Insulin Inhibits Hepatocellular Glucose Production by Utilizing Liver-enriched Transcriptional Inhibitory Protein to Disrupt the Association of CREB-binding Protein and RNA Polymerase II with the Phosphoenolpyruvate Carboxykinase Gene Promoter*

Hormones regulate glucose homeostasis, in part, by controlling the expression of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK). Insulin and glucocorticoids reciprocally regulate PEPCK expression primarily at the level of gene transcription. We demonstrate here that glucocorticoids promote, whereas insulin disrupts, the association of CREB-binding protein (CBP) and RNA polymerase II with the hepatic PEPCK gene promoter in vivo. We also show that accessory factors, such as CCAAT/enhancer-binding protein β (C/EBPβ), can recruit CBP to drive transcription. Insulin increases protein levels of liver-enriched transcriptional inhibitory protein (LIP), an inhibitory form of C/EBPα, in a phosphatidylinositol 3-kinase-dependent manner. LIP concomitantly replaces liver-enriched transcriptional activator protein on the PEPCK gene promoter, which can abrogate the recruitment of CBP and polymerase II, culminating in the repression of PEPCK expression and the attenuation of hepatocellular glucose production.

Glucose homeostasis is maintained, in part, by the hormonal regulation of hepatic gluconeogenesis. In response to hypoglycemia, glucagon-induced elevations in cAMP, as well as glucocorticoids, induce glucose production in the liver by increasing the expression of genes encoding hepatic enzymes that drive gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK). Conversely, in response to rising blood glucose levels, insulin rapidly inhibits PEPCK gene expression in a dominant fashion. Thus, tight control over hepatic gluconeogenesis is maintained by the opposing actions of hormones in response to constantly fluctuating plasma glucose concentrations.

These hormones regulate PEPCK expression primarily at the level of gene transcription. Meticulous study of the PEPCK gene promoter has revealed an overlapping set of hormone response elements, each one consisting of a specific array of hormone response elements. Glucocorticoids, for example, induce PEPCK gene transcription through the glucocorticoid response unit, which is comprised of two glucocorticoid receptor binding sites (GR1 and GR2), three glucocorticoid accessory factor binding sites (gAF1, gAF2, and gAF3), and a cAMP response element (CRE) (1–3). Hepatic nuclear factor 4α (HNF4α) and chicken ovalbumin upstream promoter transcription factor bind to gAF1, hepatic nuclear factor 3β (HNF3β) binds to gAF2, chicken ovalbumin upstream promoter transcription factor binds to gAF3, and CCAAT/enhancer-binding protein β (C/EBPβ) binds to the CRE to mediate the glucocorticoid response (2, 4, 5). Likewise, cAMP signaling increases PEPCK gene transcription through the cAMP response unit, which is comprised of a CRE, two C/EBP binding sites (P3I and P4), and an AP-1 binding site (P3II) (6).

In contrast, the mechanism by which insulin dominantly represses PEPCK gene transcription has not been fully elucidated. Insulin signals through PI3K to inhibit PEPCK expression (7). Also, an insulin response sequence on the PEPCK gene promoter, which coincides with the location of gAF2, has been identified (8). However, deletion of this site in stably transfected cell lines blocks only part of the ability of insulin to repress PEPCK expression, suggesting that there might be a more proximal insulin response sequence through which insulin could act (9). Extensive analysis of the promoter has yet to reveal such a site. Alternatively, insulin might negatively regulate PEPCK gene transcription by disrupting the interactions of proteins involved in the communication among transcription factors, coactivators, and the general transcription machinery. To explore this possibility, we tested the effect of insulin on the assembly of transcription factors, coactivators, and the general transcription machinery on the endogenous PEPCK gene promoter. Our results demonstrate that glucocorticoids and cAMP increase, whereas insulin markedly decreases, the occupancy of hepatic nuclear factor; LAP, liver-enriched transcriptional activation protein; LIP, liver-enriched transcriptional inhibitory protein; LUC, luciferase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; pol II, polymerase II; RSV, Rous sarcoma virus.
CREB-binding protein (CBP) and RNA polymerase II (pol II) on
the PECPK gene promoter in vivo. In addition, the accessory
effect of C/EBPβ can help to recruit CBP to drive transcription.
Our results also show that insulin increases the protein levels of
LIP, a natural inhibitory form of C/EBPβ, in a PI3K-dependent
manner. Remarkably, insulin causes the concomitant, spe-
cific dissociation of LAP, the activating form of C/EBP
binding to the promoter disrupt the recruitment of CBP and pol
II in response to activating hormones, resulting in the inhibi-
tion of PECK expression and the reduction of hepatocellular
glucose production.

**Materials and Methods**

**Adenovirus Construction**—The Ad-LIP recombinant adenovirus
was prepared by excising the LIP cDNA from the REC-LIP plasmid by
digestion with HindIII and EcoRV. The excised LIP cDNA was then ligated into the HindIII/EcoRV sites in the AdTrack-CMV plasmid,
which was subsequently transfected to the reconstructed embryonic adenovirus as described (10). The recombinant Ad-LIP virus was purified by freeze-
extraction and cesium banding as described previously (11).

**Antibodies**—To generate the LAP-specific 2952 antibody, a 19-amino
cysteine peptide from the carboxyl terminus of rat C/EBPβ encompassing
amino acids 278–296 was synthesized by Bio-Synthesis, Inc. as a key-
hole limpet hemocyanin conjugate. This material was then sent to East
Acres Biologicals where antibodies were raised in rabbits according to
the company’s recommended protocol. Antiserum from production bleeds
of one rabbit (rabbit 2952) was incubated with purified, bacteri-
ally expressed rat p34-C/EBPβ (LAP) that had been immobilized on
Immobilon-P filter strips overnight at 4 °C. The antigen-antibody-con-
taining strips were washed at room temperature four times in Tris-
buffered saline plus 0.05% Tween 20 (TBS-T), once with TBS-T con-
bran (1% bovine) at 37
C, 5% CO2. For hormone treatments, cells were incubated
in 0.2M glycine, buffered saline plus 0.05% Tween 20 (TBS-T), once with TBS-T con-
bovine) at 37
C. To reverse cross-linking and digest
DNA present in the samples, NaCl (200 m M final concentration) and
RNase mixture (Ambion) were added, and the samples were incubated
for 10 min at 37
C. For hormone treatments, cells were incubated
in 0.2M glycine, buffered saline plus 0.05% Tween 20 (TBS-T), once with TBS-T con-
bovine) at 37
C. To reverse cross-linking and digest
DNA present in the samples, NaCl (200 m M final concentration) and
RNase mixture (Ambion) were added, and the samples were incubated
at 65 °C for ≥ 6 h. To digest proteins, samples were incubated at 45 °C
for 90 min after the addition of the following at their final concentra-
tions: 10 mM EDTA, 40 mM Tris-HCl pH 6.5, and 50 µg/ml protease K.

**Chromatin Immunoprecipitation Assay**—The ChIP assay protocol
was adapted from methods described previously (13–16). H4IE cells
(1 X 106 cells/condition) were washed with phosphate-buffered saline and
preincubated in serum-free Dulbecco’s modified Eagle’s medium at
37
C for 48 h. After hormone treatments, cells were cross-linked with
1% formaldehyde (Fishers Scientific) in serum-free Dulbecco’s modified
Eagle’s medium at 37 °C for 5 min. To arrest cross-linking, glycine was
added directly to the medium at a final concentration of 125 mM, and
the cells were rinsed with ice-cold phosphate-buffered saline. Cells were
harvested with cell scraping buffer (1 ml/plate of ice-cold phosphate-
buffered saline with protease and phosphatase inhibitors: 1 mM phen-
ethylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, 20 mM NaCl, 1 mM Na3VO4, 10 mM Na3P04, pH 8, 0.4 mM Na2Mo04, 125 mM okadaic acid). Cells were then pelleted by cen-
trifugation at 700 × g for 4 min at 4 °C, resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, with protease and phos-
phatase inhibitors), and incubated on ice for 10 min. The lysates were
preincubated in serum-free Dulbecco’s modified Eagle’s medium at
37
C for 1 h on a rotator, after which the beads were pelleted. The supernatant was transferred to a new tube. Chromatin complexes were immunoprecipitated for 12–18 h at
4 °C while rotating with amounts (5–10 µg) of primary antibody opti-
mized for selective immunoprecipitation of signal, or without antibody
(control) to provide negative controls. Immune complexes were collected
with 40 µl of protein A/G–agarose (100 µg/ml, Santa Cruz) supplemented with 100 µg/ml sonicated salmon sperm DNA, while rotating for 3 h at 4 °C, followed by centrifugation at 1,000 × g for
1 min at 4 °C. Secondary goat IgM anti-mouse IgG (Santa Cruz) anti-
body was added to samples immunoprecipitated with BABCO mouse asciites fluid. The beads were washed for 5 min at 4 °C with 1 ml of each of the
following buffers in succession: low salt wash buffer (0.1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, 20 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 200 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA pH 8, 10 mM Tris-HCl pH 8), and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Chromatin complexes were eluted from the beads in three consecutive 30-min rotating incubations with 200 µl of elution buffer
(10 mM NaHCO3, 1 M NaCl, 1% SDS) at 65 °C. To reverse cross-linking
RNA present in the samples, NaCl (200 mM final concentration) and
RNase mixture (Ambion) were added, and the samples were incubated
at 65 °C for ≥ 6 h. To digest proteins, samples were incubated at 45 °C
for 90 min after the addition of the following at their final concentra-
tions: 10 mM EDTA, 40 mM Tris-HCl pH 6.5, and 50 µg/ml protease K.

**Glucose Production Assay**—Glucose production from H4IE cells
treated with hormones for 8 h was measured essentially as described
by Wang et al. (12) using a glucose assay kit (Sigma 510-A). The glucose
production buffer was modified slightly to include glucose-free Dulbec-
co’s modified Eagle’s medium containing 20 mM sodium lactate, 1 mM
sodium pyruvate, and 15 mM HEPES, pH 7.4, without phenol red.

**Primer Extension Analysis**—RNA was isolated using TRI Reagent
(Molecular Research Center), as detailed in the manufacturer’s proto-
col. The oligonucleotides PC28 and ACT25, designed to be comple-
mlementary to positions 102–129 and 42–67 in the rat PECPK and β-actin
mRNAs, respectively (7), were radiolabeled with γ–32P]ATP (Amer-
sham Biosciences) before use in primer extension assays. To measure
the accumulation of PECPK and β-actin mRNA, primer extension analy-
sis was performed as described by Forest et al. (9).
Assayed by primer extension of RNA preparations from cells treated for least three independent experiments. Glucocorticoids and cAMP stimulate, whereas insulin represses, glucose production and PEPCK gene expression. A glucose production was measured by incubating hepatoma cells in glucose-free medium supplemented with lactate and pyruvate, after 8-h hormone treatments: no hormone (N), 500 nM dexamethasone (D), 100 nM 8-CPT-cAMP + 500 nM dexamethasone (CD), and 500 nM dexamethasone + 10 nM insulin (DI). The bars depict the mean ± S.E. of at least three independent experiments. B, PEPCK mRNA levels were assayed by primer extension of RNA preparations from cells treated for 2 h under the above hormone conditions. β-Actin mRNA levels were assayed for internal controls.

**RESULTS**

**Hormonal Regulation of Glucose Production and PEPCK Gene Expression**—The production of glucose from gluconeogenic precursors in H4IE hepatoma cells, subjected to the indicated hormone treatments (Fig. 1A), was assayed by incubating the cells in glucose-free medium and measuring the amount of glucose released into the medium. The synthetic glucocorticoid, dexamethasone, stimulates glucose production more than 5-fold over basal conditions. Additionally, the cAMP analog, 8-CPT-cAMP, along with dexamethasone, is able to induce glucose production -8-fold over basal conditions. In contrast, when the cells are treated with dexamethasone and insulin together, glucose production drops precipitously to levels below that observed in basal conditions. Thus, hormones are able to regulate glucose production from non-carbohydrate precursors, lactate and pyruvate, in these hepatoma cells in a manner that reflects normal physiology.

Plasmid Construction, Transient Transfection, and Luciferase Assay—To construct the PEPCK-luciferase (PEPCK-LUC) reporter plasmid, the PEPCK gene promoter (−467 to +65) was excised from pPL32-CAT (18) with HindIII and BglII (New England Biolabs), blunt end digested with exonuclease, and cloned into the Smal site of pGL3-Basic (Promega). To make C/U-PEPCK-LUC, an A/III/BglII fragment was excised from pAα-GAL4 (19) and inserted into the MluI/BglII sites of the pGL3 vector. Transfections were performed using the calcium phosphate precipitation method described by Hall et al. (20). Cells were transfected, as indicated, with 10 μg of a reporter plasmid (PEPCK-LUC or C/U-PEPCK-LUC), 0.1 μg of pRL-SV40 (Promega), 5 μg of RSV-GR, 5 μg of RSV-PAK (or RSV-neo for plates not treated with 8-CPT-cAMP), and 2 μg of a GALA construct (GALA DBD, GALA-C/EBPβ (118), or GALA-CBP) to provide an equal amount of total plasmid DNA/plate. GALA-C/EBPβ (118) contains amino acids 1–118 of C/EBPβ, whereas GALA-CBP contains full-length CBP. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega).

Western Blot Analysis—H4IE cell nuclear extracts were prepared as described by Waltner-Law et al. (21), subjected to electrophoresis through a 12% acrylamide gel, and transferred to a 0.45-μm nitrocellulose membrane using a Mini-PROTEAN II apparatus (Bio-Rad). After thorough washing, the membrane was exposed first to primary antibody and then to alkaline phosphatase-conjugated secondary antibody. Protein bands were resolved by chemiluminescence using the Western Breeze immunodetection system (Novex). Individual bands were analyzed with a Fluor-S MultiImager using Quantity One software.

**In Vivo Assembly and Disassembly of Factors of the PEPCK Gene Promoter in Response to Hormones**—The ChIP assay was used to determine whether the endogenous PEPCK gene promoter is occupied by a variety of factors under the above hormone conditions. Antibodies specific for pol II, CBP, GR, HNF4α, HNF3β, C/EBPβ, C/EBPα, acetylated histone H3, and acetylated histone H4 were used to immunoprecipitate chromatin fragments (Fig. 2A). PCR amplification was conducted using primer pairs specific for the proximal, regulatory region (1) and a distal, nonregulatory region (2) of the PEPCK gene promoter. The latter primer set was included as a control to ensure that the signals observed were specific for the more proximal, regulatory region of the PEPCK gene promoter. Each input sample was examined to ensure efficient chromatin shearing (Fig. 2G), yielding fragment sizes in the range of 100–600 bp. The sensitivity range of the assay was assessed in parallel PCRs using increasing amounts of a control template (Fig. 2F). For some factors (e.g., pol II), we tested the ability of different antibodies to immunoprecipitate chromatin fragments and found results similar to those presented, thereby diminishing the possibility that the disappearance of a particular band might be the result of epitope masking during hormone treatment and cross-linking (data not shown).

Upon stimulation with either glucocorticoids alone or glucocorticoids and cAMP together, there is an increased association of pol II, CBP, and GR with the PEPCK gene promoter, presumably to drive transcription. In contrast, insulin markedly diminishes the association of pol II and CBP with the promoter (Fig. 2A). This may be one of the mechanisms through which insulin exerts its inhibitory effect on PEPCK expression and hepatic glucose production. Interestingly, GR occupancy remains constant as long as receptor ligand is present. Thus, insulin does not seem to inhibit PEPCK gene transcription by disrupting the binding of GR to the promoter, although this does not rule out the possibility of insulin-induced post-translational modifications of GR. The association of accessory factors, such as HNF4α, HNF3β, C/EBPβ, and C/EBPα, does not change across the hormone conditions tested, suggesting that these factors are bound constitutively to the PEPCK gene promoter. Likewise, the acetylation state of local histone proteins does not appear to be affected by different hormone treatments. This is consistent with previous work demonstrating the presence of hypersensitive sites and in vivo footprinting patterns in the proximal region of the PEPCK gene promoter which are not altered either by induction of transcription with cAMP or glucocorticoids or by inhibition with insulin (22, 23).

A recent study using synchronized MCF-7 cells showed that activating factors cycle on and off estrogen-responsive promoters on a time scale of 30–60 min to drive transcription (24). To test this model of hormone activation, the ChIP assay was used to examine the assembly of pol II, C/EBPα, and C/EBPβ on the PEPCK gene promoter during a glucocorticoid time course (Fig.
Polymerase II occupancy on the PEPCK gene promoter increases in the presence of dexamethasone but is not altered with prolonged hormone exposure. In addition, the binding of both C/EBP family members does not change over time. Thus, we do not observe a cyclic assembly of factors on the PEPCK gene promoter in response to glucocorticoids.

To contrast the pattern of occupancy found with the PEPCK gene promoter in response to glucocorticoids, cAMP, and insulin, we examined gene 33 and β-actin using PCR primers specific for those gene promoters. Gene 33 was chosen because its expression in H4IIE cells is stimulated by all of the aforementioned hormones, including insulin (25, 26). Consistent with this pattern of regulation, and unlike that of PEPCK, the association of pol II and CBP with the gene 33 promoter is enhanced in the presence of glucocorticoids, cAMP, and also insulin (Fig. 2D). β-Actin was chosen as a control; accordingly, the occupancy of pol II on the β-actin promoter does not significantly change across the different hormone conditions (Fig. 2E). Therefore, insulin does not disrupt the association of pol II at either the gene 33 promoter or the β-actin promoter, as it does at the PEPCK gene promoter.

Role of Accessory Factors and Coactivators in Hormone-regulated Gene Transcription—Accessory factors play important roles in the hormonal regulation of PEPCK gene transcription. Indeed, they are absolutely necessary for a response to glucocorticoid stimulation. Mutation or deletion of any one of the glucocorticoid-inducible accessory elements leads to the loss of approximately half of the glucocorticoid induction, whereas mutation of any pair of ac-
cessory elements abolishes the response altogether (2, 3, 27). But because their binding does not change in response to hormones, how might they help to mediate hormone-induced gene transcription? One possible role might be to assemble a platform upon which coactivators and the preinitiation complex may be recruited to drive transcription. Among the array of accessory elements on the PEPCK gene promoter, we chose to examine further the CRE because it is a pleiotropic element important for several aspects of PEPCK gene regulation, including basal, cAMP-, and glucocorticoid-induced gene transcription (28–30). Furthermore, we know from previous work that C/EBPβ functions well at this element to help mediate a glucocorticoid response (19).

To test whether C/EBPβ can serve as an accessory factor in PEPCK gene regulation under different hormone conditions when tethered to the CRE, H4IIE hepatoma cells were transiently transfected with either a wild-type PEPCK gene promoter-luciferase reporter construct (PEPCK-LUC) or a mutant construct in which the CRE is replaced by an upstream activating sequence to which cotransfected, chimeric GAL4 proteins bind (C/U-PEPCK-LUC) (Fig. 3A). After transfection, the cells were treated with hormones and harvested for luciferase assays. The wild-type PEPCK gene promoter construct exhibits a strong 16-fold induction by glucocorticoids and a 28-fold induction by glucocorticoids and cAMP together. Insulin suppresses the glucocorticoid induction by 77%. These results reflect the hormonal regulation patterns observed with endogenous PEPCK mRNA levels and hepatocellular glucose production (compare with Fig. 1). When GAL4-C/EBPβ is cotransfected with C/U-PEPCK-LUC, the magnitude of reporter gene expression is very similar that of the wild-type construct. There is a 17-fold induction by glucocorticoids and a 25-fold induction by glucocorticoids and cAMP together when C/EBPβ is tethered to the PEPCK CRE. Likewise, insulin suppresses glucocorticoid induction by 80%. However, when only the GAL4 DBD is cotransfected with C/U-PEPCK-LUC, the magnitude of hormone-induced reporter expression drops by about 50%. Similar results are observed when the empty parent vector is used for cotransfection (data not shown). This is consistent with previous studies in which approximately half of the basal, glucocorticoid, and cAMP induction is lost when the CRE is deleted or mutated (28, 30).

C/EBPβ and p300 interact both in vitro and in vivo, a functional collaboration that results in enhanced gene transcription (31). The region of C/EBPβ which interacts with p300 coincides with both its amino-terminal transactivation domain and an adjacent accessory factor domain, essential for glucocorticoid induction of PEPCK gene expression (19). To determine whether C/EBPβ can help to recruit the coactivator CBP (closely related to p300) to the PEPCK gene promoter, transient transfection assays similar to those described above were performed with the addition of an expression plasmid for CBP (Fig. 3B). For direct comparison, the horizontal line inside each bar denotes the reporter strength exhibited in the absence of exogenous CBP (results from Fig. 3A). The presence of exogenous CBP generally induces reporter expression a further 2-fold. Once again, the magnitude of the hormone responses when C/EBPβ, but not the GAL4 DBD, is tethered to the CRE mimics that of the wild-type reporter construct. Thus, C/EBPβ is able to function as an accessory factor not only to help bring about these various hormone responses from the PEPCK CRE, but also to help recruit CBP to the promoter to induce these responses further.

If indeed one of the roles of accessory factors is to recruit coactivators to the promoter, then it should be possible to circumvent this task by tethering coactivators directly to the promoter using the GAL4 fusion system. To test this hypothesis, GAL4-CBP was transfected into cells along with the C/U-PEPCK-LUC construct (Fig. 3B, far right). Remarkably, basal transcription increases 4-fold over that seen with the wild-type construct. Thus, when tethered directly to the CRE, CBP can induce hormone-driven gene transcription, thereby lending credence to the idea that accessory factors function, at least in part, to help recruit coactivators to the promoter. On top of that, glucocorticoid induction rises to 22-fold over basal, and glucocorticoid/cAMP induction is 25-fold over basal. This suggests that hormones affect not only the recruitment of coactivators to gene promoters but also their ability to interact with other proteins to affect transcription. In fact, when we tether the coactivator SRC-1 (via the GAL4 DBD) to other elements within the glucocorticoid response unit, we observe a similar induction with the addition of dexamethasone (32).

The ChIP assay results (Fig. 2A) demonstrate that insulin
blocks the association of CBP with the endogenous PEPCK gene promoter. The transfection assay results (Fig. 3B) show that accessory factors, such as C/EBPβ, can act to help recruit CBP to the PEPCK gene promoter and thereby drive transcription. Together, this would suggest a potential mechanism: perhaps insulin inhibits PEPCK gene transcription, at least in part, by somehow disrupting the ability of accessory factors to recruit coactivators to the promoter.

**Effect of Insulin on LIP—**In H4IIE cells, C/EBPβ can be found in two predominant forms: p34-LAP, liver-enriched transcriptional activator protein, and p20-LIP, liver-enriched transcriptional inhibitory protein. Synthesized from an internal translation initiation site (33), LIP has the same amino acid sequence as the corresponding region of LAP. Hence, LIP contains the carboxyl-terminal dimerization and DNA binding domains but lacks the amino-terminal activation domain of LAP, and it is thought to function as a natural negative regulator of transcription. Together, this would suggest a potential mechanism: per-...

**Exchange of LAP by LIP in Response to Insulin—**An increased LIP:LAP ratio could provide a mechanism for insulin to disrupt LAP function. Prior work has shown that LIP can compete successfully with LAP for binding to DNA recognition sequences at substoichiometric levels (34). Perhaps by increasing the amount of available LAP protein, insulin causes the displacement of LAP from the PEPCK gene promoter, thereby disabling its accessory factor function. To test this hypothesis, we examined the association of LAP with the PEPCK gene promoter in response to insulin. To distinguish between the actions of endogenous LIP and LAP, something that has heretofore not been possible, we developed a unique antibody (2952) that recognizes only LAP. The peptide used to generate the LIP-specific 2952 antibody contains a 19-amino acid sequence that is shared by both LIP and LAP. However, the antibody recognizes only a specific conformation of this epitope, one that is found in LAP, but not LIP, a fortuitous and very useful happenstance. The Western blot results show that in contrast to the sc-150 antibody, which recognizes both forms of C/EBPβ, the 2952 antibody recognizes LAP specifically and does not recognize LIP (Fig. 5A). As before (see Fig. 2A), the ChIP assay results show that CBP and pol II are recruited to the promoter upon glucocorticoid induction and are not associated with the promoter in the presence of insulin, this time at 30 min (Fig. 5B). The dissociation of pol II from the promoter with the insulin time course starkly contrasts the recruitment of pol II observed with a glucocorticoid time course (compare with Fig. 2B). Use of the sc-150 antibody shows that C/EBPβ is present at the promoter regardless of hormone treatment; however, this result does not specify which form of C/EBPβ is associated under these different hormone conditions. In contrast, use of the 2952 antibody demonstrates that LAP binding is disrupted *in vivo* with insulin treatment. Importantly, insulin-induced dissociation of pol II, CBP, and LAP is reversed in cells treated with the PI3K inhibitor LY 294002 (Fig. 5C). Taken together, these results clearly demonstrate that LIP replaces LAP at the endogenous PEPCK gene promoter in response to insulin and that this process depends on an intact PI3K signaling pathway. Unfortunately, a LIP-specific antibody is currently not available, so we are unable to perform the converse experiment.

**Effects of LIP on Recruitment of Activators, PEPCK Gene Expression, and Glucose Production—**To test whether LIP can disrupt the ability of hormones to recruit coactivators, activate gene transcription, and drive hepatocellular glucose production, an adenovirus construct was created to express T7-tagged LIP. Applying $4 \times 10^{10}$ optical particle units of either Ad-GFP or Ad-LIP virus to each plate of H4IIE cells resulted in the successful infection of more than 90% of the cell population as indicated by GFP expression (Fig. 6A). Cells infected with the Ad-LIP virus exhibited dose-dependent expression of the T7-tagged LIP construct (p24-T7-LIP is the p20-LIP protein with the addition of a 4-kDa T7 tag) as measured by Western blot...
Insulin Disrupts Recruitment of CBP and pol II

The data presented in this study demonstrate that at least one of the functions of hormone response unit accessory factors is to recruit coactivators to the promoter to drive transcription in response to hormones. Our results offer a model by which glucocorticoids induce, whereas insulin inhibits, PEPCK gene transcription (Fig. 7). In this model, the accessory factors bind constitutively to the promoter and assemble a platform that can serve to recruit activating factors in response to hormonal signals. Upon stimulation by glucocorticoids (dexamethasone), several factors assemble on the promoter to drive transcription, including GR, CBP, and the pol II holoenzyme. When glucocorticoids and the dominant inhibitor insulin are present simultaneously, the occupancy of CBP and pol II is lost, even though ligand-bound GR remains associated with the promoter.

In addition, our results show that insulin not only increases the protein levels of LIP, but also that it does so in a manner that relies on an intact PI3K signaling pathway. This correlates well with previous work in which insulin was shown to repress PEPCK gene expression in a PI3K-dependent manner (7). Furthermore, this increase in LIP leads to the concomitant recruitment of CBP and pol II by glucocorticoids, which culminates in the inhibition of PEPCK gene expression and hepatocellular glucose production.

Over the past few years, several different groups, including ours, have proposed a variety of putative insulin response factors, many of which are able to mimic the hormonal affects on PEPCK gene expression (20, 21, 35-38). To date, however, none affords the global regulation so characteristic of the ability of insulin to repress PEPCK gene transcription dominantly, along with a defined mechanism of action specific to a particular region of the PEPCK gene promoter. Others have recently alluded to, though not proven, a role for C/EBPβ in repressing PEPCK expression by insulin (39). In fact, we cannot claim C/EBPβ to be the one and only factor that acutely inhibits PEPCK gene transcription in response to insulin because new protein synthesis is not necessary for the immediate actions of insulin (40). Nevertheless, our results reveal a novel mechanism by which insulin does act, that is, by raising the protein...
levels of LIP (a natural inhibitory transcription factor) to compete away LAP (its activating counterpart) and thereby abrogating the ability of LAP to support the assembly of a CBP-pol II complex on the promoter. Loss-of-function experiments would be decisive, but these are not yet possible. To disrupt the function of LIP specifically, transgenic animals would need to be designed in such a way as to substitute the internal methionine, which serves as an alternative translation initiation site (33), with a carefully chosen amino acid. Additionally, a cell line would need to be established from such animals to conduct experiments in which glucose and counterregulatory hormones, all of which also affect PEPCK gene transcription, could be carefully controlled. Unfortunately, there is no guarantee that such a mutation would not alter the function of LAP; and, even if such a design were fruitful, LAP conversion to LIP by a post-translational processing enzyme has been proposed as an alternative mechanism for LIP expression (41, 42). When a LIP-specific antibody becomes available, it would be interesting to continue this work by testing the association of LIP itself with the PEPCK gene promoter in vivo.

**Fig. 6.** LIP abrogates the recruitment of CBP and pol II, represses PEPCK gene expression, and attenuates hepatocellular glucose production. A, the efficiency with which the adenovirus constructs (Ad-LIP or Ad-GFP) infected H4IIE cells was determined by measuring GFP expression levels. B, expression of p24-T7-LIP was confirmed by Western blot (using C/EBPβ antibody sc150) of nuclear extracts from cells infected with increasing amounts of the Ad-LIP virus. C, in vivo association of T7-LIP, pol II, CBP, LAP, GR, and C/EBPβ with the PEPCK gene promoter was measured using the ChIP assay on cells infected with Ad-LIP and treated with the following hormones: no hormone (N), 500 nM dexamethasone (D), 100 μM 8-CPT-cAMP + 500 nM dexamethasone (CD), and 500 nM dexamethasone + 10 nM insulin (DI). All results shown are representative of at least three independent experiments. D, PEPCK gene expression levels were measured by primer extension of RNA preparations from cells treated with dexamethasone (Dex) and infected with increasing amounts of Ad-GFP or Ad-LIP. β-Actin mRNA levels were measured for internal controls. E, glucose production was measured by incubating cells with glucose-free medium supplemented with lactate and pyruvate after dexamethasone treatment and adenovirus infection. The bars depict the mean ± S.E. of at least three individual experiments.

**Fig. 7.** Glucocorticoids promote, whereas insulin disrupts, the assembly of a transcriptionally active PEPCK gene promoter. Across different hormonal environments, accessory factors bind to the endogenous PEPCK gene promoter. C/EBPβ is shown in green, whereas other factors that bind constitutively to the PEPCK gene promoter are shown in gray. Basal transcription is maintained by weak or transient recruitment of the pol II holoenzyme. Glucocorticoids induce transcription by recruiting GR, CBP, and pol II to the promoter. Insulin represses transcription by promoting the replacement of LAP (ovals) with LIP (half-ovals) on the PEPCK gene promoter, thereby disrupting the recruitment of CBP and pol II. Meanwhile, GR remains bound in the presence of insulin as long as glucocorticoids are present. GR is shown in orange, CBP is shown in yellow, and the pol II holoenzyme is shown in blue.
Insulin is a central regulator of metabolism, opposing the effects of many different hormones (e.g., glucagon, glucocorticoids, retinoic acid, thyroid hormone) that can induce the expression of gluconeogenic enzymes, such as PEPCK. Each of the activating hormones up-regulates PEPCK gene transcription through separate, albeit overlapping, hormone response units assembled on the promoter. Therefore, insulin must possess either the capacity to counteract each hormone response (and basal transcription) individually through different means or the ability to disrupt a common mechanism of gene transcription, shared by all activating signals. The results from this study support the latter and provide evidence to suggest that insulin inhibits gene transcription, at least in part, by disturbing the interactions between promoter-bound transcription factors and the coactivator-general transcription machinery complex recruited to drive transcription. This novel mechanism could account for the action of insulin at the PEPCK gene promoter and other genes involved in the control of glucose homeostasis.

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REFERENCES

1. Sugiyama, T., Scott, D. K., Wang, J. C., and Granner, D. K. (1998) Mol. Endocrinol. 12, 1487–1498
2. Scott, D. K., Mitchell, J. A., and Granner, D. K. (1996) J. Biol. Chem. 271, 31909–31914
3. Imai, E., Stromstedt, P. E., Quinn, P. G., Carlstedt-Duke, J., Gustafsson, J. A., and Granner, D. K. (1999) Mol. Cell. Biol. 19, 4712–4719
4. Wang, J. C., Stromstedt, P. E., O'Brien, R. M., and Granner, D. K. (1996) Mol. Endocrinol. 10, 794–800
5. Hall, R. K., Sladek, F. M., and Granner, D. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 412–416
6. Rosier, W. J. (2000) Mol. Cell. Endocrinol. 162, 1–7
7. Sutherland, C., O'Brien, R. M., and Granner, D. K. (1995) J. Biol. Chem. 270, 15501–15506
8. O'Brien, R. M., Lucas, P. C., Forest, C. D., Magnuson, M. A., and Granner, D. K. (1990) Science 249, 533–537
9. Forest, C. D., O'Brien, R. M., Lucas, P. C., Magnuson, M. A., and Granner, D. K. (1990) Mol. Endocrinol. 4, 1302–1310
10. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 95, 2509–2514
11. Nevins, J. R., DeGregori, J., Jakoi, L., and Leone, G. (1997) Methods Enzymol. 283, 205–219
12. Wang, J. C., Stafford, J. M., Scott, D. K., Sutherland, C., and Granner, D. K. (2000) J. Biol. Chem. 275, 14717–14721
13. Nissen, R. M., and Yamamoto, K. R. (2000) Genes Dev. 14, 2314–2329
14. Parekh, B. S., and Maniatis, T. (1999) Mol. Cell 3, 125–129
15. Boyd, K. E., Wells, J., Gutman, J., Bartley, S. M., and Farnham, P. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13887–13892
16. Orlando, V., Strutt, H., and Paro, R. (1997) Methods 11, 205–214
17. Rozen, S., and Skaletsky, H. (1998) www.genome.wi.mit.edu/genome_software/other/primer3.html
18. Petersen, D. D., Magnuson, M. A., and Granner, D. K. (1988) Mol. Cell. Biol. 8, 96–104
19. Yamada, K., Duong, D. T., Scott, D. K., Wang, J. C., and Granner, D. K. (1999) J. Biol. Chem. 274, 5880–5887
20. Hall, R., Yamazaki, T., Rozera, T., Walnt-Law, M., O'Brien, R., and Granner, D. K. (2000) J. Biol. Chem. 275, 30169–30175
21. Walnt-Law, M., Daniels, M. C., Sutherland, C., and Granner, D. K. (2000) J. Biol. Chem. 275, 31847–31856
22. Faber, S., O'Brien, R. M., Imai, E., Granner, D. K., and Chalkley, R. (1993) J. Biol. Chem. 268, 24976–24985
23. Ip, Y. T., Granner, D. K., and Chalkley, R. (1989) Mol. Cell. Biol. 9, 1289–1297
24. Shang, Y., Hu, X., Difrenzo, J., Lazar, M. A., and Brown, M. (2000) Cell 103, 843–852
25. Chu, D. T., Davis, C. M., Chrapkiewicz, N. B., and Granner, D. K. (1988) J. Biol. Chem. 263, 13007–13011
26. Johnson, A. C., Lee, K. L., Isham, K. R., and Kenney, F. T. (1988) J. Cell. Biochem. 37, 249–255
27. Mitchell, J., Noisin, E. L., Suwanichkul, A., Yamasaki, T., Lucas, P. C., and Diasio, R. B. (1994) Mol. Endocrinol. 8, 585–594
28. Imai, E., O'Brien, R. M., Mitchell, J. N., and Granner, D. K. (1999) Mol. Cell. Biol. 19, 14717–14725
29. Liu, J. S., Park, E. A., Gurney, A. L., Roesler, W. J., and Hanson, R. W. (1991) J. Biol. Chem. 266, 19095–19102
30. Quin, G. W., Wong, T. W., Magnuson, M. A., Shabb, J. B., and Granner, D. K. (1988) Mol. Cell. Biol. 8, 3467–3475
31. Mink, S., Haenig, B., and Klempnauer, K. H. (1997) Mol. Cell. Biol. 17, 6609–6617
32. Stafford, J. M., Walnt-Law, M., and Granner, D. K. (2001) J. Biol. Chem. 276, 3811–3819
33. Calkhoven, C. F., Muller, C., and Leutz, A. (2000) Genes Dev. 14, 1920–1932
34. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
35. Chakravarty, K., Leahy, P., Becard, D., Hakimi, P., Foretz, M., Ferre, P., Foufelle, F., and Hanson, R. W. (2001) J. Biol. Chem. 276, 34816–34823
36. Guo, S., Rena, G., Cichy, S., He, X., Cohen, P., and Unterman, T. (1999) J. Biol. Chem. 274, 17164–17172
37. O'Brien, R. M., Noisin, E. L., Suwanichkul, A., Yamazaki, T., Lucas, P. C., Wang, J. C., Powell, D. R., and Granner, D. K. (1995) Mol. Cell. Biol. 15, 1747–1758
38. O'Brien, R. M., Lucas, P. C., Yamazaki, T., Noisin, E. L., and Granner, D. K. (1994) J. Biol. Chem. 269, 30419–30428
39. Ghosh, A. K., Lascor, R., Liu, P., Cichy, S. B., Danilkovich, A., Gou, S., and Unterman, T. G. (2001) J. Biol. Chem. 276, 8507–8515
40. Sasaki, K., Cripe, T. P., Koch, S. R., Andreone, T. L., Petersen, D. B., Beale, E. G., and Granner, D. K. (1984) J. Biol. Chem. 259, 15242–15251
41. Baer, M., and Johnson, P. F. (2000) J. Biol. Chem. 275, 26582–26590
42. Welm, A. L., Timchenko, N. A., and Darlington, G. J. (1999) Mol. Cell. Biol. 19, 1695–1704