THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 7, pp. 4132–4141, February 17, 2006

Profiles of Growth Hormone (GH)-regulated Genes Reveal Time-dependent Responses and Identify a Mechanism for Regulation of Activating Transcription Factor 3 By GH*[S]

Received for publication, August 2, 2005, and in revised form, October 24, 2005 Published, JBC Papers in Press, December 1, 2005, DOI 10.1074/jbc.M508492200

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In examination of mechanisms regulating metabolic responses to growth hormone (GH), microarray analysis identified 561 probe sets showing time-dependent patterns of expression in GH-treated 3T3-F442A adipocytes. Biological functions significantly over-represented among GH-regulated genes include regulators of transcription at early times, and lipid biosynthesis, cholesterol biosynthesis, and mediators of immune responses at later times (48 h). One novel GH-induced gene encodes activating transcription factor 3 (ATF3). *Atf3* mRNA expression and promoter activity were stimulated by GH. Genes for ATF3 and growth arrest and DNA damage-inducible gene 45 gamma (GADD45γ) showed similar time-dependent patterns of responses to GH, suggesting similar regulatory mechanisms. A conserved sequence in the promoters of the *Atf3* and *Gadd45γ* genes contains a CCAAT/enhancer-binding protein (C/EBP) site previously observed in the *Gadd45γ* promoter, suggesting a novel corresponding C/EBP site in the *Atf3* promoter. C/EBPβ was found to bind to the predicted *Atf3* C/EBP site, and C/EBPβ enhanced the activation of the wild-type *Atf3* promoter. Mutation of the predicted *Atf3* C/EBP site disrupted *Atf3* promoter activation not only by C/EBPβ but also by GH. These findings suggest that GH regulates transcription of *Atf3* through a mechanism utilizing factors, such as C/EBPβ, which bind to a novel C/EBP site.

Metabolic responses to GH[4] show distinctive timing both in patients and *in vitro*. Chronic anti-insulin responses are consistent with GH-induced insulin resistance (1, 2), whereas acute responses can be "insulin-like" (1). Because changes in gene expression underlie many actions of GH, one can gain insight into the distinctive timing of metabolic responses to GH by identifying genes regulated by GH at different times. Genes regulated by GH in cultured 3T3-F442A adipocytes were analyzed, because these cells exhibit metabolic responses typical of *in vivo* responses to GH, including insulin resistance (1, 3).

The molecular mechanisms controlling GH-regulated gene expression have been studied in several genes, including *Fos* (c-*fos*), which encodes a transcription factor implicated in growth regulation (4); *Spi2.1*, which encodes serine protease inhibitor 2.1 (5); and *Igf1*, which encodes insulin-like growth factor 1, which mediates many of the growth-promoting effects of GH (6, 7). Several transcription factors have been identified that mediate GH-regulated gene expression, including signal transducer and activator of transcription (STAT) 1, 3, 5a, and 5b, CCAAT/enhancer-binding protein β (C/EBPβ), and Elk-1 (4). In contrast to analysis of individual target genes for information on transcription, this study took a global approach to identify GH-regulated transcriptional mechanisms.

Microarrays provide a tool by which changes in expression of thousands of genes can be measured simultaneously. Here, microarray analysis identified 561 probe sets representing adipocyte genes regulated by GH, including many not previously identified as being GH-responsive. Examining temporal patterns of gene expression provided insight into mechanisms potentially regulating metabolic responses of adipocytes to GH, and involvement of GH in other physiological events.

Profiles of GH-regulated genes in adipocytes were used to examine whether GH-regulated genes with similar expression patterns share similar mechanisms of transcriptional regulation and corresponding functional regulatory sites in their promoters. Computational methods were applied to sequences of GH-regulated genes to identify potential binding sites for transcription factors in conserved sequences of the promoters of genes sharing similar temporal patterns of gene expression in response to GH (8). Hierarchical clustering of microarray data from 3T3-F442A adipocytes treated with GH for different times identified clusters of genes sharing similar temporal patterns of response to GH. One cluster contains highly GH-responsive genes encoding activating transcription factor 3 (ATF3) and growth arrest and DNA damage-inducible gene 45 gamma (GADD45γ). Gibbs motif sampling (9)

* This work was supported in part by National Institutes of Health (NIH) Grants DK46072 (to J. S.), LM008106 (to D. J. S.), and DK59605 (to T. H.), by National Science Foundation (NSF) Grant IBN-0080193 (to J. S.), a Bioinformatics Pilot Research Grant from the Howard Hughes Medical Institute (to J. S.), and a grant from the Biomedical Research Council at the University of Michigan (to J. S.). Microarray analysis was performed through the Michigan Diabetes Research and Training Center (NIH Grant DK58771). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3.

[1] Supported by the Cellular and Molecular Biology Training Grant (NIH Grant T32-GM07315), a National Defense Science and Engineering Graduate (NDSEG) predoctoral fellowship from the U.S. Dept. of Defense, and an NSF Graduate Fellowship.

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[4] The abbreviations used are: GH, growth hormone; ATF3, activating transcription factor 3; GADD45γ, growth arrest and DNA damage-inducible gene 45 γ; C/EBP, CCAAT/enhancer-binding protein; STAT, signal transducer and activator of transcription; CHO, Chinese hamster ovary; GHR, growth hormone receptor; WSR, Wilcoxon's signed rank; MASv5.0, (Affymetrix) MicroArray Suite version 5.0 software; QT-PCR, semi-quantitative real-time PCR; GO, Gene Ontology; wt, wild type; mut, mutant; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; pol II, RNA polymerase II; SOCS, suppressors of cytokine signaling; PI3K, phosphatidylinositol 3-kinase; MHC, major histocompatibility complex; LMP7, large multifunctional proteosome 7; TAP-1, transporters associated with antigen processing-1; SREBP, sterol regulatory element-binding protein; MRP, MIP-related protein.

Supplemental Material can be found at: http://www.jbc.org/cgi/content/full/M508492200/DC1

was used to search for conservation in the promoters of *Atf3* and *Gadd45γ* among human, mouse, and rat sequences, leading to the prediction of a novel binding site for C/EBPβ in the *Atf3* promoter. Analysis of binding to the predicted ATF3 C/EBP site and its ability to mediate transcriptional activation support a role for C/EBPβ in GH-stimulated *Atf3* promoter activity.

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 (GHR) containing the N-terminal half of the cytoplasmic domain (GHR_{1-454}, referred to as CHO-GHR cells) (10) were provided by Drs. Gunnar Norstedt (Karolinska Inst.) and Nils Billestrup (Steno Diabetes Center). 293T cells were provided by Dr. M. Lazar (University of Pennsylvania). Recombinant human GH was provided as a gift from Eli Lilly, Inc. Culture medium, calf serum, fetal calf serum, insulin, L-glutamine, and antibiotic-antimycotic were purchased from Invitrogen. Fetal calf serum for use in differentiation of 3T3-F442A cells into adipocytes was purchased from Atlanta Biologicals. Bovine serum albumin (bovine serum albumin, CRG7) was purchased from Intergen. Dexamethasone and isobutylmethylxanthine were purchased from Sigma. The Acetyl histone H4 Chromatin Immunoprecipitation Assay kit was purchased from Upstate. Formaldehyde was purchased from Sigma. Immobilized Protein A was purchased from Repligen, sonicated salmon sperm DNA from Stratagene, and the PCR Purification Kit from Qiagen. Luciferin was purchased from Promega, and β-galactosidase chemiluminescence reagents from Tropix. [α-^{32}P]dATP was purchased from ICN.

Cell Culture and Hormone Treatment—3T3-F442A preadipocytes, CHO-GHR cells, and 293T cells were grown, and 3T3-F442A preadipocytes were differentiated into adipocytes, as described previously (11). For experiments involving treatment with GH, cells were deprived of serum overnight in the appropriate medium containing 1% bovine serum albumin instead of serum. Cells were then incubated with or without GH at 500 ng/ml (22 nM) for the indicated times. To control for potential effects of serum starvation as well as account for the survival effects of GH, RNA was harvested from corresponding sets of untreated and GH-treated adipocytes at each time point.

Microarray Analysis—Total RNA was isolated from control and GH-treated adipocytes using RNA Stat60 (Tel-Test B, Inc.) and RNeasy spin columns (Qiagen) according to manufacturer instructions. For microarray analysis, preparation of cRNA, hybridization, and scanning of the mouse genome U74Av2 oligonucleotide arrays (Affymetrix) were performed according to the manufacturer's protocol by the Cell and Molecular Biology Core of the Michigan Diabetes Research and Training Center. The arrays were scanned at 3 μm with a GeneArray scanner (Affymetrix). Affymetrix® MicroArray Suite Version 5.0 (MASv5.0) software was used to analyze the .cel files. RNA was prepared and analyzed separately in three independent experiments.

Probe sets considered differentially expressed in response to GH were identified based on *p* values obtained through application of the Wilcoxon's signed rank test as implemented in MASv5.0. Expressed probe sets were considered to be changed by GH if the probe set, for at least one time point, was both significantly expressed (present) in the adipocytes ($p < 0.025$) and also showed statistically significant ($p < 0.05$) changes in response to GH in the same direction in all three experiments. Thresholds for expression and for changes in response to GH were based on *p* values observed for 7 genes (*Atf3*, *Bcl6*, *Cish*, *Ccl6 (C-10)*, *Fos*, *Gadd45γ*, and *Igf1* (data not shown)) measured on microarray that were confirmed by QT-PCR to be GH-responsive. Because in many cases multiple probe sets identify the same gene, the number of unique transcripts identified is smaller than the number of probe sets identified. Clusters of probe sets sharing similar patterns of expression over time in response to GH were identified by average linkage hierarchical clustering using the Cluster and Treeview Software packages (12, 13).

Semi-quantitative Real-time PCR—Expression of *Atf3* and *Gadd45γ* mRNA was confirmed by QT-PCR using SYBR green I (Applied Biosystems) and the iCycler system with iCycler iQ Real-time Detection System software (Bio-Rad Laboratories). Sequences of probes and primers were as follows: for murine *Atf3*, 5′-CGAAGACTGGAGCAAAATG-ATG-3′ (forward) and 5′-CAGGTTAGCAAAATCCTCAAATAC-3′ (reverse) (14); for murine *Gadd45γ*, 5′-TGCTGGCTGCCGATGA-A-3′ (forward) and 5′-ACGCCTGAATCAACGTGAAAT-3′ (reverse) (designed using Primer Express software (Applied Biosystems)), for murine *Gapdh*, 5′-ATGTTCCAGTATGACTCCACTCACG-3′ (forward) and 5′-GAAGACACCAGTAGACTCCACGACA-3′ (reverse) (15). All readings were normalized to *Gapdh*, which is not responsive to GH under the conditions used. Relative gene expression is expressed as GH-treated/control. Statistical analysis of combined experiments by *t* test compared increments above control due to GH at each time point.

Functional Classification of GH-responsive Genes—To classify the functions of genes differentially expressed by GH, genes were sorted based on categories defined by the Biological Process Ontology of the Gene Ontology (GO) hierarchy (16), using two tools, the Affymetrix NetAffx GO Mining Tool (Affymetrix) (17) and the GenMAPP2 software package (Gladstone Inst., University of California at San Francisco) (18). The Affymetrix NetAffx GO Mining Tool, containing the December 2004 Affymetrix GeneChip annotations, determines whether a given biological function is statistically overrepresented by calculating χ^2 score-derived *p* values. The GenMAPP2 analysis uses a hypergeometric distribution to calculate standardized difference scores (Z-scores) as a measure of the statistical significance, and non-parametric methods to calculate the permute *p* value. Of categories identified by both tools as significantly overrepresented ($p < 0.01$), those containing at least 2% of the total number of GH-regulated probe sets are discussed.

Identification of Potential Regulatory Elements in Atf3 and Gadd45γ Promoters—To identify potential regulatory elements in the *Atf3* and *Gadd45γ* promoters, conserved sequences were identified from orthologous human, mouse, and rat *Atf3* and *Gadd45γ* promoters. Sequences were obtained using the University of California, Santa Cruz (UCSC) Genome Browser (19) spanning 2500 bp upstream and 500 bp downstream of the transcription start site documented in Genome Browser. The Gibbs Motif Sampler was used to search for regions of conservation using subsequence lengths (8–25 bp), with 10^6 iterations for each of 10 random number seeds (9).

Plasmids and Antibodies—A plasmid containing the human *Atf3* promoter (−2050 to +34) upstream of the luciferase gene (referred to as WT ATF3-Luc) was constructed in the vector pXP2 (from A. Seasholtz, University of Michigan) (20). The *Atf3* promoter sequence from the −2050 to +34 region of plasmid pLuc-1850 *Atf3*-luciferase (21) (from S. Kitajima, Tokyo Medical and Dental University) was cut out using HindIII and ligated into pXP2. ATF3-Luc with a mutation in the predicted C/EBP site (wt: TTGCATCACCA, mut: TTG**GTG**CACCA) was constructed from WT-ATF3-Luc using the QuikChange II XL mutagenesis kit (Stratagene) and is referred to as mC/EBP ATF3-Luc. The mutation was based on an inactivating mutation in the C/EBP site in the gene for interleukin-6 (22). The plasmid for full-length rat C/EBPβ driven by the CMV promoter (referred to as CMV-C/EBPβ) was a gift from U.

Schibler (University of Geneva) and L. Sealy (Vanderbilt University). The vector pcDNA3 (Invitrogen, from K. Guan, University of Michigan), in which the CMV-C/EBPβ vector was constructed, was used to normalize total amounts of transfected DNA. The plasmid for full-length rat GHR was from N. Billestrup (Steno Diabetes Center). Antibody against a peptide in the C terminus of C/EBPβ (C-19), antibody against the N terminus of RNA polymerase II (pol II) (N-20), and normal rabbit immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology Inc. Anti-acetyl-histone-H4 was purchased from Upstate.

Electrophoretic Mobility Shift Assay—For preparing extracts enriched in C/EBPβ, 293T cells in 100-mm plates were transfected as described previously (23) with plasmids encoding C/EBPβ (1 μg), and cell extracts were prepared as previously described (24). Nuclear extracts were prepared from 3T3-F442A adipocytes at designated times after GH treatment and EMSA was performed as described previously (25). Oligonucleotide probes contained the following sequences: predicted *Atf3* C/EBP site: 5′-CGAACTTGCATCACCAGTGCCC-3′; *Gadd45γ* C/EBP site: 5′-CGCAGCCTCGCGCCAGCTGGCGGCGCCGCAC-3′; *Fos* C/EBP site: 5′-GGATGTCCATATTAGGACATC-3′; mutation in predicted *Atf3* C/EBP site (changes from wild-type are underlined): 5′-CGAACTTGGTGCACCAGTGCCC-3′. 50× or 100× unlabeled probe was added as indicated. Where indicated, extracts were incubated with anti-C/EBPβ (0.3 μg) for 20 min at room temperature prior to binding reactions. Complexes were resolved by nondenaturing PAGE (7%) followed by autoradiography, as described (24).

Chromatin Immunoprecipitation—ChIP was performed as described previously (26). For immunoprecipitation, samples containing 100 μg of nuclear protein were incubated overnight at 4 °C with 4 μg of the following antibodies individually: anti-C/EBPβ, anti-acetylated histone H4, or anti-pol II. Normal rabbit IgG and samples with no antibody served as negative controls. A single 259-bp fragment of the mouse *Atf3* promoter or a 330-bp fragment of the mouse *Fos* promoter was amplified with 35 or 31 cycles of PCR, respectively (94 °C 20 s, 60 °C 20 s, and 72 °C 30 s) using ChIP primers (*Atf3* forward: 5′-GAACACGCAGCGGCGAATAC-3′; *Atf3* reverse: 5′-CAATCCCAGGCTGACGTAATG-3′; *Fos* forward: 5′-GGCTGCAGCCGGCGAGCTG-3′; *Fos* reverse: 5′-AGAAGCGCTGTGAATGGATG-3′ (26)). 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.

Transcriptional Activation—CHO-GHR cells (1×10^5 cells/35 mm well) were pre-incubated for 4 h with Dulbecco's modified Eagle's medium supplemented with 8% calf serum, and then transiently transfected as described previously (23) with WT or mC/EBP ATF3-Luc plasmids (500 ng/well), and plasmids for C/EBPβ (10 ng/well) or full-length rat GHR (500 ng/well) as indicated. For C/EBPβ co-transfection experiments, cells were transfected for 18h in Dulbecco's modified Eagle's medium with 8% calf serum, followed by 18h in Ham's F-12 medium with 8% fetal calf serum. For GH treatment experiments, cells were transfected for 18 h, followed by 4 h in Ham's F-12 medium with 8% fetal calf serum, 18 h in Ham's F-12 medium containing 1% bovine serum albumin instead of serum, to which GH was added for 8 more hours. Transfected cells were lysed for measurement of luciferase activity using an Opticomp Luminometer as described previously (11, 24). Results show the means ± S.E. for relative luciferase units of at least four experiments, with each condition performed in triplicate. Statistical significance was assessed using a Student's *t* test.

RESULTS

GH-induced Genes in Adipocytes Are Expressed in Time-dependent Patterns—For insight into mechanisms for time-dependent responses to GH, genes regulated by GH at key time points were analyzed by microarray. To identify GH-regulated genes potentially involved in insulin resistance, cells were treated with GH for 48 h when 3T3-F442A adipocytes show impaired responses to insulin. For insight into earlier triggering events, cells were treated with GH for 30 min or 4 h. Affymetrix U74Av2 GeneChips were used to examine changes in gene transcript levels in RNA from 3T3-F442A adipocytes. Of the 12,488 probe sets represented on the chip, 561 probe sets identified transcripts which were differentially expressed in response to GH at one or more time points.[5]

While many of the genes corresponding to the 561 probe sets identified by this microarray analysis have not previously been identified as responsive to GH in adipocytes, documented GH-responsive genes were also identified. For example, GH induced the expression of the gene for insulin-like growth factor-I at 48 h, as seen in liver and adipose tissue from rodents (27, 28). Expression of the genes for GHR (29) and the acid labile subunit, which complexes with circulating insulin-like growth factor-I (30), also increased after 48 h of GH. Expression of early response genes, including *Fos*, *Jun*, and *Egr1*, increased rapidly and transiently within 30 min of GH, as reported previously (31–35). GH also increased expression of genes encoding negative regulators of GH signaling, including the genes for SOCS-1, SOCS-2, SOCS-3, and cytokine-inducible SH2-containing protein (36–39).

The metabolic responses of adipocytes to GH change over time. Similarly, adipocyte gene expression in response to GH changes in distinct patterns over time. When GH-responsive genes were sorted using an unsupervised hierarchical clustering strategy, which makes no prior assumptions regarding number or types of clusters (12), temporal patterns of changes in gene expression were evident. Three "waves" of expression (Fig. 1) corresponding to timing of GH treatment were identified in seven clusters. The early wave includes genes stimulated by GH only at 30 min (*Cluster A*); no genes were decreased with 30 min GH. An intermediate wave is comprised of genes which are transiently regulated by GH at 4 h, either positively (*Cluster B*) or negatively (*Cluster E*). Prolonged changes in expression in response to GH were evident at 48 h. In some cases the GH-induced changes seen at 48 h begin at 30 min or 4 h (*Clusters C* and *F*). The majority of prolonged GH-induced changes are observed only at 48 h (*Clusters D* and *G*).

Early GH-regulated Genes Encode Transcription Factors—A systematic, unbiased survey of biological functions regulated by GH in adipocytes was undertaken to determine to what extent genes for established responses to GH in adipocytes were regulated and to identify novel functions regulated by GH. The Gene Ontology (GO) hierarchy provides a standard classification system by which genes can be sorted into groups with associated biological functions (16). Categories of biological function that are statistically overrepresented ($p < 0.01$) within each wave (early, intermediate, or prolonged) of GH-induced gene expression were identified using the NetAffx GO Mining Tool (17) and the GenMAPP software package (18). The most specific GO categories identified by both tools as overrepresented among GH-responsive genes (Table 1) during early and intermediate waves of GH-induced gene expression identify biological functions related to regulation of transcription. Genes associated with regulation of transcription, lipid bio-

[5] The unprocessed microarray data are accessible through Gene Expression Omnibus Series accession number GSE2120 in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/). The complete list of GH-regulated probe sets (supplemental Data Set 1) is available at www.umich.edu/~jschwrtz/.

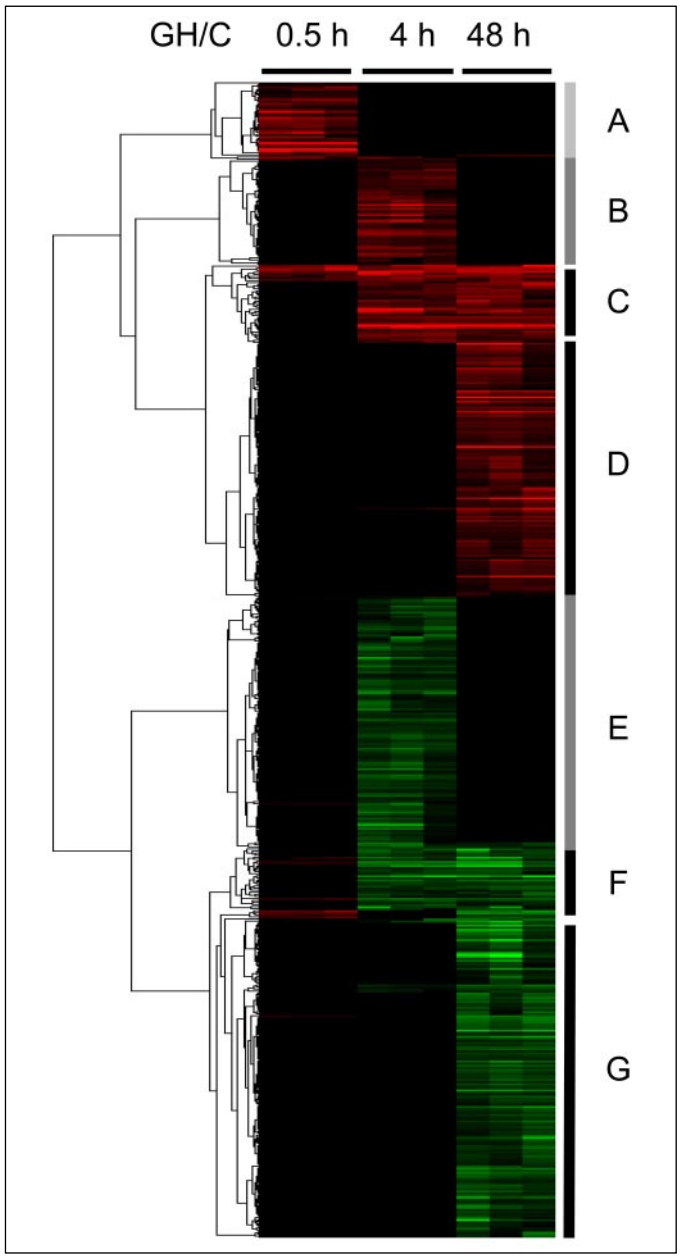

FIGURE 1. **Gene expression profiles reveal time-dependent patterns during GH treatment.** RNA from 3T3-F442A cells treated with GH for 0.5, 4, or 48 h was analyzed by microarray. RNA transcript levels are expressed as ratios of GH-treated and control (*GH/C*). Probe sets are represented in *horizontal rows* showing three independent experiments per time point, with a colorimetric scale ($\log_2(x)$) to indicate relative expression levels: *red* for GH/C $>$ 1 (increased expression), *green* for GH/C $<$ 1 (decreased expression). A total of 561 transcripts showing statistically significant changes in expression in response to GH ($p < 0.05$) were analyzed by hierarchical clustering. Times of GH treatment are indicated above each column. The seven clusters are identified at *right*; early, intermediate, and prolonged waves of gene expression are represented by *light gray*, *medium gray*, and *black bars*, respectively. Cluster trees are shown at the *left*.

synthesis, carbohydrate metabolism, and immune function are among genes statistically overrepresented in the prolonged wave of GH-induced gene expression (Table 1). Genes encoding regulators of transcription were highly represented in GH-treated cells at 30 min; all of these genes were stimulated (supplemental Table S1). Regulation of transcription is also the most highly represented function among genes regulated by GH only at 4 h (supplemental Table S2), but at this time, expression of the majority of genes was decreased by GH (25 of 27). At both 30 min and 4 h, regulators of transcription is the only functional

TABLE 1
Gene ontology categories overrepresented among GH-regulated genes

GO category	No. of genes
A) Early wave (Cluster A)	
Regulation of transcription, DNA-dependent (#6355)[a]	13
B) Intermediate wave (Clusters B and E)	
Regulation of transcription, DNA-dependent (#6355)	27
C) Prolonged wave (Clusters C, D, F, and G)	
Immune response (#6955)	23
Carboxylic acid metabolism (#19752)	18
Regulation of transcription, DNA-dependent (#6355)	17
Carbohydrate metabolism (#5975)	15
Alcohol metabolism (#6066)	13
Lipid biosynthesis (#8610)	13

[a] GO identification nos. are indicated in parentheses.

category of GH-regulated genes statistically overrepresented. A third set of genes encoding transcriptional regulators respond to GH at 48 h (supplemental Table S3A).

Prolonged GH Treatment Inhibits Genes Associated with Lipid and Cholesterol Biosynthesis—GH inhibits genes associated with lipid and cholesterol biosynthesis at 48 h. Genes whose products are associated with lipid biosynthesis are highly represented among genes inhibited by 48 h of GH in 3T3-F442A adipocytes (Table 1). These genes are part of a broader category of genes associated with lipid metabolism, which is also statistically overrepresented (supplemental Table S3B). Many of these lipid metabolism genes participate in pathways for fatty acid biosynthesis (Fig. 2). GH inhibits expression of multiple genes of the fatty acid biosynthesis pathway, in keeping with known inhibition of lipid synthesis by GH at 48 h (40). In contrast, GH increases the transcript level of one gene, which encodes acyl-CoA synthetase, long-chain family member 5, which catalyzes the first step of fatty acid metabolism. This is consistent with the role of GH in increasing fatty acid oxidation (41). In addition, genes related to cholesterol biosynthesis are also highly represented (Fig. 2), suggesting a role for GH in regulation of cholesterol synthesis in adipocytes.

Among genes associated with carbohydrate metabolism (supplemental Table S3C), GH decreases expression at 48 h of genes associated with the pentose phosphate pathway (phosphogluconate dehydrogenase, transaldolase), the tricarboxylic acid cycle (succinate-CoA ligase), and glycolysis (enolase and lactic acid dehydrogenase), while stimulating expression of a key enzyme in ketone body breakdown (3-oxoacid-CoA transferase (succinate-CoA ligase)). GH decreases expression of the gene for brain glycogen phosphorylase, associated with glycogen breakdown, while increasing expression of the gene for glycogenin 1, associated with glycogen synthesis. Most of the genes that fell into the GO categories "carboxylic acid metabolism" or "alcohol metabolism" (Table 1) also fell into either the "carbohydrate metabolism" or "lipid biosynthesis" categories. The observation of GH-induced changes in expression of genes associated with carbohydrate biosynthesis and breakdown is consistent with the ability of GH to regulate carbohydrate metabolism (1).

Prolonged GH Treatment Regulates Genes Associated with Immune Response—Interestingly, genes associated with immune responses are the most numerous of those responsive to GH at 48 h (Table 1 and supplemental Table S3D). In this category, the gene for the p85α subunit of the signaling molecule phosphatidylinositol 3-kinase (PI3K-p85α) is increased by GH. Although PI3K is associated with immune function (42), its general function in signaling is consistent with a broader role in GH action. Based on microarray, GH also increases to varying extents expression of the genes for the MHC class-I molecules H2-K1, H2-D1, H2-Q7, and H2-T3, as well the genes encoding large

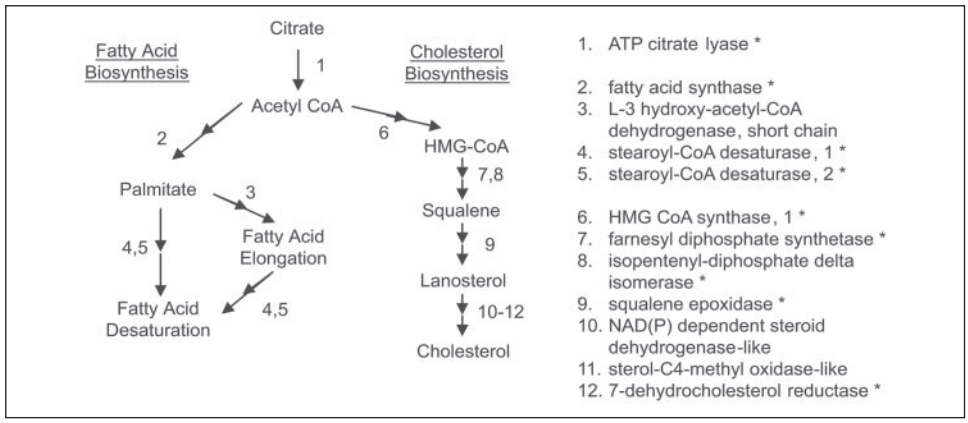

FIGURE 2. **GH suppresses the expression of genes involved in fatty acid and cholesterol biosynthesis at 48 h.** Among genes that show decreases in response to prolonged GH treatment (Fig. 1, *Clusters F* and *G*), many are involved in lipid or cholesterol biosynthesis. Schematic diagram of the pathways of lipid and cholesterol biosynthesis is at the *left*, with *numbers* representing GH-regulated genes, which are listed on the *right*. *Asterisks* mark genes reported to be regulated by SREBPs (60).

multifunctional proteosome 7 (LMP7) and transporters associated with antigen processing-1 (TAP1), which are necessary for normal cell surface expression of MHC Class I molecules (43, 44). The genes encoding the chemokines CCL6 (C10 and MRP-1) and CCL9 (MIP-1γ and MRP-2), two structurally similar members of the CC family of chemokines (45), show increased expression after 48 h of GH. Expression of genes for several other chemokines is also regulated by GH (data not shown). Overall, these changes suggest links between GH and regulation of immune function in adipocytes.

Temporal Pattern of GH-stimulated Expression of Atf3 Suggests a Mechanism of Transcriptional Regulation—Hierarchical clustering revealed temporal patterns among GH-regulated genes. It was reasoned that genes with similar patterns of expression over time in response to GH might share similar mechanisms of transcriptional regulation and utilize similar regulatory elements in their promoters. Genes with similar temporal patterns of response to GH were analyzed for shared sequences in their promoters to gain insight into molecular mechanisms for GH-regulated gene expression.

The genes encoding ATF3 and GADD45γ were stimulated by GH at all three time points with a greater expression at 4 h. Their stimulation by GH and this pattern of expression (within *Cluster C*, Fig. 1) were confirmed using semi-quantitative real-time PCR (QT-PCR) (Fig. 3). *Atf3* (supplemental Table S3C) and *Gadd45γ* (supplemental Table S3D) have among the largest overall increases in transcript level in response to GH at all three time points of the GH-responsive genes identified. *Atf3* is a stress-inducible gene that encodes a member of the ATF/CREB family of transcription factors (46) and may be involved in glucose homeostasis (14). *Gadd45γ*, also known as cytokine response 6, is a stress-inducible gene involved in growth suppression and apoptosis (47–49). A member of the GADD45 family has previously been reported to be regulated by GH (50).

To assess whether the distinctive temporal pattern of GH-stimulated expression of *Atf3* and *Gadd45γ* is mediated by regulatory elements shared by the *Atf3* and *Gadd45γ* promoters, the sequences of the human, mouse, and rat *Atf3* and *Gadd45γ* promoters were analyzed for sequence conservation using the Gibbs motif sampling algorithm (9). These searches were performed without prior knowledge of known or predicted regulatory elements. One region of sequence conservation identified was a TATA box shared by all six sequences. In addition, a highly conserved sequence was identified in the *Atf3* and *Gadd45γ* promoters (at −193 to −203 in human *Atf3*) (Fig. 4*A*). This sequence aligns with a non-consensus C/EBP site that had been identified in the human *Gadd45γ* promoter (51). The presence of a C/EBP site in this highly conserved region leads to the prediction that a functional C/EBP site is also present in the *Atf3* promoter.

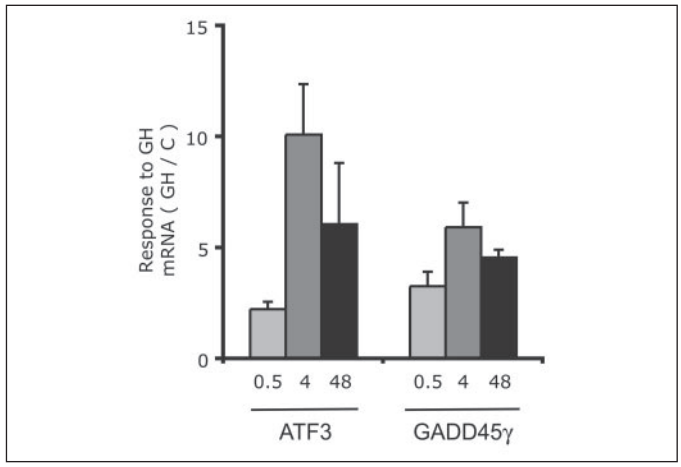

FIGURE 3. **GH stimulates *Atf3* and *Gadd45γ* mRNA transcript levels with similar temporal patterns.** RNA from adipocytes treated with GH for 0.5, 4, or 48 h was analyzed using QT-PCR. Relative mRNA transcript levels for *Atf3* and *Gadd45γ* are expressed as ratios of GH/Control. *Bars* represent mean ± S.E. for three independent experiments. The increment above control due to GH is significant for *Atf3* ($p < 0.05$ at 30 min, 4 h, $p < 0.075$ at 48 h) and for *Gadd45γ* ($p < 0.05$ at all times).

C/EBPβ is known to mediate GH-induced expression of c-*fos* (11, 26, 52). To examine whether C/EBPβ binds to the predicted C/EBP site in the *Atf3* promoter, EMSA was performed using a probe based on the predicted *Atf3* C/EBP site, with extracts enriched in C/EBPβ from 293T cells. A protein complex was found to bind efficiently to the predicted C/EBP site of *Atf3* (Fig. 4*B*, *lane 8*), and was supershifted with the addition of anti-C/EBPβ antibody (Fig. 4*B*, *lane 9*), indicating the presence of C/EBPβ in the complex. Binding to the known C/EBP site from *Gadd45γ* appeared to be weaker than binding to the predicted *Atf3* C/EBP site when equivalent amounts of protein were tested (Fig. 4*B*, *lane 5*). Binding of C/EBPβ to the *Atf3* C/EBP site was also greater than to the *Fos* C/EBP site (Fig. 4*B*, *lane 2*). The binding to the *Atf3* C/EBP probe was greatly reduced when excess unlabeled probe (50× or 100×) was added as a competitor (Fig. 4*C*, *lanes 2* and *3*). In contrast, a probe containing a mutation in the *Atf3* C/EBP site that prevents C/EBPβ binding (data not shown) does not compete for binding when added as an unlabeled competitor (Fig. 4*C*, *lanes 4* and *5*). The predicted *Atf3* C/EBP site also bound endogenous nuclear proteins from 3T3-F442A adipocytes (Fig. 4*D*, *lane 3*), and binding was almost completely supershifted with anti-C/EBPβ antibody (Fig. 4*D*, *lane 4*), indicating that endogenous C/EBPβ in adipocytes binds to the *Atf3* C/EBP site *in vitro*. There was no detectable difference in binding of C/EBPβ in nuclear extracts from cells with or without GH treatment (data not shown).

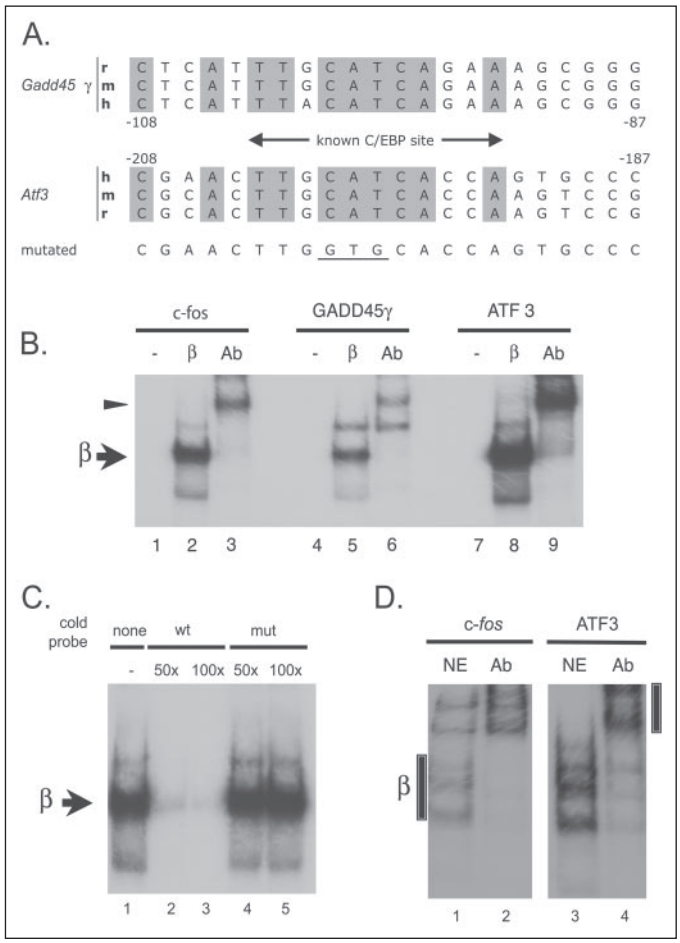

FIGURE 4. **C/EBPβ binds to the predicted C/EBP site in the *Atf3* promoter.** *A*, conserved sequences for the proximal promoters of the human (*h*), mouse (*m*) and rat (*r*) orthologues of the *Atf3* and *Gadd45γ* genes are indicated (*shaded*). A C/EBP site in the *Gadd45γ* promoter is indicated by *arrows*. *Numbering* is based on the transcription start site in the human orthologues of *Atf3* or *Gadd45γ*. The human sequences shown were used for the human *Gadd45γ* and *Atf3* EMSA probes; for mutated *Atf3* probe (*bottom*), mutated sequence is underlined. *B*, C/EBPβ expressed in 293T cells was used in EMSA with oligonucleotide probes based on the predicted C/EBP site in the *Atf3* promoter (*lanes 7–9*) as well as the C/EBP sites from the promoters of *Gadd45γ* (*lanes 4–6*) or *Fos* (*lanes 1–3*). Complexes containing C/EBPβ (*β*) are indicated by the *arrow* at the *left*. The supershifted complexes in presence of anti-C/EBPβ antibodies (*Ab*) are indicated by the *arrowhead* at the *left*. Results with *Fos* and *Atf3* C/EBP probes are representative of at least three experiments. Results for the *Gadd45γ* C/EBP site are representative of results reported by other investigators (51). *C*, probe representing the predicted C/EBP site in the *Atf3* promoter was used in EMSA with C/EBPβ expressed in 293T cells, without (*lane 1*) or with unlabeled WT probe at 50 or 100× excess (*lanes 2* and *3*, respectively). Competition by unlabeled probe representing *Atf3* C/EBP site mutated as in Fig. 4*A* (*mut*) was also tested (*lanes 4* and *5*, respectively). Results are representative of two experiments. *D*, nuclear extracts (*NE*) from 3T3-F442A adipocytes treated with GH for 30 min were used in EMSA with probes based on the predicted C/EBP site from the *Atf3* promoter (*lanes 3* and *4*) or the C/EBP site from the *Fos* promoter (*lanes 1* and *2*). Anti-C/EBPβ antibody (*Ab*) was added to NE (*lanes 2* and *4*) to identify C/EBPβ in complexes. Complexes containing C/EBPβ are indicated by the *bar* on the *left* (*β*), and supershifted complexes in presence of anti-C/EBPβ (*Ab*) are indicated by the *bar* on the *right*. Results are representative of three experiments.

Chromatin immunoprecipitation (ChIP) demonstrates that endogenous C/EBPβ binds to the *Atf3* promoter *in vivo*. Chromatin-bound proteins were immunoprecipitated with anti-C/EBPβ from nuclei of 3T3-F442A pre-adipocytes treated without or with GH for 15 or 60 min. QT-PCR shows that in 3T3-F442A pre-adipocytes GH induces *Atf3* mRNA expression in 30–60 min. (data not shown). DNA fragments associated with immunoprecipitated proteins were amplified by PCR using primers based on a promoter sequence containing the predicted *Atf3* C/EBP site. Endogenous C/EBPβ was found to immunoprecipitate with *Atf3* promoter DNA *in vivo* at all times tested (Fig. 5, *right*). A lack

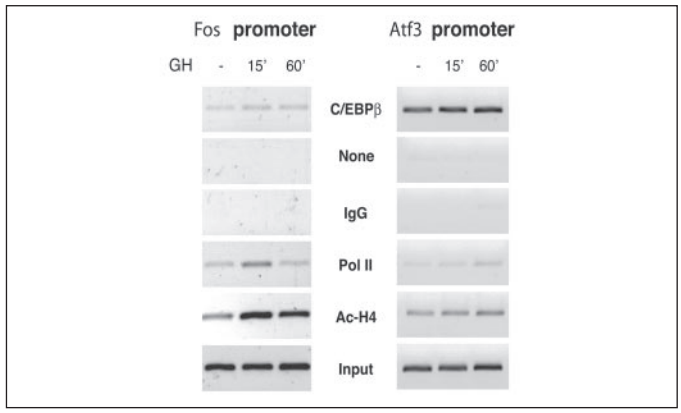

FIGURE 5. **Endogenous C/EBPβ binds to the *Atf3* promoter.** For ChIP, 3T3-F442A preadipocytes were treated with GH for the indicated times (min). ChIP was performed using anti-C/EBPβ, no antibody, normal rabbit IgG, anti-pol II, or anti-acetylated histone 4. 1% input is shown in *bottom panel*. Purified DNA was used for PCR (31 or 35 cycles for *Fos* and *Atf3*, respectively). Similar data were obtained in two other experiments.

of signal is observed when no antibody or normal rabbit IgG is used in place of anti-C/EBPβ. Similar findings for C/EBPβ were observed in pre-adipocytes and adipocytes (data not shown). The amount of RNA polymerase II (pol II) occupying the *Atf3* promoter increased at 60 min, and acetylated histone 4 occupied the *Atf3* promoter at all times tested. Interestingly, the timing of occupancy of pol II on the *Atf3* promoter differs from that on the *Fos* promoter (Fig. 5, *left*). The slower increase in pol II occupancy on the *Atf3* promoter coincides with the slower rise in transcription of *Atf3* compared with the rapid, transient increase in *Fos* expression in response to GH (26). Together, these findings suggest that endogenous C/EBPβ binds avidly to the predicted *Atf3* C/EBP site *in vivo*.

Predicted C/EBP Site in the Atf3 Promoter Mediates Its Stimulation by GH—If the predicted C/EBP site in *Atf3* is functional, it would be expected that it mediates transcriptional activation in the presence of C/EBPβ. Consistent with this, when C/EBPβ was co-expressed with a construct containing the wild-type *Atf3* promoter (from −2050 to +34) upstream of the luciferase gene (WT ATF3-Luc) in CHO-GHR cells, C/EBPβ increased *Atf3* promoter activity (Fig. 6*A*). In contrast, activation of an *Atf3*-promoter with a mutation in the C/EBP site, which prevents C/EBPβ binding (mC/EBP ATF3-Luc), is not increased when C/EBPβ is coexpressed (Fig. 6*A*). Basal activity of mC/EBP ATF3-Luc was more than five times greater than that of the empty pXP2 vector (data not shown), although it was lower than basal activity of WT ATF3-Luc. Together, these results support a role for the predicted C/EBP site in the *Atf3* promoter in mediating its transcriptional activation.

Because GH increases *Atf3* mRNA it was considered likely that GH would also increase *Atf3* promoter activity. When this was tested in CHO cells co-expressing full-length GHR, GH was found to increase the activation of wild-type *Atf3* promoter activity (Fig. 6*B*). In contrast, ATF3-Luc mutated in the predicted C/EBP site (mC/EBP ATF3-Luc) failed to mediate a response to GH (Fig. 6*B*). mC/EBP ATF3-Luc was stimulated by serum (>50% increase *versus* control, data not shown). Taken together, these findings suggest that a C/EBP site in *Atf3*, predicted from the identification of conserved sequences between *Atf3* and *Gadd45γ*, is required for activation by GH of the *Atf3* promoter.

DISCUSSION

This work identifies time-dependent patterns of expression of genes regulated by GH in adipocytes. Early (change expression only at 30 min), intermediate (change expression only at 4 h), and prolonged (change at 48 h) waves of gene expression were observed. When GH-regulated

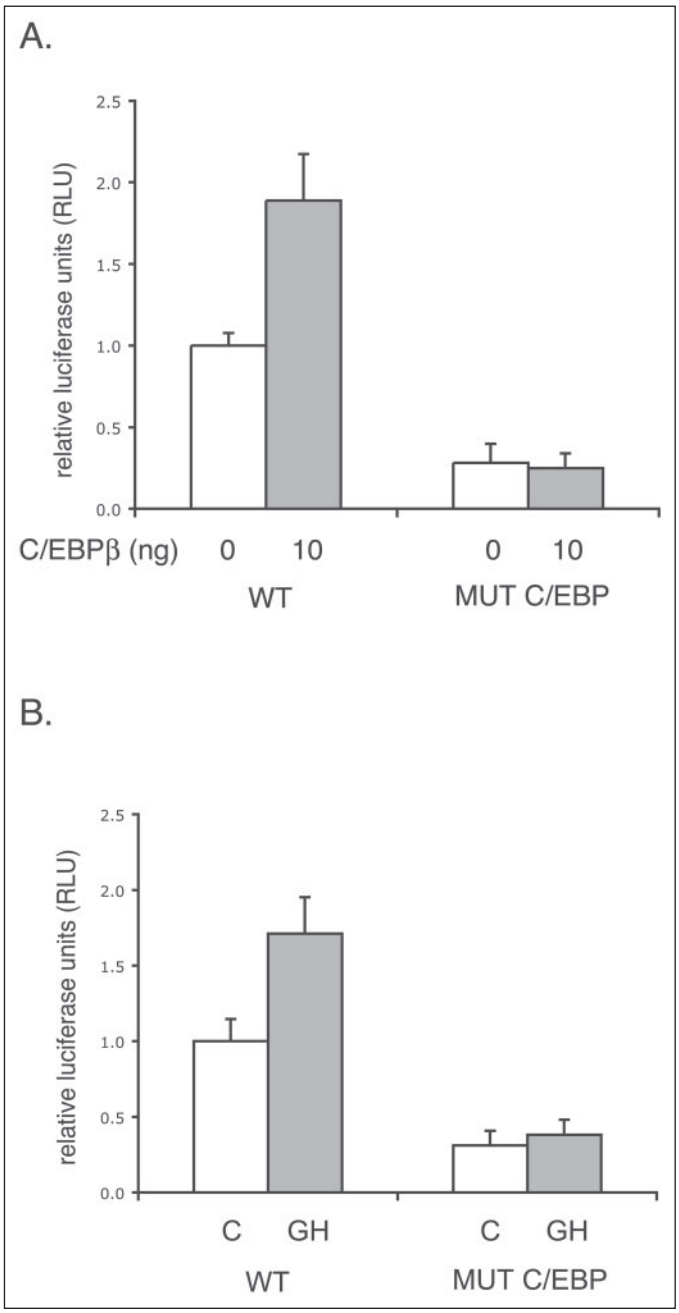

FIGURE 6. **The predicted C/EBP site mediates GH-stimulated *Atf3* promoter activation.** *A*, CHO-GHR cells were transfected with plasmids WT ATF3-Luc or mC/EBP ATF3-Luc (MUT C/EBP) without (*open bar*) or with (*gray bar*) 10 ng of CMV-C/EBPβ. Cell lysates were analyzed for luciferase activity (RLU) set to 1.0 for WT ATF3-Luc without C/EBPβ. *Bars* show mean ± S.E. for seven experiments. The increase in WT ATF3-Luc activity with C/EBPβ ($p < 0.002$) and the decrease in basal activity of MUT *versus* WT C/EBP ATF3-Luc ($p < 0.002$) are statistically significant. *B*, CHO-GHR cells were transfected with plasmids for WT ATF3-Luc or mC/EBP ATF3-Luc, as well as for GHR. Cells were treated without (*open bar*) or with (*gray bar*) GH. RLU as for Fig. 5*A*, $n = 4$ experiments. Response to GH is significant ($p < 0.002$) for WT ATF3-Luc but not for MUT C/EBP. In the absence of GH, activation of MUT C/EBP ATF3-Luc is significantly ($p < 0.002$) less than WT ATF3-Luc.

genes sharing temporal patterns of gene expression were sorted into groups by biological function, the role and timing of biological processes regulated by GH in adipocytes were suggested. Interestingly, many of the genes and biological processes regulated by GH are associated with regulation of insulin sensitivity and energy metabolism. It will be of interest to verify by QT-PCR to what extent these genes are regulated by GH, particularly for those with relatively small responses to GH.

GH Regulates Expression of Genes for Transcription Factors—Genes encoding transcriptional regulators predominate among those with early and intermediate responses to GH treatment. After 30 min of GH, transient stimulation of expression of early response genes for transcription factors such as FOS, EGR1, EGR2, JUN-B, and KLF4, may activate genes induced by GH at later times, such as those mediating chronic metabolic responses to GH in adipocytes. The gene for the transcription factor XBP1 is one of the genes increased by GH at 4 and 48 h (supplemental Table S3A). XBP1 has recently been associated with protection against obesity-induced insulin resistance (53). The gene encoding the transcription factor ATF3, which is stimulated by GH at all times tested, regulates expression of genes involved in carbohydrate metabolism (14) and is associated with insulin resistance and diabetes (see below).

Prolonged GH Treatment Decreases Expression of Genes in Pathways of Fatty Acid and Cholesterol Biosynthesis—It is well recognized that GH is an important regulator of lipid metabolism (1, 54). Here, GH coordinately decreases expression of five genes that encode enzymes of the pathway for fatty acid biosynthesis after 48 h (Fig. 2). These delayed changes in the fatty acid synthesis pathway are consistent with inhibition by GH of lipid accumulation in adipocytes (40). GH also increases expression of the gene encoding acyl-CoA synthetase, the enzyme catalyzing the first step of fatty acid metabolism. Among the different isoforms of acyl-CoA synthetase, each thought to have specific functional roles (55), GH increases expression of the gene for *Acsl5*, an isoform that is thought to be associated with β-oxidation (56), consistent with GH-induced fatty acid oxidation (41). It is also of interest that GH increases expression of the gene encoding adiponectin receptor 2 in 3T3-F442A adipocytes as early as 4 h and continuing through 48 h (supplemental Table S3B). This agrees with a recent report that expression of the gene for adiponectin receptor 2, which together with adiponectin modulates insulin sensitivity, is increased by GH in 3T3-L1 adipocytes (57).

GH also coordinately decreased at 48 h the expression of seven genes associated with cholesterol biosynthesis (Fig. 2), suggesting that GH decreases cholesterol synthesis in adipocytes. This is intriguing in light of recent work showing that decreases in membrane cholesterol can trigger insulin resistance in adipocytes (58, 59). Further, expression of five of these genes has been shown to be regulated by sterol regulatory element-binding proteins (SREBPs) (Fig. 2) (60), which regulate the expression of many genes associated with the biosynthesis of lipids, cholesterol, and the NADPH cofactor involved in lipid and cholesterol biosynthesis (60). GH at 48 h also decreases expression of four SREBP-regulated genes associated with fatty acid biosynthesis and the SREBP-regulated gene for phosphogluconate dehydrogenase, which participates in NADPH synthesis. It is of note that GH-regulated genes in this study fell into pathways such as these, as current analyses of microarrays through methods including gene set enrichment reveal changes in pathways even when individual genes are not substantially changed (61). These data suggest that gene expression coordinated by SREBPs may be modulated by GH. Expression of genes for SREBP-regulated lipogenic enzymes was also decreased in livers of mice overexpressing GH (62, 63).

GH also regulates after 48 h a set of genes categorized for their association with carbohydrate metabolism (supplemental Table S3C), in keeping with established effects of GH on carbohydrate metabolism (1). A decrease in the demand for NADPH produced by the pentose phosphate pathway due to GH-induced decrease in synthesis of lipids and sterols is consistent with observations here that GH inhibits genes for transaldolase 1 and phosphogluconate dehydrogenase, two enzymes involved in the pentose phosphate pathway. The GH-induced increase

in expression of the gene for glycogenin 1, which initiates glycogen synthesis, and decrease in the gene for brain glycogen phosphorylase together suggest a net increase in glycogen in GH-treated adipocytes. These findings are intriguing in light of previous work indicating a complex role of GH in glycogen metabolism (1, 64, 65).

One of the genes associated with carbohydrate metabolism encodes the transcription factor ATF3. In addition to being stimulated by GH at all time points tested, expression of *Atf3* increases in many tissues, including heart, liver, and brain, in response to a variety of stress-inducing stimuli (46). With regard to glucose metabolism, ATF3 has been associated with stress-induced pancreatic β-cell apoptosis (66) and with defects in glucose homeostasis (67). Further, *Atf3* expression is elevated in the pancreatic islets of patients with type 1 or 2 diabetes, the islets of non-obese NOD diabetic mice (66), and the sensory neurons of streptozotocin-induced diabetic mice (68). In contrast, both islets and mouse embryonic fibroblasts derived from ATF3 −/− mice are partially protected from stress-induced apoptosis (66).

Prolonged GH Treatment Regulates Expression of Genes Associated with Immune Responses—A variety of genes associated with immune responses are regulated by GH at 48 h, including several that have potential roles in regulation of insulin signaling (supplemental Table S3D). GH increases expression of genes for four MHC class-I molecules. Expression of the genes encoding LMP7 and TAP1, shown to be necessary for cell surface expression of MHC class I molecules (43, 44), also increased in response to prolonged GH treatment. Among their non-immune functions, individual members of the MHC Class I are reported to interact with and enhance the function of insulin receptor (69, 70).

GH increases expression of the gene that encodes PI3K-p85α >600% at 4 h and >200% at 48 h. PI3K-p85α, in addition to its roles in immune function, has also been linked to regulation of insulin sensitivity. Mice with disruption of the gene encoding PI3K-p85α show improved insulin sensitivity (71, 72). In mice chronically overexpressing human placental GH, increased levels of PI3K-p85α in muscle coincided with insulin resistance. It was suggested that excess p85α monomer might cause insulin resistance by competing in a dominant negative fashion with the insulin-activated p85-p110 heterodimer for binding to insulin receptor substrate-1 protein, leading to decreased insulin receptor substrate-1-associated PI3K activity (73).

GH causes a prolonged increase in expression of genes encoding the related chemokines CCL6 (MRP-1) and CCL9 (MRP-2), both powerful attractants for immune cells, including macrophages (74, 75). Because macrophage accumulation in adipose tissue has been observed in obesity and has been proposed to contribute to obesity-induced metabolic disorders (76, 77), it is intriguing that GH increases expression in adipocytes of genes for macrophage chemoattractants. The chemokine CCL2 can decrease insulin-stimulated glucose uptake in adipocytes (78). Growing evidence of association between adipocytes, inflammatory responses, and development of insulin resistance suggests that these chemokines and other regulators of immune function whose genes are regulated by GH may also be involved in development of insulin resistance or other responses of adipocytes to GH (79).

GH-regulated Genes Correspond in Adipocytes and in Vivo—The relevance of the present observations in GH-treated 3T3-F442A adipocytes is supported by similarities with genes profiled in GH-treated animals. In microarray data from rat adipose tissue, chronic treatment with GH led to a decrease of >30% in expression of the genes for fatty acid synthase and stearoyl-CoA desaturase 1 (80), which are also decreased in GH-treated 3T3-F442A adipocytes. In adipose tissue from mice that became insulin-resistant after 8 weeks on a high fat diet, gene transcripts for interferon γ-induced GTPase (expressed sequence tag AW111922) and chemokine CCL6 (C10) (81) increased >300%. These two genes were also among those with the largest increases in response to GH treatment after 48 h in 3T3-F442A adipocytes (data not shown), further suggesting their possible role in GH-induced insulin resistance. Further, in rats made insulin-resistant by chronic tumor necrosis factor-α infusion, adipose tissue showed increased expression of genes for glycogenin, acyl-CoA synthase 5, and GM2 activator protein, and decreased expression of fatty acid synthase (82). These genes were also changed in GH-treated adipocytes in the present study. The data presented here also correspond with microarray studies of GH-induced hepatic gene expression in transgenic knock-in mouse models with deletions of specific domains of the GHR (83). Changes in genes involved in lipid and cholesterol metabolism as well as inflammation were observed in those as well as the present studies. Expression of *Bcl6*, observed in the present microarray results to have the largest decrease in response to 48 h GH, is increased in GHR −/− mice compared with WT GHR mice. Caution must be used in directly comparing data from different microarray platforms, from different species, and from homogeneous cell lines *versus* heterogeneous cell populations in tissues. Nevertheless, similarities among of GH-regulated genes in this analysis of adipocytes *in vitro* and analysis of insulin-resistant or GH-treated animals *in vivo* suggest that their products participate in GH action.

The present discussion has focused on categories of biological function in which GH-responsive genes are statistically overrepresented, providing an unbiased, systematic approach to analyze responses of adipocytes to GH. Products of other GH-responsive genes that are not members of the categories discussed likely also contribute to GH action in adipocytes. Some genes with large changes in response to GH are in GO categories that are not statistically overrepresented among GH-responsive genes but individually might underlie adipocyte responses to GH. Other GH-regulated genes only recently cloned have not yet been assigned biological functions. GH-induced changes in gene transcript levels may reflect not only regulation by GH of transcription but also regulation of RNA half-life. Further, GH activates multiple signaling cascades, which can regulate enzyme or transcription factor activity through post-translational modifications (4). Coordinated GH-induced changes in gene transcript levels are likely only one component of the mechanism of GH action in adipocytes. Using microarrays to identify these coordinated GH-induced changes in this and other GH-responsive systems can provide important insights into GH-regulated physiological events (84).

Temporal Pattern of GH-induced Gene Expression Leads to the Identification of a Novel Transcriptional Regulatory Mechanism for Atf3—The genes encoding ATF3 and GADD45γ were found by hierarchical clustering of microarray data to share a similar pattern of response to GH: increased expression at all three time points with greatest expression observed at 4 h in these experiments. ATF3 is of particular interest because of potential involvement in regulation of glucose homeostasis and stress-induced β-cell apoptosis (14, 66). The similarity in pattern led to their comparison for analysis of GH-regulated transcriptional regulatory mechanisms. By examining conserved DNA sequences among human, mouse, and rat orthologs of the *Atf3* and *Gadd45γ* promoters, a potential C/EBP site in the *Atf3* promoter was predicted. Assays of C/EBPβ binding and promoter activity demonstrated that the predicted *Atf3* C/EBP site binds C/EBPβ *in vitro* and *in vivo* and mediates the stimulation of *Atf3* promoter activity by C/EBPβ.

The importance of the C/EBP site for the ability of GH to activate the fragment of the *Atf3* promoter tested is demonstrated by the complete loss of GH response when the C/EBP site in the *Atf3* promoter is

mutated such that the site no longer binds C/EBPβ. Disruption of the *Atf3* C/EBP site also leads to a decrease in basal promoter activity, suggesting that the predicted *Atf3* C/EBP site is important for basal *Atf3* promoter activity. However, even with a disrupted C/EBP site, the *Atf3* promoter is stimulated by serum while the promoterless pXP2 vector is not (data not shown). This suggests loss of GH response by the mutated *Atf3* promoter is not due to an overall disruption of promoter activity but reflects a specific role of the *Atf3* C/EBP site in responsiveness of the *Atf3* promoter to GH. Further experiments will determine whether GH regulates C/EBPβ occupancy on the *Atf3* C/EBP site and how C/EBPβ mediates *Atf3* transcription in response to GH. Because multiple phosphorylation states of C/EBPβ appear to modulate its ability to mediate *Fos* transcription in response to GH (23, 24), it will be of interest to determine whether similar mechanisms contribute to regulation of *Atf3* transcription by GH.

Acknowledgments—QT-PCR was performed with advice from D. Vanheyningen and Dr. R. Mortensen. We thank D. Rhodes and Dr. G. Piwien-Pilipuk for consultations on experimental design and Dr. O. A. MacDougald, Dr. L. Argetsinger, and D. Rhodes for comments on the manuscript.

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