Activation of SIRT1 by Resveratrol Represses Transcription of the Gene for the Cytosolic Form of Phosphoenolpyruvate Carboxykinase (GTP) by Deacetylating Hepatic Nuclear Factor 4α[*]

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The SIRT1 activators isonicotinamide (IsoNAM), resveratrol, fisetin, and butein repressed transcription of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (PEPCK-C). An evolutionarily conserved binding site for hepatic nuclear factor (HNF) 4α (−272/−252) was identified, which was required for transcriptional repression of the PEPCK-C gene promoter caused by these compounds. This site contains an overlapping AP-1 binding site and is adjacent to the C/EBP binding element (−248/−234); the latter is necessary for hepatic transcription of *PEPCK-C*. AP-1 competed with HNF4α for binding to this site and also decreased HNF4α stimulation of transcription from the PEPCK-C gene promoter. Chromatin immunoprecipitation experiments demonstrated that HNF4α and AP-1, but not C/EBPβ, reciprocally bound to this site prior to and after treating HepG2 cells with IsoNAM. IsoNAM treatment resulted in deacetylation of HNF4α, which decreased its binding affinity to the PEPCK-C gene promoter. In HNF4α-null Chinese hamster ovary cells, IsoNAM and resveratrol failed to repress transcription from the PEPCK-C gene promoter; overexpression of HNF4α in Chinese hamster ovary cells re-established transcriptional inhibition. Exogenous SIRT1 expression repressed transcription, whereas knockdown of SIRT1 by RNA interference reversed this effect. IsoNAM decreased the level of mRNA for PEPCK-C but had no effect on mRNA for glucose-6-phosphatase in AML12 mouse hepatocytes. We conclude that SIRT1 activation inhibited transcription of the gene for PEPCK-C in part by deacetylation of HNF4α. However, SIRT1 deacetylation of other key regulatory proteins that control PEPCK-C gene transcription also likely contributed to the inhibitory effect.

The cytosolic isoform of phosphoenolpyruvate carboxykinase (GTP) (PEPCK-C)[4] (EC 4.1.1.32) is a key enzyme in gluconeogenesis in the liver and kidney cortex, and glyceroneogenesis in the liver, white and brown adipose tissue, and skeletal muscle (1). Ablation of this gene in adipose tissue caused lipodystrophy in mice, due to an alteration in the rate of glyceroneogenesis in that tissue (2). Conversely, overexpression of the gene for PEPCK-C in adipose tissue resulted in obesity (3) and diabetes when mice were fed a high fat diet (4). PEPCK-C thus plays a critical role in the genesis of Type 2 diabetes by regulating both hepatic glucose output and the rate of triglyceride synthesis and deposition in adipose tissue (5); the aberrant expression of this gene has been proposed to result in diabetes and obesity in humans (6).

Transcription of the gene for PEPCK-C is acutely regulated by hormones, diet, and a number of small effector molecules (7). Insulin represses PEPCK-C gene transcription via a mechanism that involves PGC-1α, FOXO1, and HNF4α (8). In addition, SREBP-1c has also been implicated in the repression of transcription of the gene by insulin (9, 10). Glucocorticoids stimulate transcription of the gene for PEPCK-C in the liver (11, 12) but inhibit this process in adipose tissue (13). Various small molecules also provide potentially valuable tools for controlling PEPCK-C gene transcription and thus in regulating hepatic glucose output. For example, rosiglitasone induces PEPCK-C gene transcription in adipose tissue by activating peroxisome proliferator-activated receptor-γ2 (14), whereas epigallocatechin gallate, an active ingredient of green tea, has a strong inhibitory effect on transcription from the PEPCK-C gene promoter (15). In the current study, the effect of SIRT1 chemical activators,

including IsoNAM and resveratrol, on hepatic PEPCK-C gene transcription has been investigated.

Sir2 (silencing information regulator 2) is critical for survival during calorie restriction in *Saccharomyces cerevisiae* (16) and *Caenorhabditis elegans* (17). SIRT1, the mammalian homologue of Sir2, is a NAD^+-dependent deacetylase (18) and has also been shown to regulate a number of metabolic processes during calorie restriction in mammals (19). SIRT1 deacetylates a variety of proteins, including histones, transcription factors (20), and transcriptional co-regulators (21, 22).

There are conflicting reports of the effect of SITR1 activation on transcription of the gene for PEPCK-C. It has been proposed that an increase in SIRT1 activity stimulates PEPCK-C gene transcription, presumably due to deacetylation of specific transcription factors and co-regulators, such as FOXO1 and PGC-1α. Rodgers *et al.* (22) presented data showing that SIRT1 deacetylates, and thereby activates PGC-1α, which in turn induces transcription of the genes for PEPCK-C and glucose-6-phosphatase. There is also evidence that diabetic rats, in which the mRNA for SIRT1 in the liver was reduced to 25% of control values, have a marked decrease in the level of PEPCK mRNA (23). These and other studies (24) contrast with other reports in the literature (25–27) and with the results of the present study, in which we report that activation of SIRT1 by resveratrol or IsoNAM markedly repressed transcription of the gene for PEPCK-C in HepG2 hepatoma cells and AML12 hepatocytes; it had no effect on the level of mRNA for Glc-6-Pase. The molecular mechanism that is responsible for this effect was investigated.

EXPERIMENTAL PROCEDURES

Materials—IsoNAM, resveratrol, butein, and fisetin were purchased from Sigma and c-Fos, and c-Jun were from Biomol (Plymouth Meeting, PA).

DNA Constructs and Site-directed Mutagenesis—The luciferase reporter construct, p2000-Luc, was generated by subcloning a segment of the rat PEPCK-C gene promoter (−2367/+72) into the pGL3-Basic plasmid (Promega, Madison, WI). Truncations of the gene promoter were created using restriction enzymes to delete segments of the promoter within p2000-Luc. A site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to construct mutants of the PEPCK-C gene promoter by following the manufacturer's procedure. Two HNF4α-binding sites at −272/−252 (HNF4α site-2) and −454/−430 (AF1) were mutated from CAAAG and CTTTG to ACCAT and AGGTT, respectively, using the following primers: 5′-gca tca gca aca gtc acg gtA CCA Ttt tag tca atc aaa cgt tgt gta agg ac-3′ and 5′-tca gag ctg aat tcc ctt ctc atg acA GGT Tgc cgt ggg agt gac acc tca c-3′. Plasmids that overexpress mouse SIRT1 and FLAG-tagged human HNF4α cDNAs were gifts from Dr. Duna Massillon.

Cell Culture, Transfection, Luciferase Assay, and siRNA Knock-down Studies—HepG2 (human) and FTO-2B (rat) hepatoma cells and AML12 (mouse) hepatocytes were cultured at 37 °C in Dulbecco's modified Eagle's medium/F-12 (v/v) medium, supplemented with 10% fetal bovine serum, 10 units/ml of penicillin, and 10 μg/ml of streptomycin (Invitrogen). The medium used to culture AML12 cells was also sup-

plemented with insulin-transferrin-selenium (Invitrogen) and 40 ng/ml dexamethasone. When 0.5 mM 8-bromo-cAMP was used, cells were starved with 0.5% fetal bovine serum for 24 h. After another 24 h with 8-bromo-cAMP, the cells were treated with 5 or 10 mM IsoNAM for 17 h. Transient transfection was performed in triplicate. Approximately 200,000 cells were cultured in one well of 24-well plates for 24 h and then transfected with 0.2–0.4 μg of plasmid DNA using FuGENE 6 (Roche). After 24 h, the cells were collected and lysed using a cell lysis reagent (Promega, Madison, WI) and luciferase activity was measured using a luminometer (Molecular Devices, Sunnyvale, CA). The protein content of the lysate was determined using the BCA^{TM} protein assay reagent (Pierce). The final results were expressed as relative luciferase units per μg of protein. When chemicals, such as IsoNAM and resveratrol were tested, they were added to medium 1 h after transfection to a desired final concentration. For siRNA knockdown of SIRT1, HepG2 cells were transfected with a mixture of p2000-Luc DNA and siRNA (Dharmacon, Chicago, IL) or a mixture of p2000-Luc DNA and negative control siRNA, using the DharmaFECT Duo transfection reagent (Dharmacon). IsoNAM was added to the medium 20 h after transfection, at a final concentration of 5 mM, and the activity of luciferase was determined 24 h later.

RT-PCR and Northern Blotting—HepG2 hepatoma cells, AML12 mouse hepatocytes, or FTO2B hepatoma cells were grown in 6-well plates to 70% confluence and treated with 5 or 10 mM IsoNAM. Total RNA was isolated using the Versagene^{TM} RNA purification kit (Gentra Systems, Minneapolis, MN), according to the manufacturer's protocol. Northern blotting was done following the protocol described previously (28). The One-Step RT-PCR kit (U. S. Biochemical Corp., Solon, OH) was used to reverse transcribe mRNA for PCR analysis according to the manufacturer's instructions. In each reaction, 0.5 μg of the template RNA was used. The primer sequences were as follows: PEPCK-C forward 5′-GCA TGG GGT GTT TGT AGG AG-3′, PEPCK-C reverse 5′-CCC TAG CCT GTT CTC TGT GC-3′; Glc-6-Pase forward 5′-GTG AAT TAC CAA GAC TCC CAG-3′, Glc-6-Pase reverse 5′-GCC CAT GGC ATG GCC AGA GGG-3′; glyceraldehyde-3-phosphate dehydrogenase forward 5′-CCC ATC ACC ATC TTC CAG GA-3′, glyceraldehyde-3-phosphate dehydrogenase reverse 5′-CCA CCA CCC TGT TGC TGT AG-3′.

Nuclear Extracts and Fluorescent Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared as described previously (29). Briefly, HepG2 cells from one 150-mm plate were collected and lysed by using Nonidet P-40 at a final concentration of 0.33%. Nuclei were then collected and lysed in 200 μl of lyses buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 20% glycerol, and 1× EDTA-free proteinase inhibitors). After centrifugation at 12,000 × *g* for 10 min at 4 °C, the resultant supernatant was saved as a nuclear extract. Fluorescent EMSA was performed with a protocol described previously (10). DNA fragments were generated by annealing two complementary oligonucleotides, one of which was labeled with 6-FAM at the 5′-end (Integrated DNA Technologies, Coralville, IA). The labeled oligonucleotides were: 5′-/6-FAM/-CAA CAG TCA CGG TCA AAG TTT AGT CAA TCA A-3′ for the wild type sequence of HNF4α site-2 (−272/

−252), 5′-/6-FAM/-CAA CAG TCA CGG <u>TAC CAT</u> TTT AGT CAA TCA A-3′ for a mutant sequence, and 5′-/6-FAM/-CTC CCA CGG CCA AAG GTC ATG ACC G-3′ for the wild type sequence of AF1.

Western Blotting and Immunoprecipitation—Western blotting was performed as previously described (30), using antisera raised against HNF4α (SC-8987, 1:1000 dilution) and SIRT1 (SC-15404, 1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA). For immunoprecipitation studies, 500 μg of nuclear extract was pre-cleared at 4 °C for 2 h, using 30 μl of Protein G-agarose slurry (Roche). The supernatant was then transferred to a new tube and incubated at 4 °C for 2 h with 2 μg of the antibody against HNF4α, followed by an additional 2 h of incubation with 40 μl of Protein G-agarose slurry at 4 °C. At the end of the incubation, the beads were collected by centrifugation at $1,000 \times g$ at 4 °C for 5 min, and washed three times with 1× TTBS (1 mM Tris-HCl, pH 7.5, 0.09% NaCl, 0.05% Tween 20). After the final wash, the immunoprecipitated protein was recovered from the beads by re-suspending the pellet in 20 μl of 2× electrophoresis sample buffer, and heating at 95 °C for 5 min.

Purification of HNF4α and SIRT1 and the in Vitro Deacetylation Assay—A plasmid expressing FLAG-tagged human HNF4α was transfected into HepG2 cells using FuGENE 6 (Roche). Stably transfected colonies were established by selection with medium containing 0.5 mg/ml of G418 (Invitrogen). For purification of HNF4α, cells expressing FLAG-tagged HNF4α were collected from 20 150-mm plates and proteins were extracted using the procedure described above. FLAG-tagged HNF4α was purified using EZview™ Red Anti-FLAG M2 affinity gel, and eluted with 3× FLAG peptide (Sigma) following the manufacturer's manual. Eluted protein was further concentrated to an appropriate concentration using Microcon YM-10 (Millipore, Billlerica, MA) and stored at −80 °C in lysis buffer. FLAG-tagged SIRT1 was purified from bacteria BL21(DE3)pLysS, expressing FLAG-tagged human SIRT1 as described (31). Deacetylation *in vitro* was carried out at 37 °C for 2 h in a reaction solution containing 0.2 μg of FLAG-HNF4α, 0.4 μg of SIRT1, 40 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 1 mM dithiothreitol, 2 mM IsoNAM, 0.1 mg/ml bovine serum albumin, and 1 mM NAD⁺.

Chromatin Immunoprecipitation (ChIP) Assay—HepG2 cells were treated with IsoNAM as desired. A ChIP assay was performed as described previously (10). Briefly, cells were sequentially incubated with 1% formaldehyde at room temperature for 10 min and with 125 mM glycine for another 5 min. The cells were then washed, collected, and completely lysed in 1 ml of RIPA buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 1% SDS, 1% deoxycholic acid, 2 mM EDTA, and freshly added protease inhibitors) by passing them through a 30-gauge needle. The chromatin was sheared with sonication using a Fisher Scientific 550 Sonic Dismembrator (power settings of 3, 14 cycles of 10 s on and 50 s off), and then cleared with 30 μl of Protein G-agarose slurry (Roche), 2 μg of sheared salmon sperm DNA, and 2.5 μg of preimmune serum. One hundred μg of DNA (sheared chromatin) was used for immunoprecipitation experiments by mixing the DNA with primary antibodies against HNF4α, c-Fos, C/EBPβ (Santa Cruz Bio-

technology, Santa Cruz, CA) or water overnight at 4 °C, then mixing with 30 μl of Protein G-agarose beads and 2 μg of sheared salmon sperm DNA for an additional 1 h. The immune-precipitated complex (beads) was collected and subjected to sequential washes and centrifugations at $7,500 \times g$ for 5 min at 4 °C. The buffers sequentially used for washing were 1 ml of RIPA buffer, 1 ml of RIPA-500 buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, and protease inhibitor mixture), 1 ml of a LiCl detergent solution (0.5% deoxycholic acid, 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, and protease inhibitor mixture), and then 1 ml of 1× TBS. Immunoprecipitated complexes were then eluted by incubating the beads twice with 100 μl of elution buffer (1% SDS, 1 mM Tris-HCl, pH 8.0, and 1 mM EDTA) at 65 °C for 10 min; the two elutes were then combined. DNA was purified by reversing the cross-link followed by purification using a QIAquick PCR purification kit (Qiagen, Balancia, CA). The DNA isolated from the immunoprecipitate was analyzed using PCR with primers 5′-GCC AGC AGC ATA TGA AGT CCA-3′ and 5′-AAC TTT GGG GAG ATA ATG ATT GAA C-3′ for HNF4α binding site-2, and primers 5′-AGC ACT CTT CGT CAC TCT TAT GTC TC-3′ and 5′-CAA GGC AAG GAT CTC TCT CTC G-3′ for the negative control (non-target region). The amplified DNA fragments were separated by electrophoresis using 1.5% agarose gels and stained with ethidium bromide.

Statistic Analysis—An unpaired Student's *t* test was performed using SigmaPlot (Systat Software, Chicago, IL); a $p < 0.05$ was considered statistically significant. The means of data and their standard errors were used to create bar graphs.

RESULTS

Activators of SIRT1 Inhibit PEPCK-C Gene Transcription—The effect of SIRT1 activators IsoNAM, resveratrol, butein, and fisetin on the regulation of transcription from the PEPCK-C gene promoter was determined. HepG2 cells were transiently transfected with the luciferase reporter plasmid p2000-Luc. IsoNAM addition resulted in a dose-dependent inhibition of transcription, with an IC_{50} of 1.1 mM (Fig. 1*A*, *dash line*). Overexpressing the catalytic subunit of protein kinase A (PKAc) markedly stimulated transcription. Despite this stimulation, IsoNAM repressed transcription from the PEPCK-C gene promoter (p2000-Luc) with an IC_{50} of 2.3 mM (Fig. 1*A*, *solid line*). At 5 mM IsoNAM, only about 17% of the luciferase activity remained (*i.e.* an 83% inhibition). As predicted, nicotinamide (NAM), a SIRT1 inhibitor, caused an increase in PEPCK-C activity in rat liver (Fig. 1*A*, *inset*). Treatment of the cells with other SIRT1 activators, such as resveratrol (Fig. 1*B*), butein or fisetin (Fig. 1*C*) also markedly repressed transcription from the PEPCK-C gene promoter. IsoNAM was used for most of the subsequent experiments, in part because of its excellent solubility and stability in water.

To determine how the endogenous PEPCK-C gene responded to SIRT1 activators, HepG2 cells, FTO-2B cells, and AML12 mouse hepatocytes were treated with 5 mM IsoNAM and PEPCK-C mRNA was analyzed by Northern blotting or RT-PCR. IsoNAM decreased the levels of endogenous PEPCK-C mRNA in a time- and dose-dependent manner
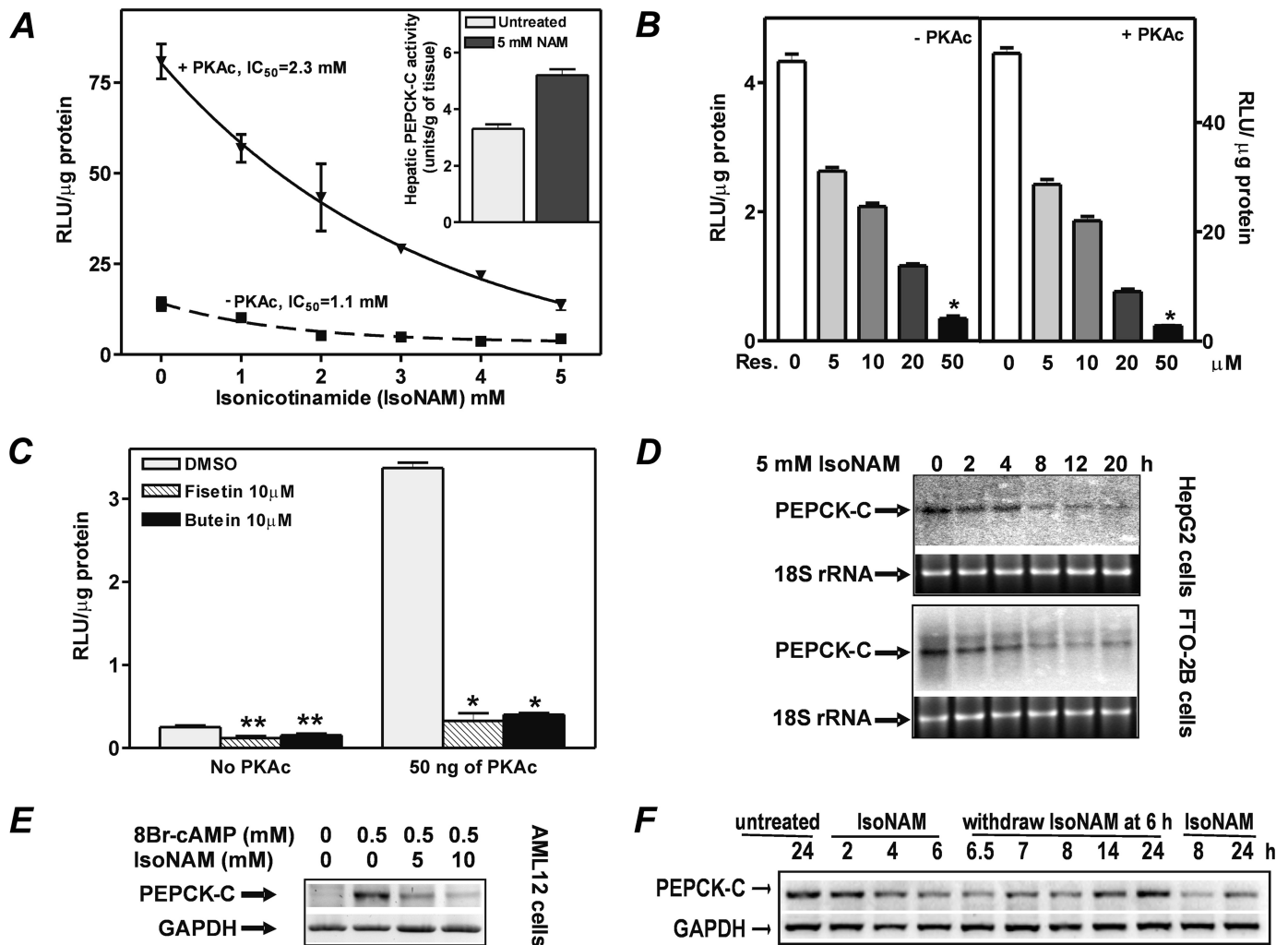
FIGURE 1. **Transcriptional repression of the PEPCK-C gene promoter by SIRT1 activators.** HepG2 cells were co-transfected with 200 ng of p2000-Luc plasmid DNA and 50 ng of PKAc plasmid DNA or 50 ng of empty vector DNA, using the protocol described under "Experimental Procedures." Cells were then treated with the indicated concentrations of SIRT1 activators, namely IsoNAM (*panel A*), resveratrol (*panel B*), and fisetin and butein (*panel C*) for 24 h. The *inset* in *panel A* was redrawn from data in Table 5 of Reshef and Hanson (58), in which fed rats were given 5 mmol of nicotinamide (NAM)/kg of body weight for 5 h and the activity of hepatic PEPCK-C determined. A unit of enzyme activity represents the conversion of 1 μmol of substrate to product per min. The IC$_{50}$ was the concentration of IsoNAM required to achieve 50% inhibition of transcription from the PEPCK-C gene promoter. Values were expressed as mean $\pm$ S.E. of triplicate determinations. *, $p < 0.001$ and **, $p < 0.023$ as compared with untreated controls. Northern blotting (*panel D*) and RT-PCR (*panel E*) were used to determine endogenous mRNA levels of PEPCK-C in FTO-2B and HepG2 cells treated with IsoNAM for various times, or in AML12 mouse hepatocytes treated with IsoNAM at the indicated concentrations. The 18S rRNA stained with ethidium bromide or glyceraldehyde-3-phosphate dehydrogenase mRNA was used as loading controls. *Panel F*, IsoNAM-induced inhibition of PEPCK-C was reversible. FTO-2B cells were cultured in Krebs-Ringer bicarbonate buffer, supplemented with 2% of bovine serum albumin and 5 mM glucose in the absence (untreated) or presence of 5 mM IsoNAM (treated). After 6 h of treatment, IsoNAM was withdrawn from culture medium. Cells were collected at the time points indicated. PEPCK-C mRNA was measured using RT-PCR; the level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was used as a control.

(Fig. 1, *D* and *E*). In contrast, there was only a marginal change in the level of Glc-6-Pase mRNA (data not shown). To rule out the possibility that decreases in PEPCK-C mRNA were due to excessive cell death, cell numbers were counted and parallel increases in cell number were noted in both IsoNAM- and water-treated cells (data not shown). Furthermore, removing IsoNAM from the cell culture medium resulted in an increase in PEPCK-C mRNA (Fig. 1*F*), indicating that the repression of PEPCK-C gene transcription by IsoNAM was reversible.

A HNF4α-containing Binding Site Cluster Is Involved in Repression of PEPCK-C Transcription by IsoNAM—To determine the specific region(s) in the PEPCK-C gene promoter that is responsible for transcriptional repression by IsoNAM, a series of sequentially truncated segments of the PEPCK-C gene

promoter, linked to the luciferase gene, were transfected into HepG2 cells. IsoNAM (5 mM) caused an ~85% inhibition in transcription from the full-length PEPCK-C gene promoter (p2000-Luc), as compared with water-treated control cells (Fig. 2*A*). Shortening the PEPCK-C gene promoter between −274 and −208 markedly decreased this inhibition to 34% (Fig. 2*A*), suggesting that the IsoNAM response element(s) resided in this segment of the gene promoter. The transcription factor binding site locator algorithm, an *in silico* method of predicting cross-species conservations of transcription factor binding sites, was used to analyze the sequence between −274 and −208 in the PEPCK-C gene promoter from seven mammalian species. Three evolutionarily conserved binding sites, HNF4α, AP-1, and C/EBP, were identified in this region (Fig. 2*B*).

Among them, the HNF4α binding site located at $-272/-252$, which was previously unknown and designated as the HNF4α site-2 to distinguish it from a previously described site, termed accessory factor 1 (AF1) at $-454/-430$ (32, 33). An EMSA demonstrated that an oligonucleotide containing sequence corresponding to the HNF4α site-2 bound to HNF4α (Fig. 2*C, lanes 2* and *3*) just as well as the AF1-containing oligonucleotide (Fig. 2*C, lanes 9* and *10*). In contrast, an oligonucleotide containing a mutated HNF4α site-2 did not interact with HNF4α (Fig. 2*C, lanes 5–8*).

To examine the involvement of the HNF4α site-2, AP-1, and C/EBP sites in the IsoNAM-mediated transcriptional repression of *PEPCK-C*, these regions in the PEPCK-C gene promoter were mutated individually or in combination, using p2000-Luc as the parent plasmid (Fig. 2*D, left side*). The AF1 site was also mutated to serve as a control. Mutation of the individual HNF4α site-2, AP-1, and C/EBP sites resulted in about a 10% decrease in IsoNAM-induced repression (Fig. 2*D*), whereas mutation in AF1 had no effect. This result was consistent with the data from truncation analysis between 490-Luc and 355-Luc, in which the AF1 site was absent (Fig. 2*A*). When all three conserved sites were mutated, IsoNAM-mediated inhibition was decreased from 90 to 57% (Fig. 2*D, triple mutant*). This result indicated that the cluster of three binding sites was required for maximal IsoNAM-induced repression. Indeed, when a triplicate of the aforementioned binding cluster was linked to a minimal TK promoter, it recapitulated IsoNAM-induced repression (Fig. 2*E*). Because the overall relief of inhibition was not complete in the triple mutant (only 57%), unknown factor(s) other than this cluster of binding sites was likely involved in IsoNAM-induced repression of transcription from the PEPCK-C gene promoter.

To further assess the role of HNF4α in the IsoNAM-induced repression of transcription of the PEPCK-C gene, Chinese hamster ovary cells, which have no detectable HNF4α, were transfected with p2000-Luc. IsoNAM or resveratrol treatment resulted in marginal

effects on transcription from the PEPCK-C gene promoter (Fig. 3, *lanes 1* and *2*, and *7* and *8*). When a plasmid encoding HNF4α was co-transfected with p2000-Luc, luciferase activity was markedly stimulated (Fig. 3, *lane 1 versus 3* and *5* and *lane 7 versus 9* and *10*). However, addition of either IsoNAM or resveratrol to the culture medium blocked this stimulation (Fig. 3).
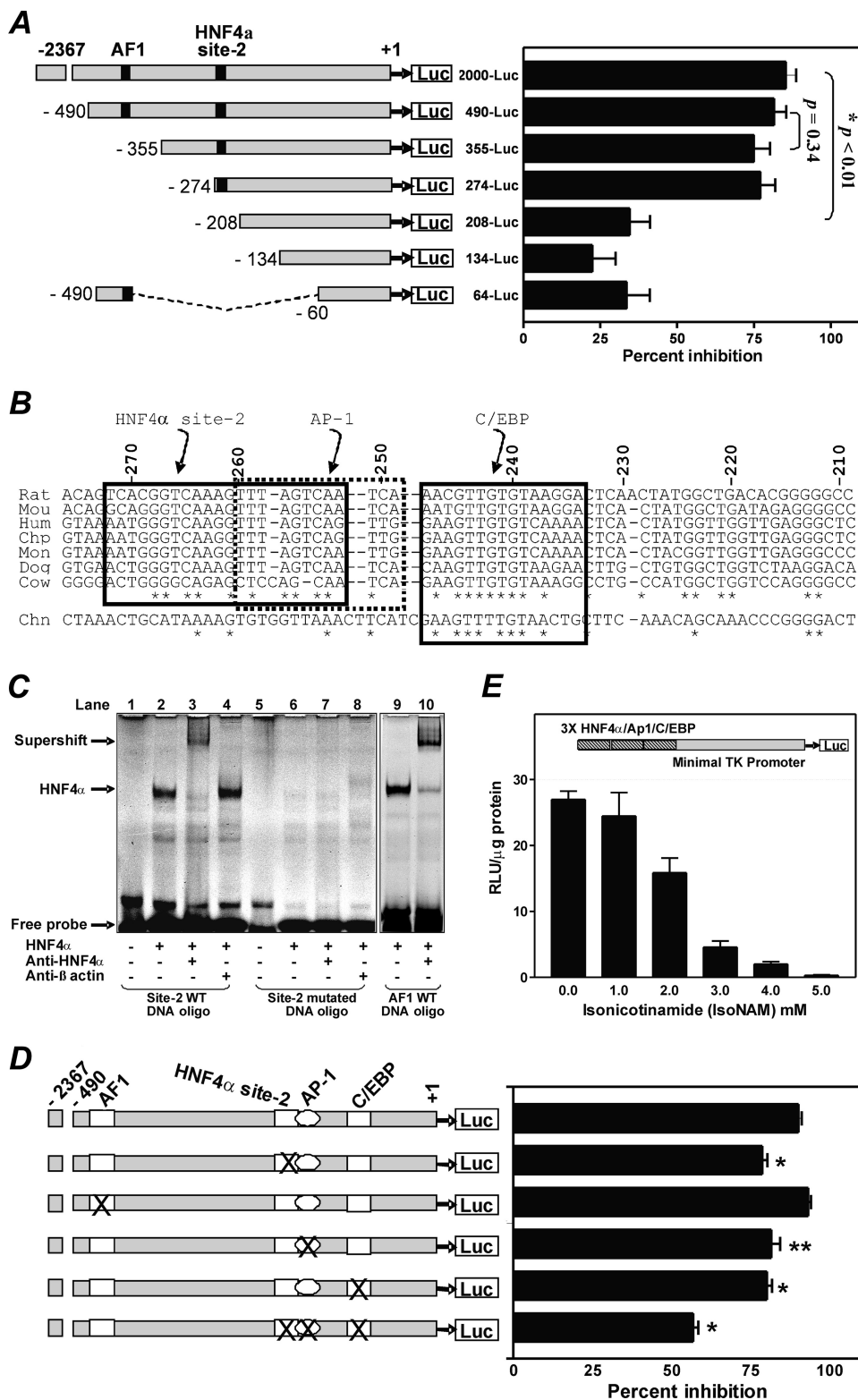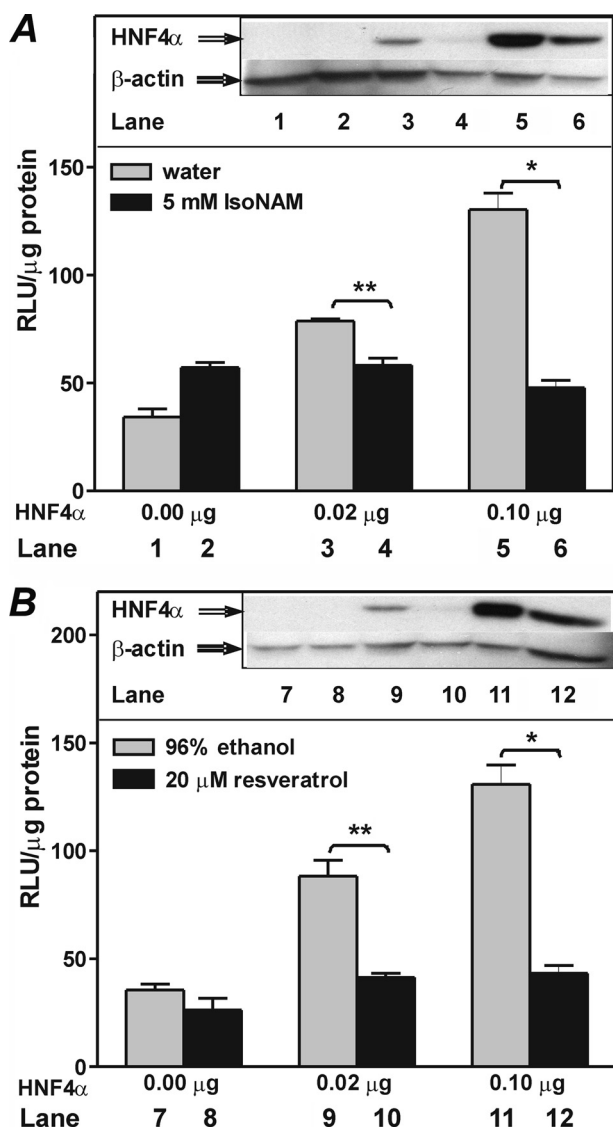
FIGURE 3. **HNF4α is required for SIRT1 activator-induced transcriptional repression of PEPCK-C.** Chinese hamster ovary cells were co-transfected with p2000-Luc, PKAc, and various amounts of plasmid overexpressing HNF4α. Cells were then treated with 5 mM IsoNAM or 20 μM resveratrol. The total amount of DNA for each transfection was adjusted to the same value by adding empty vector DNA. The results were expressed as the mean ± S.E. of triplicate measurements. *, $p < 0.001$ and **, $p < 0.004$. *Insets* show levels of HNF4α in cells as determined by Western blotting.

Western blotting analyses showed that HNF4α levels were decreased in IsoNAM or resveratrol-treated Chinese hamster ovary cells (Fig. 3, *insets*).

IsoNAM Causes Deacetylation of HNF4α and Decreases its DNA Binding Affinity—Acetylation of HNF4α enhances its nuclear retention and DNA binding affinity (34). Because IsoNAM activates SIRT1, we determined the effect of IsoNAM treatment on the acetylation status of endogenous HNF4α. Although there was only a modest decrease in the level of total nuclear HNF4α during 24 h of IsoNAM treatment, the acetylated species of HNF4α decreased markedly and reached its lowest level 14 h after adding IsoNAM to the culture medium (Fig. 4A, *lanes 1–7*); as a control, water did not alter the acetylation status of HNF4α (Fig. 4A, *lanes 8–14*). This observation suggested that SIRT1, activated by IsoNAM, likely de-acetylated HNF4α *in vivo*. To test this hypothesis, purified FLAG-HNF4α was incubated with SIRT1. Western blotting showed that HNF4α was deacetylated by SIRT1 *in vitro* (Fig. 4B). In addition, EMSA demonstrated that deacetylation of HNF4α by SIRT1 *in vitro* decreased the binding affinity of HNF4α to an oligonucleotide containing the HNF4α site-2 sequence (Fig. 4C). NAD⁺, a cofactor for SIRT1, was omitted in a control reaction. To further assess the effect of deacetylation of HNF4α on its binding affinity to the PEPCK-C gene promoter *in vivo*, a ChIP assay was performed using cells treated with 5 mM IsoNAM or water for 6, 10, or 20 h. The binding of HNF4α to the promoter was markedly decreased at 10 and 20 h (Fig. 4D), which was consistent with the low level of acetylation of HNF4α (Fig. 4A).

AP-1 and HNF4α Reciprocally Interact with the HNF4α Site-2/AP-1 Binding Site in the PEPCK-C Gene Promoter—Three AP-1 binding sites in the PEPCK-C gene promoter have been previously identified (35), one of which was located at −274/−208 (Fig. 2B). Because this AP-1 site overlapped HNF4α site-2, we speculated that only one of these transcription factors could interact with DNA at a given time, due to spatial restriction. An EMSA demonstrated that DNA containing the HNF4α site-2/AP-1 binding sites only interacted with the c-Jun/c-Fos heterodimer (*i.e.* AP-1), but not with homodimers of c-Jun (Fig. 5A). To determine whether AP-1 and HNF4α competed for the HNF4α site-2/AP-1 binding site on the PEPCK-C gene promoter, four EMSA reactions were carried out in which the amount of AP-1 was kept constant, whereas the amount of HNF4α was increased over a range of 0, 1, 2, and 3 pmol. An increase in the concentration of HNF4α

FIGURE 2. **A novel HNF4α binding site in the PEPCK-C gene promoter is required for IsoNAM-induced transcriptional repression.** *Panel A,* HepG2 cells were treated with 5 mM IsoNAM or water (untreated control) after co-transfection of PKAc DNA and luciferase reporter constructs, which harbored various segments of the PEPCK-C gene promoter from rat. IsoNAM-dependent repression of transcription from the PEPCK-C gene promoter was expressed as a percent inhibition of treated as compared with untreated cells. Values were expressed as mean ± S.E. of four separate tests, with each test performed in triplicate. *Panel B,* the sequence from −272 to −208 of the rat PEPCK-C gene promoter was aligned with corresponding sequences from the mouse (*Mou*), human (*Hum*), chimpanzee (*Chp*), monkey (*Mon*), dog (*Dog*), cow (*Cow*), and chicken (*Chn*). Evolutionary conserved sites are indicated by *boxes*. *Panel C,* HNF4α binding site-2 interacted with HNF4α. Purified FLAG-HNF4α was used to perform an EMSA with the wild type or mutated DNA oligonucleotides corresponding to the HNF4α binding site-2 sequence, or with the wild type DNA corresponding to AF1, as described under "Experimental Procedures." For the supershift assays, either anti-HNF4α serum or anti-β-actin serum was added to the reactions. *Panel D,* HNF4α binding sites were mutated individually or jointly with other conserved binding sites for AP1 and C/EBP in the p2000-Luc plasmid. The effect of IsoNAM on transcription from the PEPCK-C gene promoter, in the presence of co-transfected PKAc was measured using the procedure described under *panel A.* Values were expressed as mean ± S.E. of three separated tests, with each test done in triplicate. *, $p < 0.001$, and **, $p < 0.016$ as compared with the wild type gene promoter control. *Panel E,* an artificial promoter, composed of three copies of HNF4α-AP1-C/EBP binding sequences (−277/−218) and the TK minimal gene promoter, was cloned into pGL3-Basic vector to generate a reporter plasmid (*inset*). When this reporter plasmid was co-transfected with PKAc into HepG2 cells, IsoNAM caused a dose-dependent inhibition of transcription from the PEPCK-C gene promoter. Values are expressed as mean ± S.E. of three separate analyses.
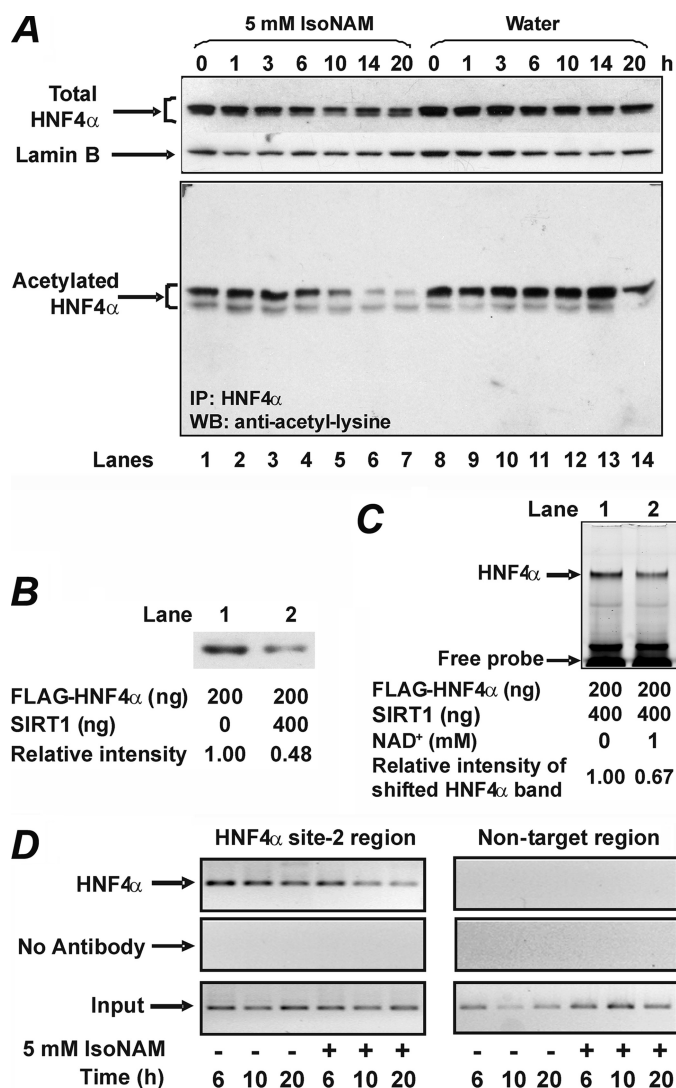
A

B

C

D

FIGURE 4. **IsoNAM-induced deacetylation of HNF4α attenuates its binding to PEPCK-C gene promoter.** *Panel A,* IsoNAM treatment resulted in a time-dependent deacetylation of nuclear HNF4α. HepG2 cells were treated with 5 mM IsoNAM or water for the times indicated in the panel. Total nuclear HNF4α was measured using Western blotting; lamin B was used as a loading control. The acetylated species of endogenous HNF4α was assayed using immunoprecipitation of HNF4α, followed by Western blotting of acetyl-lysine. *Panel B,* SIRT1 deacetylated HNF4α *in vitro*. Purified FLAG-HNF4α was mixed with reaction buffer or reaction buffer plus SIRT1. The acetylation status of HNF4α was then assayed using Western blotting for acetyl-lysine. The band density was quantified by densitometry and the value of the control was set as 1. *Panel C,* deacetylation of HNF4α attenuated its binding to DNA. Purified FLAG-HNF4α was deacetylated *in vitro* by SIRT1 in the presence or absence of NAD$^+$. The resultant proteins were used in EMSA reactions. *Panel D,* IsoNAM treatment attenuated the binding of HNF4α to the HNF4α site-2 in the PEPCK-C gene promoter as determined by a ChIP assay. HepG2 cells were treated with water or 5 mM IsoNAM for the times indicated. Binding of HNF4α to the HNF4α site-2 was assessed using a ChIP assay, as described under "Experimental Procedures."

resulted in an increase in the HNF4α·DNA complex (Fig. 5*B*, *lanes 2–5*) and a reduction in the AP-1·DNA complex. These results indicate that HNF4α competes away DNA from the AP-1·DNA complex. As a control, the addition of 21 pmol of bovine serum albumin did not disrupt the AP-1·DNA complex. In a reciprocal manner, increasing concentrations of AP-1 effectively competed with HNF4α for binding to the DNA probe (Fig. 5*B*, *lanes 7–10*).
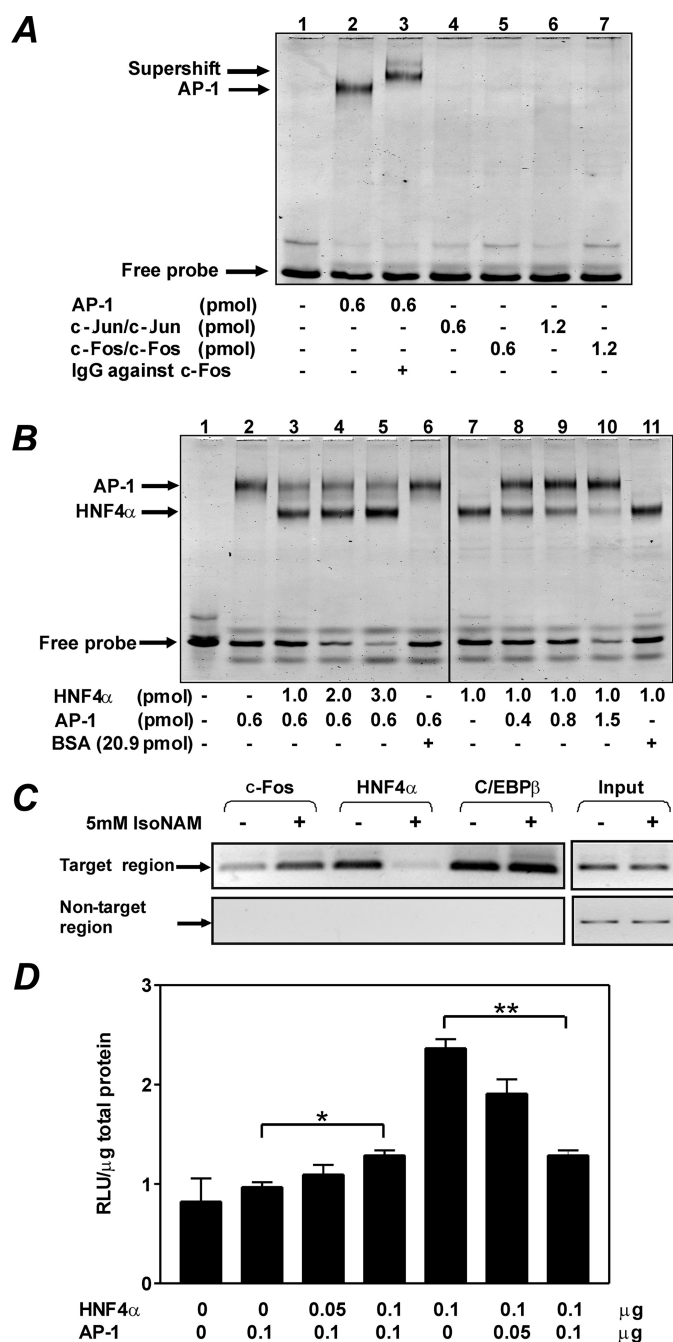
A

B

C

D

FIGURE 5. **IsoNAM enhances binding of AP-1 to the PEPCK-C gene promoter.** *Panel A,* an EMSA of the purified AP-1 components bound to the overlapping HNF4α site-2/AP-1 region in the PEPCK-C gene promoter. *Panel B,* purified AP-1 and HNF4α compete for the overlapping HNF4α site-2/AP-1 region in the PEPCK-C gene promoter. *Panel C,* IsoNAM enhanced the interaction of AP-1, but decreased the binding of HNF4α, to the PEPCK-C gene promoter. HepG2 cells were treated 5 mM IsoNAM for 10 h. A ChIP assay was used to monitor the binding of AP-1, HNF4α, and C/EBPβ to the overlapping HNF4α site-2/AP-1 region (*target region*). *Panel D,* AP-1 and HNF4α competitively regulated the CRE-mutated PEPCK-C gene promoter. HepG2 cells were co-transfected with a CRE-mutated p2000-Luc plasmid and with 0.05 and 0.1 μg of plasmids expressing HNF4α or AP-1 and luciferase activity was measured. The values are expressed as the mean ± S.E. of triplicate measurements. *, $p < 0.05$ and **, $p < 0.01$. *BSA*, bovine serum albumin.

Because binding of HNF4α to the PEPCK-C gene promoter was attenuated by IsoNAM treatment of cells (Fig. 4*D*), and because AP-1 and HNF4α competed for the HNF4α site-2/ AP-1 sequence (Fig. 5*B*), we hypothesized that AP-1 would

occupy the binding site in IsoNAM-treated cells. A ChIP assay showed that binding of AP-1 to the HNF4α site-2/AP-1 site was greatly enhanced after 10 h of treatment with IsoNAM, whereas binding of HNF4α markedly decreased (Fig. 5*C*). At the same time, binding of C/EBPβ to its site did not change significantly.

Gurney *et al.* (35) reported that AP-1 stimulated PEPCK-C gene transcription. One of three AP-1 binding sites, the cyclic AMP regulatory element (CRE), played a critical role for such stimulation. With the CRE site present in p2000-Luc, it is therefore difficult to determine whether there was a competition between AP-1 and HNF4α at the HNF4α site-2/AP-1 site. When the CRE was mutated in the p2000-Luc reporter plasmid, HNF4α and AP-1 competed with each other in regulating transcription from the PEPCK-C gene promoter (Fig. 5*D*). Taken together, our data indicate that when SIRT1 is activated by IsoNAM, the subsequent deacetylation of HNF4α decreases its affinity for the HNF4α site-2/AP-1 site, thus permitting AP-1 to bind to its overlapping site on the PEPCK-C gene promoter.

SIRT1 Represses Transcription from the PEPCK-C Gene Promoter—Because SIRT1 activators activate SIRT1, and SIRT1 deacetylated HNF4α (Fig. 4*B*), we assessed the effect of the overexpression of SIRT1 on transcriptional regulation of the PEPCK-C gene in HepG2 cells. Exogenous expression of SIRT1 repressed luciferase activity from the co-transfected p2000-Luc reporter plasmid (Fig. 6*A*). The extent of repression for transcription was directly related to the quantity of SIRT1 transfected. In addition, when endogenous SIRT1 was knocked down using SIRT1 RNAi, IsoNAM-induced repression of the PEPCK-C gene promoter was greatly attenuated (Fig. 6*B*). This result demonstrates that factors that activate SIRT1, such as IsoNAM and resveratrol, do so by deacetylating, and thus inactivating, key transcription factors, such as HNF4α, which are required for hepatic PEPCK-C gene transcription.

DISCUSSION

SIRT1 is a member of a class of NAD^+-dependent deacetylases that ADP-ribosylates acetyl-lysine protein substrates to remove acetate (see Ref. 36 for a detailed review of the enzymology involved). SIRTs are found in a wide variety of organisms and are responsive to dietary restriction (37) and changes in cellular redox state (22). The involvement of SIRT1 in glucose homeostasis is controversial and requires further investigation. Studies have shown that activation of SIRT1 induces transcription of genes coding for key enzymes in gluconeogenesis, thus stimulating hepatic glucose output via deacetylation of PGC-1α (22). Erion *et al.* (23) noted a decrease in PEPCK-C mRNA in the livers of diabetic rats in which SIRT1 mRNA had been reduced to 25% of control levels using an antisense oligonucleotide. In addition, Frescas *et al.* (24) reported that activation of SIRT1 in wild type cells (an immortalized liver cell line) by resveratrol had no effect of PEPCK-C mRNA but stimulated the expression of the gene for Glc-6-Pase. The introduction of FOXO1, into wild type cells, via an adenoviral vector, resulted in a marked elevation of PEPCK-C mRNA. However, Banks *et al.* (26) found that overexpression of SIRT1 did not result in hyperglycemia in transgenic mice, but instead improved insulin sensitivity. In addition, analogues of resveratrol, which are 1,000 times more potent SIRT1 activators, have been developed
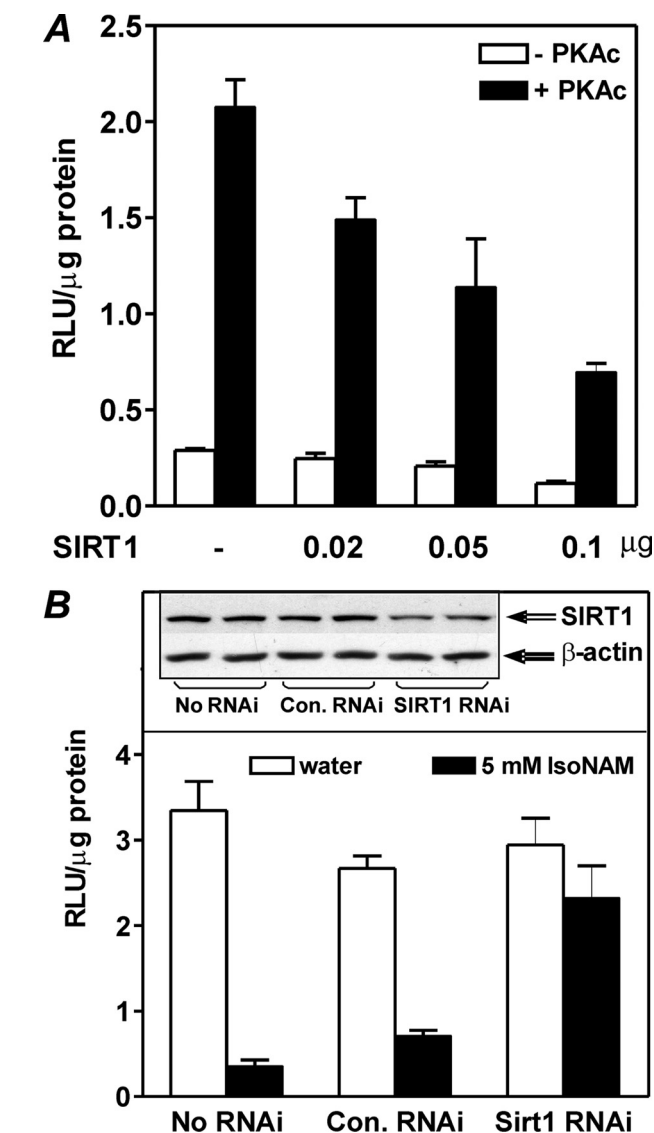

FIGURE 6. **SIRT1 represses transcription of the PEPCK-C gene in HepG2 cells.** *Panel A*, mouse SIRT1 was overexpressed in HepG2 cells in the absence (*open bar*) or presence of PKAc (*black bar*). Transcription of the PEPCK-C gene promoter was determined using a luciferase reporter assay. *Panel B*, RNAi knock-down of SIRT1 attenuated IsoNAM-induced transcriptional repression of *PEPCK-C*. HepG2 cells were co-transfected with p2000-Luc and no RNAi, control RNAi, or Sirt1 RNAi, and then treated with water (*open bar*) or 5 mM IsoNAM (*black bar*). Luciferase activity was measured 24 h later. The *insert* is a Western blot of SIRT1 in the lysates of the transfected cells. The luciferase activities were expressed as the mean ± S.E. from triplicate measurements.

(25). These compounds act by lowering the K_m of acetylated proteins for SIRT1 and have been shown to improve insulin sensitivity, lower the concentration of glucose in the blood of dietary-induced and genetically obese mice, and to generally improve whole body glucose homeostasis in the animals (25). Although not measured directly, the implication of these studies is that expression of the gene for PEPCK-C in the liver would be decreased to account for a lowered rate of gluconeogenesis noted in that tissue. Finally, Liu *et al.* (27) reported that disrupting SIRT1 activity resulted in an increase in glucose output. There are thus a series of studies that come to opposite conclusions regarding the effect of SIRT1 on hepatic gluconeogenesis in the liver.
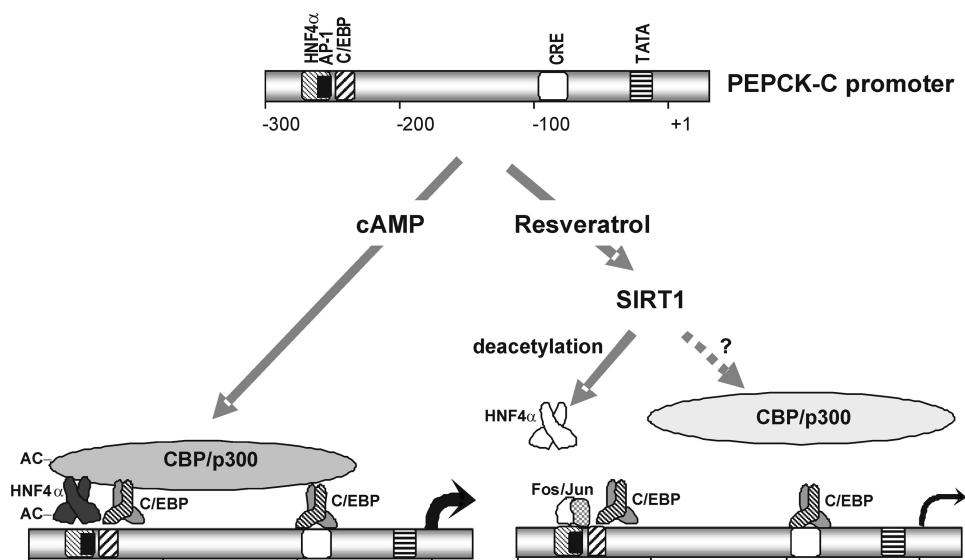
FIGURE 7. **A model for the SIRT1-induced transcriptional repression of PEPCK-C gene promoter.** PEPCK-C gene promoter and several key transcription factor binding sites are drawn to scale.

HNF4α is a component of the triad of regulatory proteins involved in the effect of insulin on PEPCK-C gene transcription. The forkhead protein FOXO1, HNF4α, and the co-activator PGC-1α have been shown to interact at the AF1 site within glucocorticoid regulatory unit of the PEPCK-C gene promoter (41). Insulin alters this interaction and inhibits gene transcription, in part by initiating the PKB-catalyzed phosphorylation of FOXO1, which results in dissociation of the complex. HNF4α is acetylated (34), which increases its binding affinity for both CBP and its binding domain on a target gene promoter; deacetylation decreases binding. Thus, the AF1 site within the glucocorticoid regulatory unit of the PEPCK-C gene promoter would be a logical candidate as a site in the promoter responsible for its negative effect on PEPCK-C gene transcription caused by the activation of SIRT1 by Iso-NAM. However, mutation of this site had no effect on the inhibition of transcription caused by either IsoNAM or resveratrol. We have identified a hitherto unknown HNF4α regulatory element in the PEPCK-C gene promoter (HNF4α site-2), which maps between −272 and −248 (Fig. 7). Because this region of the gene promoter does not bind FOXO1, we conclude that the SIRT1-catalyzed deacetylation of HNF4α noted in this study does not involve an interaction with the complex of FOXO1 and PGC-1α, which occurs at the AF1 site of the glucocorticoid regulatory unit. However, the lack of inhibition by SIRT1 activation caused by a deletion of the AF1 site could also be explained by the fact that endogenous FOXO1 and PGC-1α are undetectable in HepG2 cells by Western blotting (data not shown). It is also clear that factors acting at the HNF4α site-2 are not entirely responsible for the dramatic reduction in PEPCK-C gene transcription noted upon activation of SIRT1 by resveratrol or IsoNAM, because mutation of the HNF4α 2-site only partly reversed IsoNAM-induced inhibition. However, the deacetylation of other key regulatory transcription factors or co-activators is likely involved in inhibition of the PEPCK-C gene transcription noted in this study. For example, SIRT1 represses the action of CBP/p300, an important co-regulator of PEPCK-C gene transcription, by deacetylating lysines 1020 and 1024, within the cell cycle regulatory domain (21). It would be of interest to determine the effect of deacetylating CBP/p300 on transcription of this gene.

The HNF4α site-2 maps to a region of the PEPCK-C gene promoter as shown by Roesler *et al.* (42), using DNase I footprinting, to bind nuclear proteins; this area of the promoter is highly conserved in a number of mammalian species (see Fig. 2) and is in a region of the gene promoter that is critical for control of PEPCK-C gene transcription (43). Our original analysis of the PEPCK-C gene promoter (42, 43) identified two regulatory elements in this region: P3(I), which binds members of the

It is likely that the activation of SIRT1 will have a broad effect on transcription factors and more directly by deacetylating target proteins. Lin *et al.* (38) reported that PEPCK in yeast was acetylated at lysines 19 and 514, and that deacetylation of the enzyme caused by the activation of SIRT2 resulted in a loss of enzyme activity of the enzyme in yeast. Acetylation of lysine 514 was required both for enzyme activity and for the ability of yeast to grow in non-fermentable carbon sources. In addition, the deacetylation of PEPCK-C in yeast blocked the expected extension of lifespan caused by water starvation (38). If this effect of SIRT1 can be duplicated in the livers of mice, it would be consistent with a decrease in the rate of gluconeogenesis and in hepatic glucose output and supports our finding that PEPCK-C gene transcription is decreased when SIRT1 is activated by resveratrol.

Here we report a detailed study on the effect of activating SIRT1 using resveratrol, butein, fisetin, or IsoNAM on the transcription of *PEPCK-C*. We find that activation of SIRT1 causes a marked inhibition of transcription from the PEPCK-C gene promoter via deacetylation of a transcription factor/co-activator(s) required for expression of the gene in the liver (Fig. 7). Our data demonstrated that a major target of SIRT1-mediated deacetylation is HNF4α, an orphan nuclear receptor that is closely linked to diabetes. HNF4α was originally isolated from the liver, but is present in a number of tissues, including small intestine, kidney, and pancreatic islet. A number of mutations in the gene for HNF4α have been identified and associated with an atypical form of diabetes, termed maturity onset diabetes of the young (39). HNF4α regulates transcription of a broad variety of genes in the liver, including HNF1α, glucose-6-phosphatase, and PEPCK-C. HNF4α binds to the AF1 site (−454 to −430) in the glucocorticoid regulatory unit of the PEPCK-C gene promoter, where it is involved in glucocorticoid-stimulated transcription of the PEPCK-C gene (12, 32). The carboxyl-terminal, zinc finger domain of HNF4α binds to the PEPCK-C gene promoter and interacts with co-regulatory molecules such as CBP/p300 (40) and PGC-1α (41).

C/EBP family of transcription factors, and P3(II), which binds AP-1 (a Fos/Jun heterodimer) and stimulates PEPCK-C gene transcription (35). P3(I) is required for liver-specific transcription of the gene (44) and acts together with the CRE (-128 to -77), in cAMP stimulation of hepatic PEPCK-C gene transcription (43). C/EBPβ, which binds to the P3(I) site, itself has multiple acetylation sites (45). However, the ChIP data shown in Fig. 5*C* did not detect an IsoNAM-induced alteration in binding of C/EBPβ to the PEPCK-C gene promoter.

The AP-1 site identified in this study overlaps the HNF4α regulatory domain in the PEPCK-C gene promoter, suggesting a potential competition between the two transcription factors for binding to this site. We have demonstrated that increasing concentrations of AP-1 competes with HNF4α binding to the gene promoter (Fig. 5*B*) and markedly reduces the induction of PEPCK-C gene transcription by HNF4α in HepG2 cells (Fig. 5*D*). Our hypothesis is that deacetylation of HNF4α by SIRT1 reduces its binding affinity, allowing AP-1 to bind to the site and thus inhibit PEPCK-C gene transcription (Fig. 7). Interestingly, only the Fos/Jun heterodimer bound to the site; the c-Jun homodimer did not bind to the HNF4a binding site in our EMSA. The transcriptional activity of AP-1 can be altered by post-translational modifications, such as sumoylation of both c-Fos and c-Jun, which decreases their activity (46). Transcription of the gene for c-Fos is also induced by cAMP (47) and insulin (48). Recently, SIRT1 was shown to interact with c-Jun and JunD, but not c-Fos (49), suggesting that acetylation of c-Jun may regulate the ability of AP-1 to modify PEPCK-C gene transcription. The physiological relevance of the proposed interaction between AP-1 and HNF4α on the PEPCK-C gene promoter remains to be established.

The potential role of C/EBP in the repression of PEPCK-C gene transcription noted in this study is not clear. C/EBP binds to the P3(I) site in the promoter (42, 43), which is within 10 bp of the AP-1/HNF4α site-2 (Fig. 2*B*). Mutation of this site in the PEPCK-C gene promoter resulted in a small but significant decrease in transcription in the presence of IsoNAM (Fig. 2*D*). This suggests that C/EBP could be involved in response of the PEPCK-C gene to activation of SIRT1. However, the ChIP assay shown in Fig. 5*C* did not detect a difference in the binding of C/EBPβ to the PEPCK-C gene promoter in HepG2 following exposure to IsoNAM. Members of the C/EBP family of transcription factors have also been shown to bind to the CRE in the PEPCK-C gene promoter, which is within 150 bp of the HNF4α site-2. The ChIP assay used in this study will not distinguish between sites in a gene promoter that are closer than 400 bp, dependent on the size of the promoter fragment created when the DNA is sheared. C/EBPβ is a potential target for functional alterations by acetylation/deacetylation, because it is acetylated at multiple lysines by CBP/p300; acetylation at lysine 39 is critical for regulating its ability to activate gene transcription (45). As an example, STAT5 induces transcription of the ID1 gene by the recruitment of HDAC1, which deacetylates C/EBPβ allowing it to stimulate transcription (50). To date there is no evidence that SIRT1 catalyzes the deacetylation of C/EBPβ.

In contrast to the results of this study, Rodgers *et al.* (22) have reported that the SIRT1-mediated deacetylation of PGC-1α, induced by the addition of 1 mM pyruvate to hepatoma cells, increased the level of PEPCK-C mRNA. They also demonstrated that knockdown of SIRT1 by siRNA completely abolished the stimulatory effect of 1 mM pyruvate on the relative concentration of PEPCK-C mRNA in Fao hepatoma cells. We find, however, that exogenous expression of SIRT1 inhibits the PKAc induction of transcription from the PEPCK-C gene promoter and that knockdown of SIRT1 results in loss of the Iso-NAM-induced inhibition of transcription. It is possible that the difference noted between the two studies are due to the fact that we have investigated the effect of SIRT1 on PEPCK-C gene transcription induced by PKAc; activation of SIRT1 only marginally repressed transcription from the PEPCK-C gene promoter in the absence of the cyclic nucleotide. Rogers *et al.* (22) used 1 mM pyruvate, which is 10 times the physiological concentration in the liver, to stimulate SIRT1, presumably by increasing the NAD/NADH ratio in the cells, in the presence of overexpressed PGC-1α. IsoNAM significantly inhibited PEPCK-C gene transcription only when PKAc was used to induce expression of the gene in cell transfection studies. This suggests that the deacetylation of transcription factors or coactivators, such as C/EBP or CBP/p300, may be involved in the repression of PEPCK-C gene transcription in cells activated by the addition of PKAc. It seems likely that multiple elements in the PEPCK-C gene promoter are required for the physiological effects of SIRT1 repression of transcription of the gene in the liver. These initial studies have not probed the complex interactions that are known to regulate transcription of this gene, to include the effect of deacetylation on the activity of C/EBPβ, c-Jun, or CBP/p300.

The overall physiological response of an intact mammal to the activation of SIRT1 is far clearer by comparison. Activation of SIRT1 in mice by resveratrol or its analogues causes a reduction in hepatic glucose output in diet-induced or genetically obese animals (25). This has been attributed to an increase in hepatic insulin sensitivity caused by the activation of SIRT1. Sun *et al.* (51) reported that SIRT1 is down-regulated in insulin-resistant cells and that expression of SIRT1 increases insulin sensitivity. Because insulin decreases transcription of the gene for PEPCK-C (52), it is likely that an increase in insulin sensitivity, caused by SIRT1 activation, will lead to a decrease in transcription of the gene for PEPCK-C and contribute to a reduction in hepatic glucose output. The effect of SIRT1 on insulin sensitivity has been attributed to repression of transcription of the gene for PTP1B (51), a well known negative regulator of the insulin signaling pathway (53). It is thus likely that resveratrol exerts its long term metabolic effects, in part, by modulating the rate of gluconeogenesis and thus controlling the overall levels of hepatic glucose output in mammals.

Because diabetes is a disease of both carbohydrate and lipid metabolism (54), it is critical that resveratrol or its analogues affect not only hepatic glucose output, but also adipose tissue to control the rate of lipolysis and/or FFA re-esterification by that tissue. Glyceroneogenesis is the major metabolic pathway for the formation of the glycerol 3-phosphate used for triglyceride synthesis in adipose tissue (55); a key enzyme in the process is PEPCK-C (2, 56). Picard *et al.* (57) reported that resveratrol increased the rate of epinephrine-induced FFA release from adipose tissues and that nicotinamide, a SIRT1 inhibitor,

decreased FFA release. If PEPCK-C in adipose tissues responds to alterations in deacetylation of transcription factors such as peroxisome proliferator-activated receptor-γ2 and HNF4α in a manner similar to the liver, one would predict that resveratrol would decrease transcription of the gene for the enzyme, decreasing glyceroneogenesis and thus diminishing the rate of FFA re-esterification, resulting in a greater release of FFA. In contrast, Reshef and Hanson (58) found that nicotinamide markedly decreased the activity of PEPCK-C in adipose tissues, whereas it increased PEPCK-C activity in the liver. Based on these results, SIRT1-mediated de-acetylation would have an inverse effect on PEPCK-C gene transcription in the two tissues: inhibition in the liver and induction the adipose tissue. This is similar to the differential effect of glucocorticoids on PEPCK-C gene transcription in adipose tissue and liver (59–61). This underscores the importance of insuring that excess FFA does not accumulate in the blood, because fatty acid metabolism in the skeletal muscle inhibits both the uptake of glucose from the blood and its subsequent oxidation in that tissue (62), often leading to insulin resistance and Type 2 diabetes (63). The effect of SIRT1 on the control of PEPCK-C gene transcription in adipose tissues is potentially a key factor in understanding the mechanism underlying the effect of drugs aimed at altering the rate of acetylation of transcription factors to control the clinical symptoms of Type 2 diabetes in humans. In view of the contrasting results discussed above, more extensive studies on the effect of resveratrol and its analogues on PEPCK-C gene transcription on white adipose tissue would be of interest.

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