-F442A cells treated without (*lane 2*) or with GH for 10–60 min (*lanes 3–5*) were analyzed by EMSA.

indicate that C/EBPβ and -δ are present in complexes bound to the c-*fos* promoter and that their binding is rapidly increased by GH in 3T3-F442A cells.

C/EBPβ occurs as three alternative translation products: the full-length protein (p35C/EBPβ), LAP (p32C/EBPβ), and LIP (p20C/EBPβ). LAP is more prominent than p35 and mediates transcriptional activation in response to multiple stimuli when bound to DNA (26–29). LIP retains the basic region and leucine zipper of C/EBPβ but lacks the N-terminal transcriptional activation domain of LAP. LIP can form heterodimers with LAP and is reported to inhibit transcriptional activation of genes by LAP (14).

To detect LAP and LIP in complexes bound to the C/EBP site, the EMSA was modified to improve resolution (17). Using extracts from 293T cells overexpressing LAP or LIP, homodimers of LAP and LIP and heterodimers of LAP/LIP were bound to the c-*fos* C/EBP-SRE probe (Fig. 3*B*, *lane 1*). The addition of antibodies specific for C/EBPβ causes complete disappearance of the complexes (not shown). In 3T3-F442A cells, GH increases the appearance of LAP/LAP and LAP/LIP in 10 min (*lane 3*). By 60 min, the GH-induced increase was more than 3 times the level in control cells (untreated; *lane 5 versus lane 2*). The LIP/LIP homodimer is not detectable in 3T3-F442A cells under the conditions of these experiments, yet LIP readily participates in formation of the heterodimer. Thus, these results indicate that both LAP and LIP are present in complexes bound to a C/EBP site. In 3T3-F442A cells, the GH-induced increase in binding (Figs. 2 and 3*A*) reflects in part an increase in LAP/LAP and LAP/LIP binding.

LIP, LAP, and C/EBPδ Have Different Effects on GH-stimulated c-fos Promoter Activity—To examine whether LIP, LAP, or C/EBPδ modulate GH-promoted gene expression mediated by the c-*fos* promoter, each of these proteins was overexpressed in combination with wtFos-Luc in GH-responsive CHO-GHR cells, in which endogenous C/EBPs are low (8). One might predict that since C/EBP appears to restrain GH-stimulated c-*fos* promoter activation, overexpression of the inhibitory isoform LIP might decrease GH-stimulated gene expression. Expression of LIP reduces the basal level of c-*fos* promoter activity by 50% (Fig. 4*A*, *open bars*). Moreover, LIP almost completely blocks the ability of GH to stimulate reporter expression via the c-*fos* promoter. The effect of GH on luciferase expression in the presence of LIP is not different ($p > 0.05$), while the stimulation by GH is significant in the absence of LIP ($p < 0.05$) ($n = 5$). Control data verify that the wild type c-*fos* promoter is capable of being stimulated in the presence of LIP when cells are treated with 10% calf serum (not shown). The inhibition of GH-stimulated luciferase activity by overexpression of LIP is consistent with the possibility that LIP contributes to restraining c-*fos* expression in response to GH.

In contrast to the inhibition with LIP, overexpression of LAP elevates basal c-*fos* promoter activity to 4 times the level observed in cells that do not express LAP (Fig. 4*B*, *open bars*). However, LAP does not alter the effectiveness of GH in stimulating the c-*fos* promoter, since GH stimulates reporter expression via the c-*fos* promoter to a similar extent above the respective control levels in the presence and absence of LAP. The approximately 2-fold stimulation of luciferase by GH is significant ($p < 0.05$) both in the presence and absence of overexpressed LAP.

C/EBPδ is also present in complexes bound to the c-*fos* C/EBP site. Since C/EBPδ binding is increased by GH and since C/EBPδ can heterodimerize with LIP or LAP (26), the ability of C/EBPδ to alter GH-stimulated c-*fos* promoter activation was examined (Fig. 4*C*). Overexpression of C/EBPδ, like LAP, elevates basal c-*fos* promoter activation. However, unlike LAP, GH-stimulated c-*fos* promoter activation relative to its control was consistently at least twice as great in the presence of C/EBPδ as in its absence. This activation by C/EBPδ of GH-stimulated gene expression, combined with the observation that C/EBPδ is present in the complex bound to c-*fos* to a lesser extent than is C/EBPβ, makes it unlikely that C/EBPδ contributes to the apparent restraint of GH-stimulated gene expression mediated by the c-*fos* C/EBP site. Physiologically, the level of GH-regulated c-*fos* expression may reflect in part a balance among the disparate contributions of LIP, LAP, and C/EBPδ.

GH Transiently Induces Expression of C/EBPβ and C/EBPδ mRNA—Because C/EBPβ and -δ contribute to GH-regulated c-*fos* expression, the ability of GH to regulate levels of C/EBPβ and -δ was examined. The expression of mRNA for C/EBPβ is low in untreated quiescent 3T3-F442A fibroblasts (Fig. 5*A*, *upper panel*, *lane 1*). C/EBPβ mRNA is elevated over 2-fold between 30 and 60 min after GH treatment (*lanes 3–5*) and subsides by 120 min (*lane 6*). The mRNA for C/EBPδ is elevated 5-fold according to the same time course (Fig. 5*A*, *middle panel*).

To determine whether the stimulation of C/EBP mRNA by GH leads to stimulation of the respective proteins, C/EBPβ and C/EBPδ were examined in 3T3-F442A fibroblasts by immunoblot analysis. Levels of C/EBPδ were found to increase 45 min

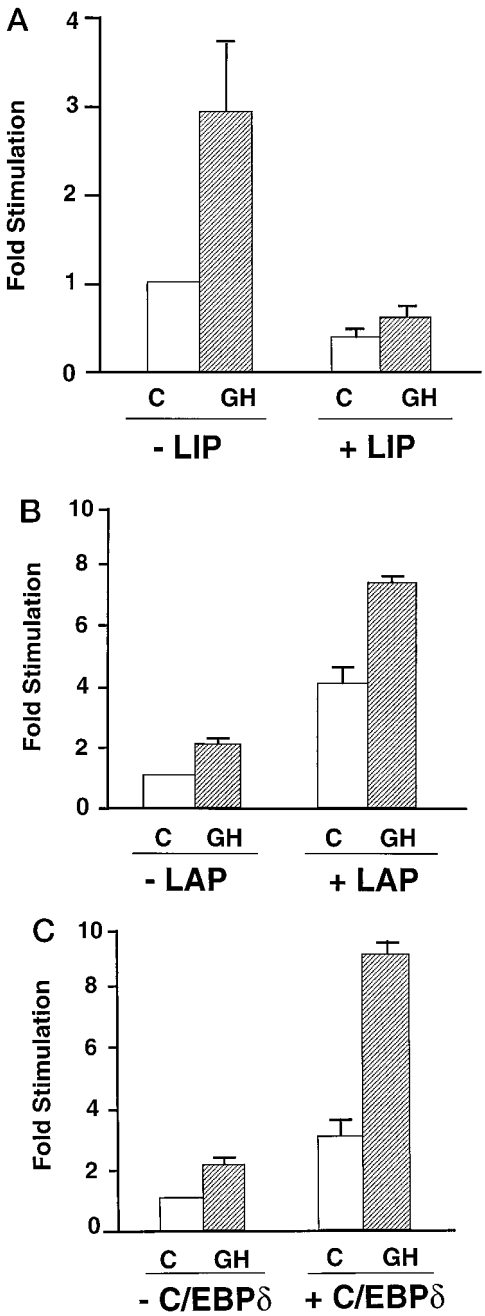

FIG. 4. *A*, expression of LIP inhibits GH-stimulated reporter expression via the c-*fos* promoter. CHO-GHR cells were transiently transfected with CMV-LIP (+*LIP*) or pcDNA (−*LIP*). After 48 h, cells were treated with GH (*hatched bars*) or vehicle (*open bars*) for 4 h and were analyzed for luciferase activity. Each *bar* represents the mean ± S.E. for five independent experiments. The response to GH is significant ($p = 0.008$) in cells that were not transfected with CMV-LIP but is not significant in cells overexpressing LIP (+*LIP*). *B*, expression of LAP stimulates basal, but not GH-stimulated, c-*fos* promoter activity. CHO-GHR cells were transiently transfected with CMV-LAP (+*LAP*) or vector pcDNA (−*LAP*) and were treated and analyzed as described for *A*. Each *bar* represents the mean ± S.E. for six experiments. The response to GH is significant ($p < .05$) in cells transfected with or without CMV-LAP. *C*, C/EBPδ augments basal and GH-stimulated gene expression. Samples were treated as in Fig. 4, *A* and *B*, using CMV-C/EBPδ for transfection. The response to GH is significant ($p < 0.05$) in the absence and presence of C/EBPδ. The response to GH in the presence of C/EBPδ is significantly ($p < 0.05$) greater than the response to GH in the absence of C/EBPδ.

after GH treatment and to subside by 120 min (Fig. 5*B*), corresponding with GH-induced changes in levels of C/EBPδ mRNA. However, levels of C/EBPβ, analyzed using an antibody

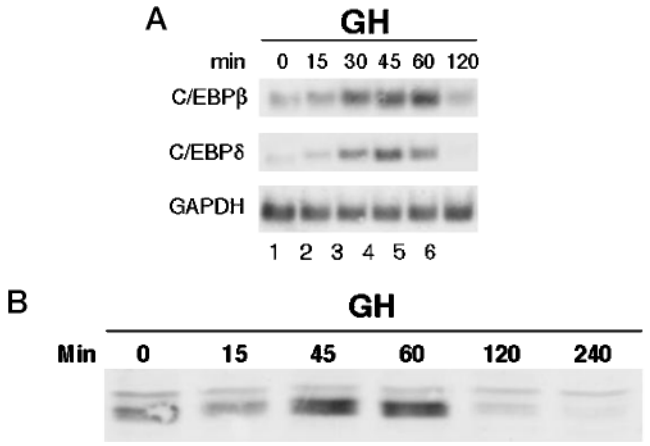

FIG. 5. *A*, GH transiently induces C/EBPβ and C/EBPδ mRNA. Total RNA from 3T3-F442A cells incubated with GH for 0–120 min was analyzed on Northern blots using cDNA specific for C/EBPβ (*upper panel*). Blots were stripped and reprobed with cDNA for C/EBPδ (*middle panel*) or GAPDH (*lower panel*) to evaluate loading. This experiment has been repeated two times. *B*, GH transiently increases the protein expression of C/EBPδ. 3T3-F442A fibroblasts were incubated with GH for varying times and then were lysed and analyzed by immunoblotting with anti-C/EBPδ (1:1000). This experiment has been repeated three times.

against the C terminus of C/EBPβ, do not appear to change in GH-treated cells (Fig. 6). Both LIP and LAP are evident in the absence (*lane 1*) and presence of GH (*lanes 2–10*). The levels of the proteins appear to be relatively constant at all time points, although expression of LIP (*band a*) may increase at 2 and 4 h after GH treatment (*lanes 8* and *9*).

GH Promotes the Dephosphorylation of LAP and LIP—In cells incubated with GH, LAP and LIP each shift to a more rapidly migrating form (Fig. 6, *bands b*). A time course reveals that the more rapidly migrating forms of LAP and LIP appear within 30 min of GH treatment (*lane 5*), peak at 60 min (*lane 7*), and then subside and are absent 24 h later (*lane 10*). The mobility shift on the immunoblots suggests that GH might promote dephosphorylation of the C/EBPβ isoforms.

To ascertain whether the more rapidly migrating bands (*bands b*, Fig. 6) represent dephosphorylated forms of LAP and LIP, lysates from GH-treated cells (60 min) were incubated with alkaline phosphatase for 1 h before the lysates were applied to the gel. Alkaline phosphatase treatment causes both LAP and LIP to migrate exclusively as the faster mobility form (Fig. 7*A*, *lane 4*, *bands b*), consistent with *bands b* representing dephosphorylated LAP and LIP. The addition of the phosphatase inhibitor vanadate blocks the mobility shift induced by alkaline phosphatase (*lane 3*). The immunoblot also shows a faint upper band, presumably the p35 form of LAP, which also migrates faster after alkaline phosphatase treatment (*lane 4*), consistent with GH-promoted dephosphorylation of p35LAP also. Migration of LAP and LIP was not altered by the addition of vanadate alone to the lysates (not shown). The dephosphorylated forms of LAP and LIP co-migrate with the faster mobility forms of LAP and LIP in lysates from cells treated with GH (*lane 2 versus lane 4*, *bands b*). These data are consistent with GH promoting the dephosphorylation of LIP and LAP.

Untreated 3T3-F442A cells contain undetectable levels of C/EBPδ, and GH rapidly and transiently increases expression of C/EBPδ (Fig. 5*B*). The C/EBPδ induced by GH in 45 min appears to contain both phosphorylated (*band a*) and dephosphorylated (*band b*) forms of the protein (Fig. 7*B*). The addition of alkaline phosphatase converts most of the C/EBPδ to the dephosphorylated form (*lane 3*), and the dephosphorylation is blocked by the simultaneous addition of vanadate (*lane 4*).

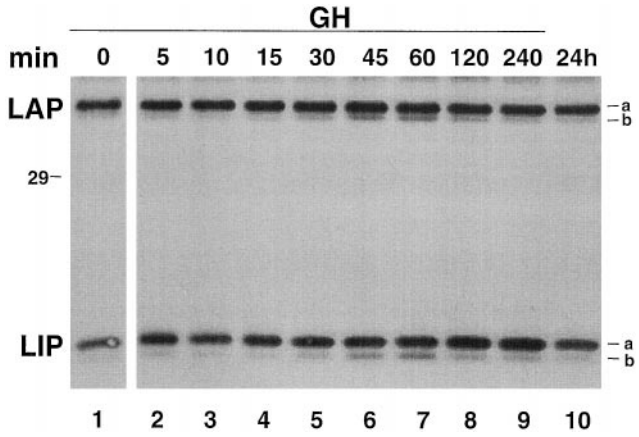

FIG. 6. **GH transiently increases the mobility of C/EBPβ.** 3T3-F442A fibroblasts were incubated with GH for varying times and then were lysed and analyzed by immunoblotting with anti-C/EBPβ (1:1000) as described under "Experimental Procedures." Bands representing LAP (p32C/EBPβ) and LIP (p20C/EBPβ) and the slower (*a*) and faster (*b*) migrating forms of LAP and LIP are indicated. $M_r \times 10^3$ is designated. This experiment was repeated six times.

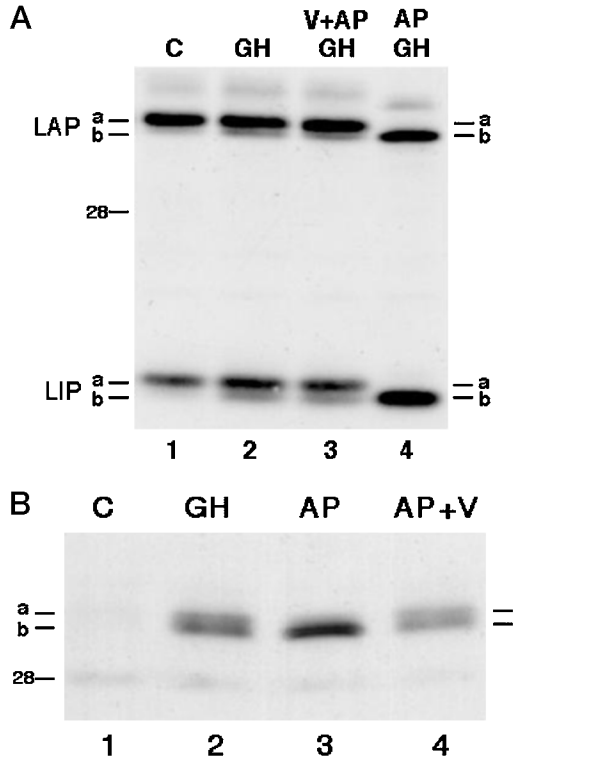

FIG. 7. **GH promotes dephosphorylation of C/EBPβ.** *A*, lysates analyzed in Fig. 6 were incubated with alkaline phosphatase (*AP*, 40 units) without (*lane 4*) or with (*lane 3*) vanadate (*V*, 10 mM) as described under "Experimental Procedures," prior to SDS-polyacrylamide gel electrophoresis and immunoblotting with antibody specific for C/EBPβ. Notations are as for Fig. 6. This experiment has been repeated four times. *B*, lysates were treated with alkaline phosphatase with (*lane 4*) or without (*lane 3*) vanadate as for *A* and were probed for C/EBPδ. Similar results were obtained in two experiments.

Thus, both phosphorylated and dephosphorylated C/EBPδ appear to be induced by GH. These observations indicate that GH promotes the dephosphorylation of C/EBPβ isoforms present in 3T3-F442A fibroblasts and induces expression of both phosphorylated and dephosphorylated C/EBPδ.

DISCUSSION

C/EBP Participates in GH-regulated Gene Expression—Mutation of the C/EBP site in the c-*fos* upstream regulatory region enhances the ability of GH to stimulate gene expression mediated by the c-*fos* promoter, suggesting that proteins bound to the C/EBP site restrain the ability of GH to stimulate c-*fos* promoter activity. C/EBPβ and to a lesser extent C/EBPδ, but not C/EBPα, were found to be present in complexes bound to the c-*fos* C/EBP site and were increased by GH. Furthermore, overexpression of LIP, an inhibitory form of C/EBPβ, interferes with the ability of GH to stimulate gene expression via the c-*fos* promoter, consistent with the possibility that LIP participates in restraining GH-stimulated c-*fos* expression. Overexpression of the stimulatory C/EBPβ form LAP elevates basal c-*fos* promoter activity but does not alter the ability of GH to stimulate the c-*fos* promoter, while expression of C/EBPδ enhances the ability of GH to stimulate promoter activation. Overall, these studies for the first time implicate C/EBPβ and -δ in GH-regulated c-*fos* expression. The different effects of the C/EBP family members on GH-regulated c-*fos* promoter activity suggest that a combinatorial effect of the various proteins bound to the C/EBP site may counterbalance each other to produce the net physiological response to a regulator such as GH.

GH Promotes the Binding of C/EBPβ and C/EBPδ—In addition to showing that C/EBP plays a role in GH-regulated c-*fos* expression, this study indicates that GH regulates several aspects of C/EBP function, including stimulating the binding of C/EBPβ and -δ to the c-*fos* C/EBP site within 5 min of GH treatment. This stimulation coincides with GH-stimulated binding of SRF and Elk-1 to the c-*fos* SRE (8) adjacent to the C/EBP site. The rapid onset of the increase in binding makes it unlikely that increased amounts of the proteins account for the stimulation of binding. Based on supershift analysis, the complex at the C/EBP site contains primarily C/EBPβ and a lesser amount of C/EBPδ. The increase in C/EBPβ reflects an increase in LAP/LAP and LIP/LAP dimers. It has been observed that the binding of C/EBPβ can be increased in the presence of the retinoblastoma protein (30). Physiological and functional interactions of C/EBPβ with NF-κB (31, 32), Sp1 (33), and p300 (34) have also been observed. Future analysis of whether the relative amounts of C/EBPβ and C/EBPδ in the complex change with GH treatment will provide insight into the regulation and significance of changes in the composition of the C/EBP-containing complex.

The regulation of C/EBPδ by GH is distinct from regulation of C/EBPβ, although GH was found here to induce mRNA for both C/EBPβ and C/EBPδ within 30 min. An earlier study reported that GH stimulates C/EBPδ but not C/EBPβ mRNA when a lower concentration of GH (50 ng/ml) was used than in the present study (35). However, while GH increases the level of C/EBPδ (both phosphorylated and dephosphorylated), the amount of C/EBPβ was not substantially altered by GH under the conditions of these experiments. Rather, the phosphorylation state of C/EBPβ appears to be regulated by GH.

Relative Roles of LIP, LAP, and C/EBPδ in GH-promoted c-fos Expression—The reciprocal roles of LIP and LAP on gene expression in the liver (26) suggest that these forms of C/EBPβ may exert opposing effects on GH-promoted c-*fos* expression. In fact, LAP was found to increase basal c-*fos* promoter activity and LIP to decrease it in CHO-GHR cells. However, the reciprocal relationship between LIP and LAP did not persist in the context of GH treatment. Overexpression of LAP did not alter the ability of GH to stimulate c-*fos* promoter activity; 2-fold stimulation by GH was observed in the absence and presence of LAP, despite the difference in basal promoter activity. In contrast, overexpression of LIP interfered with GH-stimulated reporter expression mediated by the wild type c-*fos* promoter. Although LIP overexpression reduced basal transcription, the c-*fos* promoter was still stimulated by serum in the presence of

LIP under the conditions of these experiments. This indicates that the promoter was capable of being stimulated and reinforces the lack of response to GH in the presence of LIP. The physiological function of C/EBPβ in response to GH may thus reflect a balance between the stimulatory and inhibitory effects of LAP and LIP on the c-*fos* promoter.

It is tempting to speculate that such a balance between LIP and LAP might be regulated by GH. C/EBPβ associates with the c-*fos* C/EBP site in untreated quiescent 3T3-F442A cells. A simple explanation for restraint of GH-promoted transcription via the C/EBP site is that the LIP associated with the C/EBP site on the c-*fos* promoter restrains GH-stimulated promoter activity. If so, such restraint would be expected to be relieved when the C/EBPβ site is mutated, presumably interfering with the binding of LIP and allowing enhanced stimulation in response to GH. Although levels of LIP bound to the C/EBP site appear to be lower than LAP, LIP readily participates in heterodimer formation. The function of each component of these complexes remains to be determined. Another contributing event to the overall function of C/EBPβ could involve a decrease in activation of transcription by LAP under the influence of GH, which would also be reflected in restraint of GH-stimulated c-*fos* expression. C/EBPδ appears to be present to a much lesser extent than C/EBPβ in the complex bound to the c-*fos* C/EBP site. However, enhancement of GH-stimulated promoter activity by C/EBPδ could also contribute to the net effect of LIP and LAP on the c-*fos* promoter.

Regulation of the Phosphorylation State of LIP and LAP—An intriguing explanation for the role of C/EBPβ in GH-stimulated c-*fos* promoter activity is that GH-promoted changes in the phosphorylation state of LIP and/or LAP determine their relative effectiveness in regulating the c-*fos* promoter. Since the presence of the dephosphorylated forms of both LIP and LAP increase in response to GH, one can speculate that dephosphorylation may have opposite consequences on each form of the protein. Another possibility is that the dephosphorylated LIP and LAP observed on immunoblots represent newly synthesized forms of the proteins (consistent with RNA data). If so, whether the newly synthesized dephosphorylated form or mature phosphorylated form was active in this context remains unclear.

It is well established that regulation of phosphorylation state is an important regulatory mechanism for function of transcription factors (36), including C/EBPβ. Multiple phosphorylation sites have been characterized on C/EBPβ and are reported to be phosphorylated by Ras (Thr^{235}), calcium/calmodulin-dependent protein kinase (Ser^{276}) (37) protein kinase C (Ser^{105}, Ser^{240}, and Ser^{299}), protein kinase A (Ser^{105}, Ser^{173}, Ser^{233}, and Ser^{299}) (38–41). Thus, multiple phosphorylation sites on C/EBPβ are available for regulation by GH. The data presented here indicate that GH promotes dephosphorylation of C/EBPβ, similar to observations that insulin promotes dephosphorylation of C/EBPα (12, 20). Since both LIP and LAP shift to a more rapidly migrating form, the dephosphorylated residue(s) probably lies in the C-terminal half of C/EBPβ common to both LAP and LIP rather than in the N-terminal transcriptional activation domain unique to the LAPs. Interestingly, the p35 form of C/EBPβ, which appears faintly on the immunoblot in Fig. 7*A* in addition to p32 LAP also migrates faster in the presence of alkaline phosphatase, suggesting that both p32 and p35 forms of LAP are dephosphorylated in GH-treated cells. It will be of great interest to determine what residues of C/EBPβ are dephosphorylated by GH and to identify enzymes that might mediate such regulation. Preliminary data suggest that dephosphorylation can increase binding of LAP,[2] indicating that phosphorylation state has a potent influence on behavior of the C/EBPβ proteins.

Another pressing question is what the functional consequences of phosphorylation or dephosphorylation of C/EBPβ are in GH-regulated gene transcription. In previous studies, phosphorylation of C/EBPβ was reported to activate transcription in some cases, while in others it decreases binding of C/EBPβ or has no effect. GH is known to stimulate the phosphorylation of multiple transcription factors. Both GH-promoted Ser phosphorylation of Elk-1 (8, 9) and Tyr phosphorylation of STAT 1, 3, or 5 are associated with GH-promoted transcriptional activation of target genes (3, 6, 7, 42–44). This is the first report that GH can promote dephosphorylation of a transcription factor. The GH-induced dephosphorylation of LIP or LAP may be related to a GH-regulated derepression of the c-*fos* promoter. Interestingly, the chicken homologue of C/EBPβ, NF-M, is derepressed rather than activated by phosphorylation (45), raising the possibility that derepression of LAP could be a consequence of GH treatment. Derepression is consistent with the observation that mC/EBP-Luc is enhanced by GH as compared with wtFos-Luc. How inhibition or restraint in the absence of GH and derepression in the presence of GH would occur is not yet clear, but multiple mechanisms may exist. A possible inhibitory site in the c-*fos* promoter about 216 base pairs upstream of the transcription start site has been identified, but it was not reported to be regulated by GH (46). A preliminary report suggests an inhibitory role of GH-stimulated STAT 5 on PPARγ-activated expression of the *aP2* gene in primary adipocytes (47). Such events are probably distinct from the C/EBP-mediated inhibition of GH-regulated gene expression observed here.

As our understanding grows of how GH regulates the C/EBP family of transcription factors, such information can be integrated with the present observations on the role of C/EBPβ and -δ in GH-regulated transcription. Although suggestive, the importance of the GH-stimulated increase in C/EBPδ is also not known at present. The ratios of LIP and LAP, as well as their phosphorylation states, are likely to be crucial in determining the net C/EBPβ-regulated transcription in a given cell type. In summary, these studies implicate C/EBPβ and C/EBPδ in GH-regulated c-*fos* expression and raise intriguing possibilities that GH-regulated binding of C/EBPβ and -δ and/or dephosphorylation of LAP or LIP contribute to a tonic inhibition of GH-stimulated c-*fos* expression.

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