Genes: Structure and Regulation:

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Hepatic Nuclear Factor 3 and Nuclear Factor 1 Regulate 5-Aminolevulinate Synthase Gene Expression and Are Involved in Insulin Repression*

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Although the negative regulation of gene expression by insulin has been widely studied, the transcription factors responsible for the insulin effect are still unknown. The purpose of this work was to explore the molecular mechanisms involved in the insulin repression of the 5-aminolevulinate synthase (ALAS) gene. Deletion analysis of the 5′-regulatory region allowed us to identify an insulin-responsive region located at −459 to −354 bp. This fragment contains a highly homologous insulin-responsive (IRE) sequence. By transient transfection assays, we determined that hepatic nuclear factor 3 (HNF3) and nuclear factor 1 (NF1) are necessary for an appropriate expression of the ALAS gene. Insulin overrides the HNF3β or HNF3β plus NF1-mediated stimulation of ALAS transcriptional activity. Electrophoretic mobility shift assay and Southwestern blotting indicate that HNF3 binds to the ALAS promoter. Mutational analysis of this region revealed that IRE disruption abrogates insulin action, whereas mutation of the HNF3 element maintains hormone responsiveness. This dissociation between HNF3 binding and insulin action suggests that HNF3β is not the sole physiologic mediator of insulin-induced transcriptional repression. Furthermore, Southwestern blotting assay shows that at least two polypeptides other than HNF3β can bind to ALAS promoter and that this binding is dependent on the integrity of the IRE. We propose a model in which insulin exerts its negative effect through the disturbance of HNF3β binding or transactivation potential, probably due to specific phosphorylation of this transcription factor by Akt. In this regard, results obtained from transfection experiments using kinase inhibitors support this hypothesis. Due to this event, NF1 would lose accessibility to the promoter. The posttranslational modification of HNF3 would allow the binding of a protein complex that recognizes the core IRE. These results provide a potential mechanism for the insulin-mediated repression of IRE-containing promoters.

Insulin performs a central role in homeostasis regulating the expression of over 100 genes (1, 2). Many of these genes are regulated by insulin at the transcriptional level, but the molecular mechanisms by which this regulation is achieved are poorly understood. Progress in this area has been restricted by the fact that no unique consensus insulin-responsive sequence or element (IRE)[1] has been reported, in contrast with consensus-responsive elements described for other hormones. However, an IRE with a T(G/A)TTT(T/G)(G/T) core sequence has been associated with insulin-induced transcriptional repression of a number of metabolic genes, including those that encode phosphoenolpyruvate carboxykinase (PEPCK) (3), insulin-like growth factor-binding protein 1 (4), tyrosine aminotransferase (5), glucose-6-phosphatase (6), apolipoprotein CIII (7), and aspartate aminotransferase (8). *trans*-Acting factors that interact with the IRE of these genes have been identified, but none have been shown directly to mediate the insulin response. Several genes involved in carbohydrate metabolism are negatively regulated by insulin through members of the C/EBP (9) and HNF3 transcription factor families (10). In addition, NF-1 was shown to mediate repression of the glucose transporter type 4 promoter by insulin (11). However, in no case does the binding of one of these proteins correlate with the effect of insulin. Thus, the actual function of this consensus motif and the proteins that are relevant for the negative insulin effect are still under investigation.

The first step of the heme biosynthesis in mammalian cells is catalyzed by the mitochondrial matrix and rate-limiting enzyme 5-aminolevulinate synthase (ALAS). There are two related ALAS isozymes that are encoded by two separate genes located on different chromosomes (12). The erythroid cell-specific enzyme or ALAS-2 is developmentally regulated, and it markedly increases during erythropoiesis to meet the demand for heme during hemoglobin production (13). The second enzyme, ubiquitous or liver-type ALAS (ALAS-1), is probably expressed in all tissues to provide heme for cytochromes and other hemoproteins (14).

Expression of ALAS in the liver was found to be subject to feedback regulation by heme, the end product of the pathway (14). In addition, the liver ALAS gene is under multicomponent control at the transcriptional level. Transcription of the ALAS gene is stimulated by cAMP (15) and respiratory uncoupling (16), whereas phorbol esters (17) and insulin (18) repress ALAS gene transcription. This pattern of regulation is accomplished by CREB, nuclear respiratory factor-1, and AP-1 transactivation through the *cis*-acting elements CRE, nuclear respiratory

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[1] The abbreviations used are: IRE, insulin-responsive element; AIP, acute intermittent porphyria; ALAS, 5-aminolevulinate synthase; AP-1, activation protein-1; CAT, chloramphenicol acetyltransferase; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; HNF3, hepatocyte nuclear factor 3; NF-1, nuclear factor-1; PEPCK, phosphoenolpyruvate carboxykinase.

factor-1-binding site, and 12-*O*-tetradecanoylphorbol-13-acetate-responsive element, respectively, found in the 5′-regulatory region of ALAS gene. Although evidence has been provided that insulin provokes a rapid inhibition of ALAS transcription and that 800 bp of the proximal 5′-flanking region of the ALAS gene are sufficient to confer this negative regulation (18), the elements and transcription factors involved in insulin-mediated regulation of ALAS gene expression are still unknown. In a recent report (19), we showed that activation of two signaling pathways often considered to be functionally separated during insulin action, the Ras/extracellular signal-regulated kinase/$p90^{RSK}$ pathway and the phosphatidylinositol 3-kinase/protein kinase B pathway, are jointly required for insulin-mediated inhibition of ALAS gene expression in rat hepatocytes and human hepatoma cells. This may suggest that these two pathways converge on a common transcription factor or complex that is targeted by insulin.

Because of the complexity of insulin action at the gene level, it is important to delineate the actual contribution of the regulatory sequences in several promoters to identify common themes in signaling by this hormone. The purpose of this study was to examine the molecular mechanism underlying insulin-inhibited expression of ALAS and to establish the main regulatory elements and transcription factors that determine insulin responsiveness in the ALAS gene. Our results show the presence of a functional binding site for the transcription factor HNF3 and a putative response sequence for NF1 in the proximal ALAS promoter. Both transcription factors appear to be necessary to achieve complete basal ALAS expression, although NF1 would need the presence of the HNF3 factor. In addition to the transcription factor-binding sites, this region includes an IRE-like sequence, localized at position −383 to −389 bp, overlapping the HNF3 site in an inverted orientation. Mutation of IRE is required to abrogate the insulin effect. From the data reported in this paper, it is apparent that insulin could interfere with HNF3 and NF1 binding or transactivation potential necessary, but not sufficient, to mediate its negative effect on ALAS gene transcription. Likewise, the participation of an unknown IRE-dependent binding inhibitory factor on ALAS promoter is hypothesized. These results provide a potential mechanism for the insulin-mediated repression of IRE-containing genes.

EXPERIMENTAL PROCEDURES

Expression Vectors—The following expression vectors were used as indicated in each experiment. The plasmid pACAT contains the −833 to +42 sequence of hepatic rat ALAS gene cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene in vector pBLCAT6 (18). The deletion-mutant plasmids pAΔ459CAT, pAΔ354CAT, pAΔ156CAT, pAΔ75CAT, and pAΔ38CAT were described previously (15). The A-ZIP series of expression vectors (A-Fos, A-CREB, and A-C/EBP), kindly supplied by Dr. Charles Vinson (NCI, National Institutes of Health, Bethesda), are cytomegalovirus-driven vectors, in which the normal basic region critical for DNA binding at the N terminus of the leucine zipper was replaced by an acidic sequence, and have been described before (20). The mammalian expression plasmids for HNF3α, pRBT7mHNF3α (21), and HNF3β, pCMV-HNF3β (22) were the generous gifts from Dr. Kenneth Zaret (Fox Chase Cancer Center, Philadelphia) and Dr. Robert Costa (University of Illinois, Chicago), respectively. The mammalian expression vector for NF1, pCMV-CTF/NF1, has been described previously (23) and was kindly provided by Dr. Pilar Santisteban (Universidad Autónoma de Madrid, España). Plasmid pCEFL containing the β-galactosidase gene and puroBABE vector, which conveys resistance to puromycin, were also used.

Plasmid Generation—The plasmid p4XCRECAT contains four CRE sites from the somatostatin gene cloned into the HindIII-XbaI site of the pBLCAT2 vector (provided by Dr. P. Sassone-Corsi, Institut de Génetique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). The plasmid pALIRE, containing the hepatic rat ALAS promoter fragment −459/−354 downstream of CRE sites, was created from pACAT vector by digestion with PstI and AflII. The purified fragment as well as the BamHI 4XCRECAT digested vector were blunt-ended with Klenow fragment and ligated by using T4 DNA ligase (New England Biolabs). The pALIREinv contains the same 106-bp fragment from the ALAS promoter cloned in the inverted direction. Oligonucleotides of −459/−354 fragment truncations, containing promoter region −459 to −420 (5′-GCATCTCTTCACCAGGACCATTCTATTCTTGGGCCATTCA), or −419 to −380 of the ALAS gene (5′-TCAAGTAAAGAATCCCTGTCATCGATGCAAACAAAACCAA) or mutated versions of the latter sequence, 5′-TCAAGTAAAGAATCCCTGTCATCGATGCAAACAATAG-CAA, in which underlined bases have been mutated to disrupt a putative IRE sequence, and 5′-TCAAGTAAAGAATCCCTGTCATCGCGGC-CCCCAAAACCAA-3′, in which underlined bases have been mutated to disrupt the HNF3-like binding site, were cloned into the BamHI site of p4XCRECAT to generate pALIRE I, pALIRE II, pALIREm II, and pALIREmHs II, respectively. The fidelity of all generated plasmids was checked by DNA sequencing.

Cell Culture—The human hepatoma cell line HepG2 was grown as monolayers in minimum essential medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% penicillin/streptomycin, 1% glutamax (Invitrogen), and 100 mM Hepes. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and the same additions. Both cell lines were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Transient Transfections and CAT Assay—All plasmids used for transfection assays were isolated from *Escherichia coli* strain DH5α and purified using the Wizard Maxiprep kit (Promega), and the DNA concentration was estimated spectrophotometrically. Transient transfections were described previously in detail (15, 18). In brief, cells were plated in 35-mm dishes at a density of 5×10^5 cells/well in 2.5 ml of medium. After a 24-h attachment period, transfections were performed according to the standard calcium phosphate precipitation method. Each well was transfected with a mixture containing 4 μg of pACAT or its derivatives and 6.5 μg of pCEFLβgal along with the plasmids indicated in each experiment. The amount of DNA used for transfection was kept constant in all the samples by varying the amount of nonspecific DNA carrier. Control transfections with carrier alone and carrier plus vector pBLCAT6 or p4XTRECAT were performed in parallel. After 16 h, the medium was replaced with 2.5 ml of serum-free medium, and cells were incubated for 24 h in the presence or in the absence of 10 nM insulin and/or the additions indicated in each experiment. As indicated in some of the experiments, 200 nM wortmannin and/or 10 μM PD98059 were added 30 min prior to insulin addition. Wortmannin was replenished twice during the incubation.

Analysis of CAT and β-galactosidase activities were performed in cell extracts as described previously (15, 18), according to Seed and Sheen (24) phase extraction assay for CAT activity. β-Galactosidase activity was expressed as $A_{420} \times \mu g$ of protein^{-1} h^{-1}. CAT activity was expressed as the amount of radiolabeled chloramphenicol acetylated by 1 mg of protein in 1 min and normalized for equal transfection efficiency with β-galactosidase activity. β-Galactosidase activity was not modified by any of the treatments used.

Antisense and Double-stranded Oligodeoxynucleotides Studies—HNF3 antisense oligodeoxynucleotide (5′-CGGCTCGTGCCCTTCCAT-CTTCAC-3′), corresponding to codons 5–12 of human HNF3β mRNA (25), and NF1 antisense oligodeoxynucleotide (5′-CAGTGCCTCGATG-AACGGGTGGAA-3′), complementary to codons 4–11 of human NF1 mRNA (26), were designed to block synthesis of these proteins. The HNF3 and NF1 sense oligodeoxynucleotides were used as controls. Double-stranded oligodeoxynucleotide representing the consensus sequence for PEPCK HNF3 (5′-GCCCATTGTTTGTTTTAAGCC-3′) (27) and double-stranded oligodeoxynucleotide representing the consensus sequence for NF1 (5′-GGCACCTGTTTCAATTTGGCACGGAGCCAAC-AG-3′) or mutated versions for HNF3 (5′-GCCCATTGGGCCATTTAA-GCC-3′) and NF1 (5′-GGCACCTGTTTCAATTTGTTACGGATTCAAC-AG-3′) in which underlined bases have been mutated, were used in competition experiments. To study the effects of oligodeoxynucleotides on CAT expression, transfected HepG2 cells were incubated for 24 h in serum-free medium containing 2 μM of the indicated oligodeoxynucleotide. After 12 h media were removed and replaced by fresh media containing oligodeoxynucleotides.

RNA Isolation and Northern Blot Analysis—Total cellular RNA was isolated from transfected and cultured HepG2 and HeLa cells according to Chomczinsky and Sacchi (28). Transfections were performed with LipofectAMINE Plus reagent (Invitrogen) following the manufacturer's protocol. Both cell lines were cotranfected with 2 μg of pCMV-HNF3β or 2 μg of pCMV-CTF/NF1 along with 1 μg of puroBABE. Puromycin (Sigma) (2.5 μg/ml) was added 24 h after transfection. Resistant clones were harvested 72 h later. Twenty four hours before harvesting, the

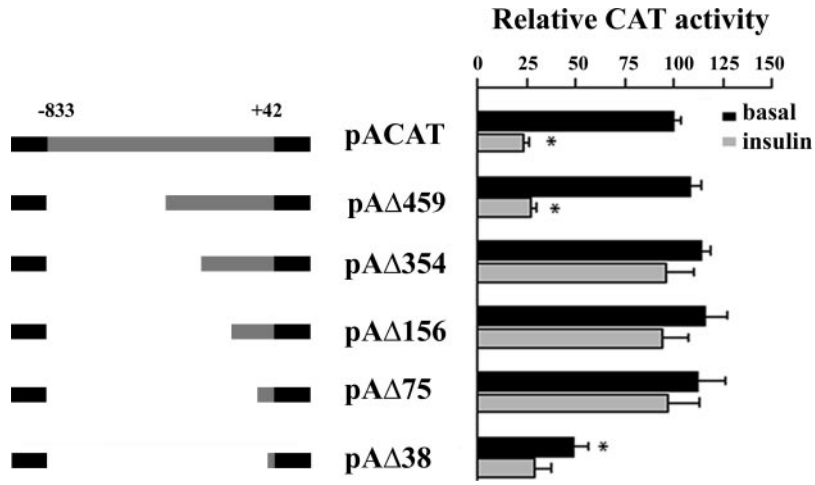

FIG. 1. **Deletion analysis reveals a region important for insulin-mediated inhibition of ALAS promoter activity.** HepG2 cells transiently transfected with 4 μg/plate of pACAT, or equivalent amounts of deletion mutants containing the 5′-flanking region of ALAS gene illustrated on the *left*, were incubated in serum-free medium alone (*black bars*) or with the addition of 10 nM insulin (*gray bars*). After 24 h, cells were harvested, and CAT activity was determined as described. Results are expressed as relative CAT activity with respect to basal value for each construction, which was set to 100. *Bars* represent mean ± S.E. of four independent experiments performed in duplicate. Student's *t* test was used to compare insulin-treated and nontreated samples (*, $p < 0.05$).

cells were placed in serum-free medium and incubated with or without insulin for the last 8 h. Insulin concentration was 10 nM for HepG2 cells and 1 μM for HeLa cells. The yield and purity of RNA samples were assessed by absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm, respectively. For Northern blot analysis, 20 μg of total RNA was denatured, electrophoresed in 1% glyoxal/agarose gels, and transferred to nylon membranes (Hybond N, Amersham Biosciences). The membranes were sequentially hybridized with ^{32}P-labeled probes to ALAS, HNF3β, NF1-X, and β-tubulin. To detect ALAS mRNA, a 26-mer oligodeoxynucleotide was synthesized complementary to bases +328 to +353 of human hepatic ALAS mRNA (29). To detect HNF3 mRNA, a 24-mer oligodeoxynucleotide was synthesized complementary to bases +13 to +36 of human HNF3 mRNA (25). The oligodeoxynucleotides were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The resulting probe had a specific activity of about $4-6 \times 10^3$ cpm/fmol. Hybridization was carried out overnight at 70 °C in the same prehybridization solution by adding the ^{32}P-labeled oligodeoxynucleotide (3.0×10^5 cpm/cm^2) as described previously (30). To detect β-tubulin mRNA or NF1 mRNA, β-tubulin or NF1-X cDNA was labeled by random priming using [α-^{32}P]dCTP and Klenow to a specific activity of about $4-6 \times 10^8$ cpm/μg. Membranes were stripped, prehybridized, hybridized, and washed in standard conditions described by Sambrook and Russell (31). The membranes were then exposed to x-ray film or scanned directly onto a Bio-Imaging Analyzer Fujifilm LAS-1000 and quantified.

Electrophoretic Mobility Shift Assays—HepG2 cells were plated in 9-cm culture plates at a density of 2.5×10^6 cells in 9 ml of medium. After 24 h of incubation in serum-free medium, the cells were treated with 10 nM insulin for 15, 30, or 60 min and then harvested. Nuclear extracts were prepared as described by Andrews and Faller (32). The double-stranded DNA probes and unlabeled competitors used were ALIRE II (fragment −419/−380 from ALAS promoter), a mutant version of ALIRE II called ALIREm II, in which bases at −385 and −383 have been mutated to disrupt a putative IRE sequence and HNF3 5′-GCACTAGCAAAACAAACTTATTTTGAACACG, containing the consensus sites for wild type HNF3 (33) transcription factor. To generate radioactive probes, the sense and antisense oligodeoxynucleotides were annealed and labeled using [γ-^{32}P]ATP (222 Tbq/mmol) (PerkinElmer Life Sciences) and T4 polynucleotide kinase (New England Biolabs) as described before (15). Binding reactions were performed by mixing 10 μg of nuclear extract with 3 μg of poly(dI-dC)·poly(dI-dC) and 100,000 cpm of the labeled probe in binding buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol) in a 20-μl final volume. The reaction was incubated for 20 min at room temperature. For competition assays, 50–200-fold excess of unlabeled competitor oligonucleotides were mixed 20 min prior to the addition of labeled probes. After the binding reactions, the samples were directly loaded onto a 5% (w/v) nondenaturing polyacrylamide gel containing 0.25× TBE (1× TBE: 50 mM Tris borate, 1 mM EDTA, pH 8.3). The gel was pre-electrophoresed at 100 V for 1 h in the cold room, and electrophoresis was performed at 180 V for 3 h in 0.25× TBE. The gel was then dried and autoradiographed by exposing overnight to Kodak XAR-5 films with an intensifying screen at −70 °C. Oligodeoxynucleotides were chemically synthesized by Bio-Synthesis Inc. (Lewisville, TX).

The supershift analyses were performed by incubating the nuclear extract with 3 μl of specific antibody at 4 °C overnight, prior to band-shift assays as described previously. Antibody against HNF3β was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Southwestern and Western Blotting Procedures—Southwestern blotting was based on the procedure described by Dong *et al.* (34). Nuclear extracts (75–100 μg) from HepG2 cells were loaded in duplicate onto an SDS-10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was cut in two. One-half was subjected to Southwestern blotting and the other half to Western blotting. For Southwestern blotting, membrane-bound proteins were allowed to renature in hybridization buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 5% of non-fat dry milk) for 3 h at room temperature. The membrane was then rinsed twice with the hybridization buffer containing 0.25% non-fat dry milk. Subsequently, the nitrocellulose was hybridized with the end-labeled probe (10^7 cpm) in 25 ml of hybridization buffer containing 0.25% non-fat dry milk and 250 μg of poly(dI-dC)·poly(dI-dC) overnight at room temperature. The filter was washed five times (10 min each) with hybridization solution containing 0.25% non-fat dry milk, dried, and exposed overnight to x-ray film or scanned directly onto a Bio-Imaging Analyzer Fujifilm LAS-1000 and quantified.

For Western blot, membrane-bound proteins were probed with polyclonal anti-rabbit anti-HNF3β (Santa Cruz Biotechnology). The antibody was detected by using horseradish peroxidase-linked goat anti-rabbit IgG (Sigma), visualized by the Pierce Super Signal Ultra Chemiluminescence signaling system and a PhosphorImager FujiFilm LAS-1000.

Data Analysis—Most of the experiments were carried out at least three times. All transfection studies were performed at least in four separate experiments, in which duplicate dishes were transfected. All data were expressed as means ± S.E. When statistical analysis was performed, data were compared by the paired Student's *t* test, and *p* values below 0.05 were considered significant.

RESULTS

The −459/−354 Fragment Is Required for Insulin-mediated Inhibition of ALAS Promoter—We reported previously that the fragment between −833 and +42 bp of the 5′-flanking region of the ALAS gene is enough for basal expression and is able to confer insulin-mediated inhibition on ALAS promoter activity (18). In order to identify specific sequences within this upstream region that are essential for insulin inhibition, the pACAT construct, containing the entire region, was deleted stepwise, and the resulting constructs were analyzed for CAT activity in transiently transfected HepG2 cells. As shown in Fig. 1, deletion of the sequence between −833 and −459 did not significantly impair the insulin-inhibited promoter activity. Further deletion of the promoter from −459 to −354 completely abolished the regulatory effect of insulin. Subsequent deletions were no longer responsive to the hormone. These results strongly suggest that the region between −459 and −354 in the ALAS promoter contains essential elements that confer insulin responsiveness. It is important to note that there were no significant differences in the basal expression of the ALAS/CAT

TABLE I
Effect of dominant negative variants of CREB, C/EBP, and Fos and coactivators CBP/p300 on the transcriptional activity of ALAS promoter

HepG2 cells were transiently transfected with 4 μg/plate of pACAT and cotransfected or not with 3 μg/plate of each of the indicated expression vectors in the presence or in the absence of 10 nm insulin. Results are expressed as relative CAT activity with respect to the basal value of pACAT, which was set to 100. Values are mean ± S.E. of three different experiments performed in duplicate.

Treatment	Basal	Insulin
	% relative CAT activity	
None	100.0 ± 3.3	35.6 ± 3.4
A-CREB	139.2 ± 7.2	37.0 ± 3.3
A-C/EBP	126.5 ± 5.5	36.5 ± 4.1
A-Fos	112.0 ± 4.6	32.4 ± 2.1
CBP	157.3 ± 6.1	36.9 ± 3.9
p300	144.7 ± 8.6	41.1 ± 5.1

fusion gene among the deletion mutants tested, except for pAΔ38CAT, in which CAT activity fell to values 50% lower than those of the entire promoter.

We next analyzed whether other sequences on the ALAS promoter, adjacent to the −459/−354 fragment, although not sufficient, would be required to exert full insulin responsiveness. Previous studies (15, 17) have demonstrated that ALAS promoter contains functional binding sites for CREB (−149 and −45 bp) and AP-1 (−261 bp) transcription factors. Other factors, like C/EBP, are predicted to bind to the proximal 459 bp in the ALAS gene 5′-flanking region, based on the similarity of their recognition sites to this sequence (35). We concentrated on examining the role of these factors, which are expressed in HepG2, because they are regulated by phosphorylation and had been reported to be involved in the regulation of several genes by insulin (3, 8). To investigate the functional contribution of CREB, AP-1, and C/EBP factors to the regulation of ALAS transcription by insulin, dominant negative variants of these proteins were transfected into HepG2 cells (20). These factors contain, instead of the DNA binding domain, an acidic domain complementary in charge distribution to the basic region of the targeted factor. As a result, when the A-ZIP factor dimerizes with a wild type factor to form a coiled coil through the leucine zipper region, the respective acidic and basic regions continue the formation of a very stable helical structure that engages the basic region of the wild type factor and prevents it from binding DNA. Overexpression of A-CREB or A-Fos or A-C/EBP had no effect upon the hormonal regulation of ALAS/CAT (Table I). However, expression of A-CREB slightly increased the basal ALAS promoter activity. Overexpression of the empty vector in which the A-ZIP factors are cloned had no effect upon the extent or the pattern of regulation (data not shown). Thus, none of these factors are essential for inhibition by insulin. Recent studies (36) have shown that insulin can disrupt the interaction of CBP/p300 with several transcription factors known to be important in the regulation of gene expression. Because CBP/p300 proteins are important for transactivation by CREB and this transcription factor is involved in the basal expression of ALAS (15), we considered the possibility that insulin signaling may modify interactions between CBP/p300 and CREB. Overexpression of coactivators CBP or p300 did not modify the inhibitory effect of insulin on ALAS promoter activity (Table I).

Predicted Regulatory Sites in the −459/−354 Fragment—Computer-aided analyses (35) of the −459/−354 region of the ALAS promoter revealed several motifs resembling consensus sequences for binding of many known nuclear factors. Of particular interest were two potential sites for HNF3, located at −417 and −396 bp, and one putative site for NF1 in reverse orientation, located at −425 bp. These nuclear proteins have been reported to be involved in insulin repression of PEPCK (37) and glucose transporter type 4 (38) promoters, respectively (Fig. 2*A*). In addition, the sequence GGTTTTG, highly homologous to the IRE found in several promoters repressed by insulin, overlaps the HNF3 potential binding sites in an inverted orientation. Based on this sequence information, several constructs were made to identify the elements required for the inhibition of ALAS promoter activity by insulin.

The −459/−354 Fragment of the ALAS Gene Promoter Is Sufficient to Confer Hormonal Regulation to a Heterologous Promoter—The aforementioned results prompted us to examine whether the region between −459 and −354 bp directly mediates the effect of insulin on basal ALAS gene transcription. To address this question, we first introduced this fragment in front of a minimal heterologous promoter from thymidine kinase gene and downstream from four CRE sites, driving the CAT reporter gene, to ensure that it was indeed responsible for the action of the peptidic hormone. This construct, pALIRE, was transiently transfected into HepG2 cells. The basal promoter activity of pALIRE was increased with respect to the empty reporter vector. Insulin was capable of inhibiting CAT expression by 56% in this chimeric promoter. However, the parental p4XCRECAT vector or the plasmid harboring the ALAS fragment in the inverted orientation (pALIREinv) were insensible to the presence of insulin (Fig. 2*B*). To refine this analysis, we dissected this region in two smaller fragments encompassing the −459 to −420 bp (pALIRE I), containing the potential NF1-binding site, and −419 to −380 bp (pALIRE II), containing the putative HNF3-binding sites and the IRE, which were cloned into the p4XCRECAT vector. As shown in Fig. 2*B*, the ALIRE I promoter activity was not modified in insulin-treated cells. On the other hand, CAT expression in HepG2 cells transfected with the construct containing two putative HNF3-binding sites and the IRE was significantly diminished in the presence of insulin, whereas a mutant version (pALIREm II), obtained by altering the IRE sequence from GGTTTTG to GGTTATC, was no longer responsive to the hormone.

In previous papers we demonstrated that the cAMP nonmetabolizable derivative 8-CPT-cAMP leads to transcriptional activation of the ALAS gene in rat hepatocytes (18) and HepG2 cells (15). This cAMP-dependent induction of ALAS expression is repressed by insulin, in a dominant fashion, in both cellular systems. In order to obtain a clearer insight about the nature of the sequences involved in the inhibitory effect exerted by the hormone, we assayed the ALIRE constructs in transient transfection assays in HepG2 cells in the presence of 8-CPT-cAMP. As shown in Fig. 2*B*, the CAT activity of all reporter vectors was increased when the cells were stimulated by cAMP. However, the cAMP effect was counteracted by the presence of insulin, only by the ALIRE fragment (−459/−354) and the smaller construct ALIRE II (−420/−380) which maintained 38 and 60% CAT activity, respectively. These results confirm that the −459/−354 region of the ALAS promoter is sufficient to confer insulin responsiveness and suggest that the potential HNF3-binding sites and the IRE are the most crucial elements. They also indicate that the region containing the putative NF1-binding site may be important to achieve the full inhibitory effect, considering the difference in CAT activity between pALIRE and pALIRE II. More important, these data also indicate that the same *cis*-element(s) required for effective insulin repression of basal ALAS gene expression may be involved in insulin inhibition of cAMP-induced transcription.

HNF3 Isoforms and NF1 Are Involved in ALAS Gene Expression—To assess further the role of HNF3 and NF1 in the

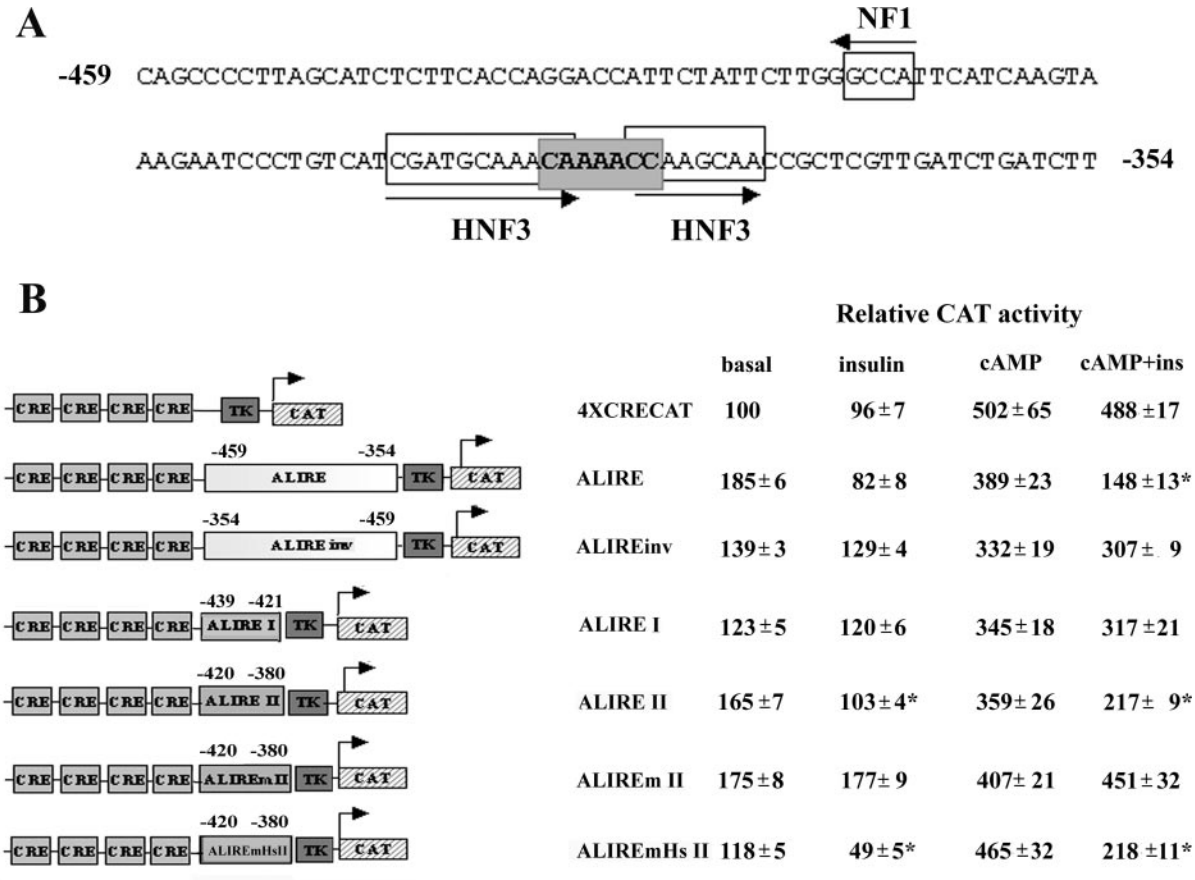

FIG. 2. **Identification of consensus sequences in the human ALAS promoter −459 to −354 fragment that confer insulin responsiveness.** *A*, schematic diagram of ALAS promoter −459 to −354-bp fragment. The MatInspector data base search was employed, and only the highest probability sites are indicated. Searches were conducted in both the forward and reverse direction. The *boxed areas* represent the potential HNF3- and NF1-binding sites, and the *gray boxed* heptanucleotide represents the putative ALAS-IRE. *B*, HepG2 cells were transiently transfected with 4 μg/plate of the chimeric expression vector illustrated on the *left*. Cell cultures were treated or not with 10 nM insulin and/or 100 μM 8-CPT-cAMP for 24 h. Results are expressed as relative CAT activity with respect to basal value for p4XCRECAT, which was set to 100. Values represent mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to compare insulin-treated and nontreated samples (*, $p < 0.05$).

repression of ALAS transcription by insulin, we examined whether these transcription factors were able to interfere or mimic this inhibitory effect in HepG2 cells. Therefore, HepG2 cells were cotransfected with pAΔ459CAT and vectors encoding HNF3α or -β isoforms or the NF1 protein. Surprisingly, overexpression of HNF3α or -β isoforms exhibited opposite behaviors on ALAS/CAT expression (Fig. 3*A*). Whereas the α isoform significantly decreased the basal ALAS promoter activity without modifying insulin action, overexpression of β isoform increased the basal expression and markedly blunted the insulin repression of ALAS/CAT activity. In contrast to what happened with HNF3 isoforms, overexpression of NF1 did not alter CAT expression in both basal and insulin-treated cells. When vectors encoding for HNF3β and NF1 were jointly cotransfected in HepG2 cells, a synergistic stimulation of basal promoter activity was achieved. Most important, the ability of HNF3β to block the insulin inhibitory effect was increased from 45 to 62% in the presence of overexpressed NF1 (Fig. 3*A*).

The ability of the HNF3β to abolish insulin-dependent inhibition of ALAS/CAT activity prompted us to examine whether overexpression of HNF3β in HepG2 cells correspondingly suppresses insulin-dependent inhibition of the endogenous ALAS gene. As shown in Fig. 3*B*, the decrease in the ALAS mRNA level observed after insulin treatment was prevented in cells that overexpressed HNF3β. A similar experiment showed that insulin inhibition of ALAS mRNA expression was not affected in HepG2 cells overexpressing the NF1 protein.

In another approach, we used HNF3β or NF1 antisense oligodeoxynucleotides or double-stranded oligodeoxynucleotides, representing the consensus sequences for HNF3 or NF1, in order to diminish the functional endogenous levels of these transcription factors either by blocking its synthesis or by offering competitor DNA harboring binding sequences, respectively. As shown in Fig. 3*C* incubation with HNF3 antisense or HNF3-binding site containing oligodeoxynucleotides inhibited the basal ALAS promoter activity mimicking the action of insulin. Remarkably, there was no additional reductions in ALAS/CAT expression when either HNF3β antisense or HNF3 double-stranded oligodeoxynucleotides were incubated in the presence of insulin. A similar effect on ALAS/CAT expression was observed when HepG2 cells transfected with pAΔ459CAT were incubated with NF1 antisense or double-stranded oligodeoxynucleotides containing the consensus site for NF1 protein (Fig. 3*D*). The inhibitory effect produced by the double-stranded oligodeoxynucleotide was not observed when mutations that disrupted the core motif of HNF3- or NF1-binding site were introduced (Fig. 3, *C* and *D*) or scrambled antisenses were used (data not shown).

Taken together, these results suggest that HNF3β and NF1 are necessary for an appropriate expression of ALAS, although the NF1 effect seems to be dependent on the presence of the forkhead transcription factor. We can hypothesize that insulin modifies the ability of HNF3β, and probably NF1, to modulate transcription driving to a repressed ALAS expression.

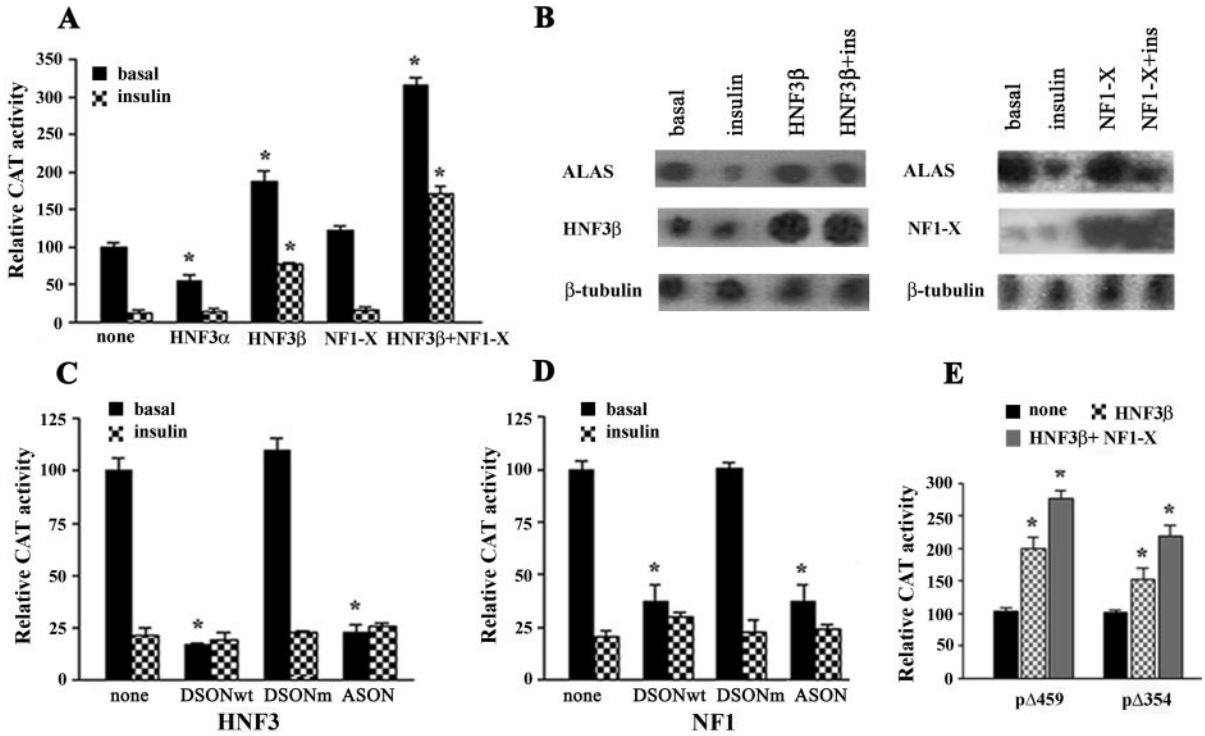

FIG. 3. **Effect of HNF3α, HNF3β, and NF1 transcription factors on insulin-mediated inhibition of ALAS promoter activity and ALAS mRNA levels.** *A*, HepG2 cells were transiently transfected with 4 μg/plate of pAΔ459CAT and cotransfected or not with 6 μg/plate of each of the expression vectors for HNF3α or HNF3β and NF1-X proteins as indicated. Samples were incubated in serum-free medium in the presence or absence of 10 nM insulin for 24 h. Results are expressed as relative CAT activity with respect to the basal value of pAΔ459CAT, which was set to 100. *B*, HepG2 cells were transfected with expression vectors encoding for HNF3β or NF1-X and puroBABE which conveys resistant to puromycin. Twenty four hours after transfection, cells were treated for an additional 60 h with puromycin. Resistant cells were placed in serum-free medium and incubated in the presence or absence of 10 nM insulin for the last 8 h. Total RNA (20 μg) was separated on denaturing agarose gels, blotted onto nylon membrane, and hybridized to ^{32}P-labeled probes specific for ALAS, HNF3β, or NF1-X as described under "Experimental Procedures." β-Tubulin served as a loading control. *C* and *D*, HepG2 cells were transiently transfected with 4 μg/plate of pAΔ459CAT and incubated for 24 h in serum-free medium containing or not 2 μM double-stranded oligodeoxynucleotide (*DSONwt*) or a mutant version (*DSONm*) or antisense oligodeoxynucleotides (*ASON*) to HNF3β or NF1, as indicated, in the presence or absence of 10 nM insulin. Results are expressed as relative CAT activity with respect to the basal value of pAΔ459CAT, which was set to 100. *E*, HepG2 cells were transiently transfected with 4 μg/plate of pAΔ459CAT or pAΔ354CAT and cotransfected or not with 6 μg/plate of each of the expression vectors for HNF3β and/or NF1-X proteins as indicated and incubated in serum-free medium for 24 h. Results are expressed as relative CAT activity with respect to basal value of pAΔ459CAT or pAΔ354CAT, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. Student's *t* test was used to compare samples cotransfected and non-cotransfected (*, $p < 0.05$) (*A*); samples treated or nontreated with double-stranded oligodeoxynucleotide (*DSONwt*), mutant version (*DSONm*), or antisense oligodeoxynucleotides (*ASON*), respectively (*, $p < 0.05$) (*C* and *D*); samples cotransfected and non-cotransfected with HNF3β (*, $p < 0.05$); and samples cotransfected with NF1-X or not (*, $p < 0.05$) (*E*).

The results of the ALAS promoter deletion analysis (Fig. 1) showed that pAΔ354CAT mutant abolished the inhibitory effect of insulin, but it did not significantly affect the basal expression of the ALAS/CAT fusion gene. This truncation remove the putative HNF3 sites located at −417 and −396 bp and the NF1 site found at −425 bp. Nevertheless, the above results indicate that HNF3 and NF1 seem to be necessary for basal ALAS gene expression. We reasoned that if HNF3 and NF1 play a role in ALAS basal expression, other binding sites for these transcription factors should be met downstream. To address this question, we examined the promoter activity of pAΔ354CAT in the presence of overexpressed HNF3β and NF1 proteins. As summarized in Fig. 3*E*, the pattern of pAΔ354CAT expression induced by HNF3 or HNF3 plus NF1 was similar to that of pAΔ459CAT, indicating the presence of binding sites for the mentioned transcription factors downstream −354 bp. These data are not consistent with a simple model proposing that insulin interferes with the ability of HNF3 and/or NF1 to bind the ALAS promoter to explain the hormone repression of ALAS expression. This evidence raises the intriguing possibility that the presence of the IRE sequence allows the interaction of an unknown inhibitory factor driving to ALAS down-regulation. In this regard, transfection experiments carried out with an expression vector containing a mutated HNF3-binding site version of the ALIRE II region (pALIREmHs II), which cannot bind HNF3 (data not shown), support this hypothesis. In contrast to what happens with pALIREm II, in which IRE disruption caused a lost of the insulin repression, the peptidic hormone was competent to repress the CAT expression of pALIREmHs II in an indistinguishable fashion from that of the wild type pALIRE II (Fig. 2*B*).

Insulin Does Not Repress ALAS Expression in HNF3-deficient HeLa Cells—To assess further the role of HNF3 on the basal ALAS promoter activity and the insulin-mediated repression, we extended the analysis to HeLa cells. These cells are devoid of HNF3 transcription factors (39) and contain high levels of NF1 proteins (40), thus providing a useful model system for gene reporter expression experiments. Most important, when pAΔ459CAT-transfected HeLa cells were incubated in the presence of insulin, no effect on ALAS/CAT expression was observed, even though a 100-fold higher hormone concentration was used (Fig. 4*A*). However, when HeLa cells were cotransfected with an expression vector encoding HNF3β, the ALAS promoter activity was significantly increase, and the ability of insulin to repress ALAS/CAT expression was restored (Fig. 4*A*).

We next asked whether HNF3 transcription factors could increase the endogenous ALAS gene in HeLa cells. For this

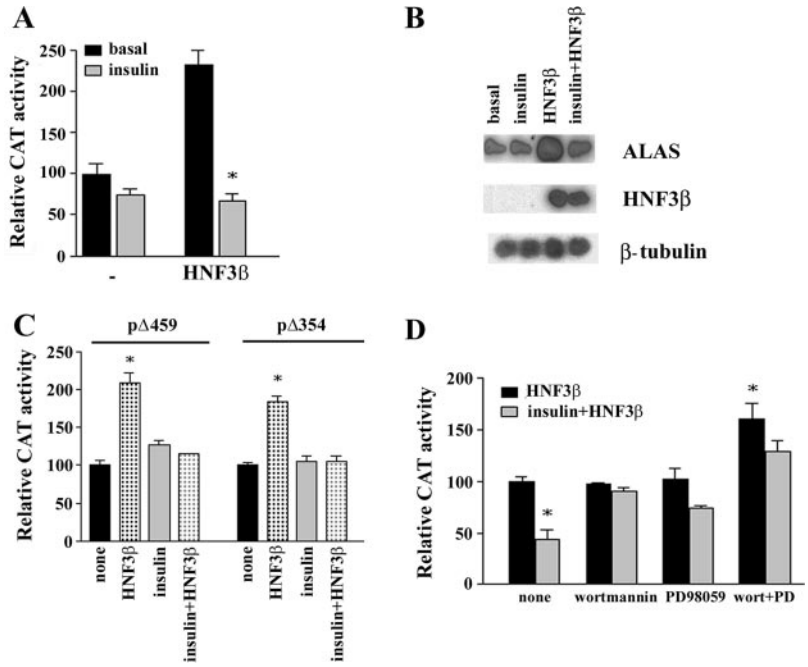

FIG. 4. **Insulin impairs HNF3β activation of ALAS promoter activity in HeLa cells.** *A*, HeLa cells were transiently transfected with 4 μg/plate of pAΔ459CAT and cotransfected or not with 6 μg/plate of the expression vector for HNF3β protein as indicated. Samples were incubated in serum-free medium in the presence or absence of 1 μM insulin for 24 h. Results are expressed as relative CAT activity with respect to basal value of pAΔ459CAT, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in duplicate. *B*, HeLa cells were transfected with the expression vector encoding for HNF3β and puroBABE, which conveys resistance to puromycin. Twenty four hours after transfection, cells were treated for an additional 60 h with puromycin. Resistant cells were placed in serum-free medium and incubated in the presence or absence of 1 μM insulin for the last 8 h. Total RNA (20 μg) was separated on denaturing agarose gels, blotted onto nylon membrane, and hybridized to ^{32}P-labeled probes specific for ALAS or HNF3β as described under "Experimental Procedures." β-Tubulin served as a loading control. *C*, HeLa cells were transiently transfected with 4 μg/plate of pAΔ459CAT or pAΔ354CAT and cotransfected or not with 6 μg/plate of expression plasmids for HNF3β. Samples were incubated in serum-free medium in the presence or absence of 1 μM insulin for 24 h. Results are expressed as relative CAT activity with respect to the basal value of pAΔ459CAT or pAΔ354CAT, which was set to 100. *D*, HeLa cells were transiently transfected with 4 μg/plate of pAΔ459CAT and cotransfected with 6 μg/plate of the expression vector for HNF3β protein. Samples were incubated in serum-free medium containing or not 1 μM insulin and/or 200 nM wortmannin (*wort*) and/or 10 μM PD98059 (*PD*) for 24 h. Inhibitors were added 30 min prior to the addition of the hormone, and wortmannin was replenished twice during incubation. Results are expressed as relative CAT activity with respect to the untreated sample cotransfected with pAΔ459CAT plus HNF3β, which was set to 100. *Bars* represent the mean ± S.E. of three different experiments performed in triplicate. Student's *t* test was used to compare insulin-treated and nontreated samples (*, $p < 0.05$) (*A*); samples cotransfected and non-cotransfected (*, $p < 0.05$) (*C*); samples containing insulin or insulin plus inhibitor(s) to samples without any addition (*, $p < 0.05$) and samples containing the inhibitor(s) to samples without any addition (*, $p < 0.05$) (*D*).

purpose, insulin-treated or untreated HeLa cells overexpressing HNF3β were used to perform Northern blot analysis to determine the ALAS and HNF3β mRNA levels. As shown in Fig. 4*B*, the overexpression of HNF3β increased ALAS mRNA and insulin impaired this induction. These results reinforce the hypothesis that HNF3 is essential for the basal expression of ALAS and provide a clue to elucidate the action of insulin-repressing ALAS promoter activity. As shown in Fig. 4*C*, the ALAS promoter transcriptional activity was induced when HNF3β was overexpressed in HeLa cells, even when pAΔ354CAT as a fusion reporter gene was used. Overexpressed HNF3-mediated induction of both pAΔ459CAT and pAΔ354CAT in HeLa cells was impaired by insulin. These results confirm that other HNF3-binding sites are localized downstream −354 bp and support the concept that insulin modifies the binding or transactivation capacity of HNF3 on the ALAS promoter in an IRE-independent manner.

In a previous paper (19) we demonstrated that both phosphatidylinositol 3-kinase and Ras/mitogen-activated protein kinase signaling pathways are required for the regulation of ALAS gene expression by insulin. Therefore, we performed HNF3 cotransfection experiments in HeLa cells in the presence of wortmannin, a phosphatidylinositol 3-kinase inhibitor, and/or the mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059. The rationale was that whether overexpression of HNF3 in HeLa cells correlates with the ability of insulin to repress ALAS promoter activity, this effect would be abolished by incubation with the mentioned inhibitors. As shown in Fig. 4*D*, the presence of wortmannin and/or PD98059 severely curtailed the effect of insulin on ALAS/CAT expression in HeLa cells overexpressing HNF3β. Although neither wortmannin nor PD98059 was able to modify HNF3β-induced expression of the ALAS/CAT fusion gene, the presence of both inhibitors increased ALAS promoter activity. This effect was observed previously in HepG2 cells (19).

HNF3 Binds ALAS Promoter at −419/−380 Region—We attempted to determine whether HNF3 transcription factors could bind to ALIRE II region of the ALAS promoter. This fragment includes putative HNF3 sites and a highly homologous IRE. We performed gel mobility shift assays with radiolabeled, double-stranded oligodeoxynucleotides containing this sequence and nuclear extracts isolated from HepG2 cells. A protein-DNA complex was visualized with ALIRE II probe, as revealed by the presence of a retarded band (Fig. 5). This complex was competed in a dose-dependent manner by unlabeled ALIRE II or by an unlabeled oligonucleotide containing the HNF3 consensus sequence. However, addition of a 200-fold molar excess of oligodeoxynucleotide ALIRE I, a region located immediately upstream of ALIRE II on the ALAS promoter, failed to prevent complex formation (data not shown). Furthermore, when the electrophoretic mobility shift assay was performed using a radiolabeled HNF3 probe, the two protein-DNA complexes formed were displaced with an excess of unlabeled ALIRE II (Fig. 5). However, we failed to detect a nucleoprotein

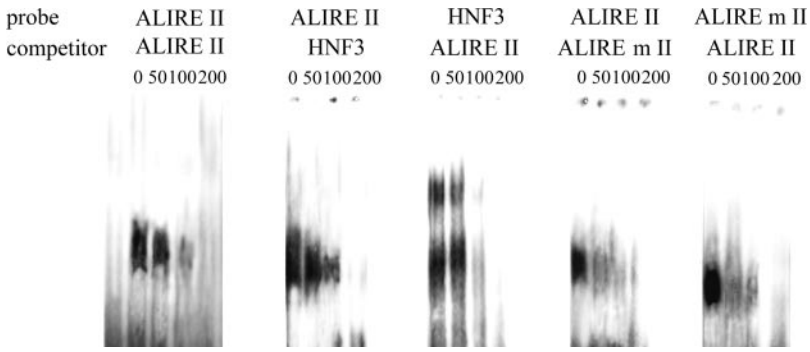

FIG. 5. **Putative HNF3 site forms a complex with proteins expressed in HepG2 nuclear extracts.** Ten μg of protein prepared from extracts of HepG2 cells were incubated with ^{32}P-labeled probes representing the putative HNF3 site at −382 (*ALIRE II*), or its mutated version (*ALIREm II*) or HNF3 consensus sequence (*HNF3*) in the presence or absence of increased quantities of unlabeled competitor oligodeoxynucleotides as indicated.

complex involving the ALIRE II region whose formation was disrupted by insulin treatment (data not shown). Finally, we examined whether mutation of the IRE, which affects the ability of ALIRE II to mediate the insulin response, correlates with a decreased binding ability. As shown in Fig. 5, the radiolabeled ALIREm II oligodeoxynucleotide, in which the IRE sequence was disrupted, formed a similar protein-DNA complex as efficiently as ALIRE II did and competed equally well with each other. These results indicate a dissociation between function and *in vitro* binding activity because the IRE mutation abolishes the ability of ALIRE II to mediate an insulin response (see Fig. 2*B*).

To assess further that HNF3 binds specifically to ALIRE II in the 5′-flanking region of the ALAS gene, two independent experimental approaches were used. Fig. 6*A* shows the results of a supershift assay, in which the incubation of HepG2 nuclear extracts with an antibody directed against HNF3β led to the appearance of a supershifted band. In the second approach, Southwestern blotting assay was performed. Samples of HepG2 nuclear extracts with binding activity were separated by SDS-PAGE and either visualized by Coomassie staining (not shown) or transferred to a nitrocellulose membrane and probed with radiolabeled ALIRE II, ALIREm II, or HNF3 consensus sequence oligonucleotides. A radioactive band, which comigrates with the 48-kDa species, was detected with all the used probes (Fig 6*B*). The identity of this protein was confirmed by Western blot with an antibody anti-HNF3β (data not shown). Most interesting, two faster migrating bands were observed when the membrane was treated with ALIRE II-radiolabeled probe but not when membrane was treated with HNF3 (Fig. 6*B*) or ALIREm II probe. These results confirm the identity of HNF3β as the transcription factor interacting with the ALIRE II region of the ALAS promoter and suggest that other smaller proteins could bind to this fragment in an IRE-dependent manner.

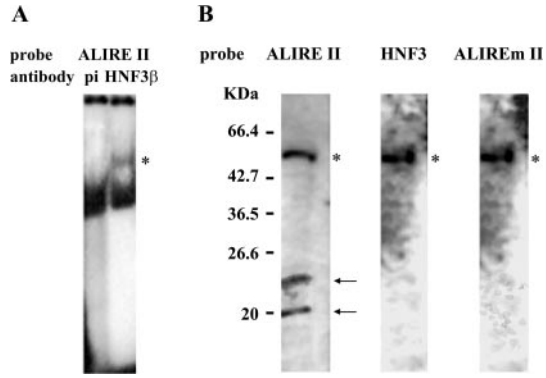

FIG. 6. **A functional HNF3β-binding site in the ALAS −419 to −380 fragment.** *A*, 10 μg of nuclear extract isolated from HepG2 cells were subjected to supershift assays. Nuclear proteins were preincubated with 3 μl of antibody against HNF3β protein, and then ^{32}P-labeled ALIRE II probe was added. Control were incubated with preimmune immunoglobulins (*pi*). The supershift is indicated by an *asterisk*. *B*, Southwestern analysis of the protein complex bound to the −419 to −380 fragment of the ALAS promoter. Nuclear extracts (100 μg) from HepG2 cells were electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membrane. Proteins on the membrane were renatured, and membrane was probed with ^{32}P-labeled ALIRE II or its mutated version ALIREm II or HNF3 (300,000 cpm) oligodeoxynucleotides. A 48-kDa DNA-binding protein was detected with the three probes that are indicated by *asterisks*. *Arrows* indicate two faster migrating bands detected only with ALIRE II probe.

DISCUSSION

Although the negative regulation of gene expression by insulin has been widely studied, the transcription factors responsible for the insulin effect are still unknown. The purpose of this work was to explore the molecular mechanisms involved in the insulin repression of the ALAS gene. Deletion analysis of the 5′-regulatory region of the ALAS gene allowed us to identify an insulin-responsive region located at −459 to −354 bp.

Insertion of this fragment into a heterologous promoter conferred insulin responsiveness confirming that this region is sufficient to drive transcriptional repression by insulin. We show that the same fragment of the ALAS promoter was able to inhibit cAMP-induced transcriptional activity when it was ligated to multiple CREB-binding sites. Yeagley *et al.* (41) have postulated that insulin inhibits basal transcription by a different mechanism than that utilized for inhibition of cAMP-dependent protein kinase-induced PEPCK transcription. They hypothesized that insulin disrupts interactions between CREB, CBP, and RNA polymerase II complex to repress cAMP-inducible PEPCK expression. With respect to ALAS regulation, however, our results suggest that the same mechanism is involved in insulin repression of both basal and cAMP-induced transcription. In this regard, the fact that the sole mutation of IRE was sufficient to abrogate the insulin effect in both conditions is highly significant. *In silico* analysis of the ALIRE fragment revealed the presence of highly homologous binding sites for HNF3 and NF1. As these factors are expressed in liver and regulated by protein kinases, they constitute promising targets for insulin action.

The data presented in this paper provide several facts indicating that HNF3β is involved in the basal expression of the ALAS gene. The observation that CAT activity was induced when a reporter gene containing the ALIRE region with two potential HNF3-binding sites was cotransfected with an expression vector encoding for HNF3β supports this hypothesis. Similar results were obtained in HeLa cells that are devoid of endogenous HNF3 isoforms. Conversely, when functional endogenous levels of HNF3β were diminished by incubation of HepG2 cells with antisense HNF3β or with double-stranded DNA representing the consensus element for this transcription factor, the activity of the ALAS promoter was strongly inhibited. Finally, Northern blot assays show that overexpression of HNF3β increases the levels of ALAS mRNA in both HepG2 and

HeLa cells. A similar approach demonstrates that transcription factor NF1 is also required for basal expression of ALAS. However, the effect of NF1 seems to be restricted to the presence of HNF3β. The HNF3 or FoxA (forkhead) family of proteins constitutes a class of transcriptional regulators originally identified as activators that coordinately regulate the expression of a number of genes in the liver by binding to their promoters and enhancers (21, 42, 44). These proteins have been implicated recently (45) as critical players during embryogenesis at the stage where body axis formation and commitment to different development fates are decided. The HNF3 family is also involved in the hormonal control of hepatic gene expression. For instance, HNF3 serves as accessory factors for glucocorticoid-mediated transcription of the hepatic PEPCK (46), tyrosine aminotransferase (47), and insulin-like growth factor-binding protein-1 (48).

What is the role HNF3β plays in transcriptional control of the ALAS gene? The HNF3s are unlikely to precipitate a cascade of further regulatory events. However, the HNF3β binding could enhance transcription from the ALAS gene promoter by recruiting components of the basal transcription complex. In addition, HNF3 may function as an architectural component by stabilizing nucleosomal structures or another protein particle that favors a particular rotational or translational setting of the surrounding chromatin. This might serve to bring other important players as NF1 and basal factors into the correct orientation to allow physical interactions. Cockell *et al.* (49) have demonstrated that binding of HNF3β or -γ was required for the induction of pancreatic α-amylase by pancreas transcription factor 1. In this regard, recent work from Zaret and co-workers (50) demonstrates that HNF3 binds their sites in compacted chromatin and opens the local nucleosomal domain in the absence of ATP-dependent chromatin-remodeling enzymes. The ability of HNF3 to open chromatin is mediated by a high affinity DNA-binding site and by the C-terminal domain of the protein, which binds histones H3 and H4 (50). Moreover, in the same paper (50), the authors show that although NF1 binds to its site in free DNA, it does not do so on the nucleosome array. When HNF3 binds and opens up the local nucleosomal domain, NF1 gains access to its binding site. A similar mechanism would be operating on the ALAS promoter and could explain the HNF3-dependent effect of NF1 upon ALAS transcription.

By having demonstrated that HNF3β and NF1 are required for an efficient expression of the basal ALAS gene, we are faced with defining their involvement on the insulin inhibitory effect. Our data show that insulin overrides the HNF3β or HNF3β plus NF1-mediated stimulation of ALAS transcriptional activity. The antisense HNF3β treatment inhibits the ALAS/CAT expression resembling insulin action. This result and the fact that no additional inhibition was observed when HepG2 cells were incubated with both antisense HNF3β and insulin suggest that common elements would be targeted in both cases. At this point of the discussion the results of gel-shift experiments merit some comments. Incubation of HepG2 nuclear extracts with an oligonucleotide containing the ALIRE II sequence of ALAS promoter formed a complex that was specifically competed with an unlabeled consensus HNF3 probe and vice versa. This result coupled with the supershift and Southwestern assay strongly demonstrate that HNF3β binds to ALIRE II. Results obtained in experiments with antisense oligonucleotides prompted us to expect that insulin treatment would be able to disrupt the DNA-protein complex. However, no obvious difference in bandshift assay was seen between nuclear extracts prepared from insulin-treated or -untreated HepG2 cells. Nevertheless, this is not uncommon and has been reported for other genes (46, 51). The simplest explanation would be that insulin does not modify the HNF3 binding but diminished its transactivation capacity. In a recent report, Wolfrum *et al.* (52) have demonstrated that activation of phosphatidylinositol 3-kinase/Akt pathway by insulin induces FoxA-2 (HNF3β) phosphorylation, nuclear exclusion, and inhibition of FoxA-2-dependent transcriptional activity in HepG2 cells. FoxA-2 physically interacts with Akt and is phosphorylated at a single conserved site (Thr-156) that is absent in FoxA-1 (HNF3α) and FoxA-3 (HNF3γ) proteins (52, 53). It is worth mentioning the possibility that insulin alters the binding of HNF3β, and this modification has not been able to be detected by us in gel-shift assays. In this regard, Shim *et al.* (54) reported that HNF3 binds to its sites on nucleosomes with an affinity that is altered from its relative affinity for the sites on free DNA and with an ~5-fold lower affinity for nucleosomal *versus* free DNA. Another possibility is that HNF3β could be removed from the DNA complex after Akt-dependent phosphorylation, but the binding site becomes occupied by another protein that forms a complex of similar electrophoretic mobility. Appropriate candidates for such protein is the HNF3α that could bind the core motif but would not be capable of transactivating the ALAS transcription. The rationale of this possibility is that because α isoform is not phosphorylated by Akt, then it could interact with the HNF3-binding site still in the presence of insulin (52, 53). The possibility that another protein that recognizes the ALIRE II region could bind, depending on the Akt-mediated phosphorylation of HNF3β, seems to be an attractive hypothesis by the light of the experiments carried out with smaller regions of the promoter. These experiments demonstrate that other HNF3-binding sites exist, downstream from the ALIRE II region, that allow the induction of ALAS expression after HNF3β overexpression. Nevertheless, this region is not able to confer insulin responsiveness. These results indicate that unbinding of HNF3 and/or its posttranslational modification is not enough to achieve the insulin-mediated inhibitory effect, and they suggest that other proteins, which are regulated by insulin or whose access to the ALAS promoter is facilitated by the hormone signaling, are necessary to repress the ALAS gene transcription. Most important, the action of this putative factor is dependent on the IRE motif integrity. Several results support this statement. First, Southwestern blotting assay shows that at least two polypeptides other than HNF3β can bind to ALIRE II and that this binding is dependent on the integrity of the IRE. Second, the results obtained in transient transfection experiments with the heterologous promoter containing the ALIRE II fragment demonstrate that mutation of the HNF3 element, which impedes the binding of HNF3β factor, did not prevent the insulin inhibition of reporter gene expression. Conversely, similar experiments show that mutation of the IRE motif, which does not appear to change the binding of HNF3β, is sufficient to abolish the inhibitory effect of insulin. This dissociation between HNF3 binding and insulin action through the ALAS-IRE provides evidence that HNF3β is not the sole physiologic mediator of insulin-induced transcriptional repression. Hall *et al.* (10) have demonstrated that an IRE sequence mediates FKHRL1-induced transcription of the insulin-like growth factor-binding protein-1 gene but does not always correlate with FKHRL1 binding. Moreover, the authors propose that the insulin response mediated by IRE must involve another protein.

Porphyrias are disorders resulting from complications due to overproduction of heme precursors. AIP is an autosomal hereditary metabolic aberration resulting from a partial defect in the activity of the third-step enzyme (porphobilinogen deaminase) during the course of heme synthesis (12). Any factor leading to

an increased enzyme requirement, using heme as a prosthetic group, or to increased degradation of heme will reduce the heme pool (55) and consequently stimulate ALAS synthesis. This in turn leads to an accumulation of porphyrin precursors prior to porphobilinogen deaminase. The pathogenesis of AIP is not known, but it is suggested that the lack of heme, or an accumulation of porphyrin precursors affecting the nervous system, is chiefly responsible for the clinical expression of AIP (12, 56). In many patients, the onset of acute AIP attacks can be aborted by adequate nutritional intake (12). Carbohydrate intake blocks ALAS, which has been demonstrated in numerous clinical and experimental studies (57, 58). However, the mechanisms by which carbohydrates modulate the heme synthesis are not elucidated to date. The present paper provides a rational basis for remission of AIP acute attacks as a consequence of carbohydrate treatment. The high levels of carbohydrate intake cause the increased production of insulin by beta cells from the pancreas. Insulin would repress the expression of ALAS driving to a minor formation of porphyrins precursors that in turn would abrogate the AIP symptoms.

The physiological significance of the role HNF3β plays in transcriptional control of the ALAS gene has not been established, but certain clues suggest that this could be important. The HNF3 proteins play critical roles in embryonic development (42). The HNF3β null mutant phenotype lacks node, notochord, and foregut. Embryos do not develop beyond E8.5 (59, 60). This family of transcription factors is also involved in other stages of development like the passage of female rats to puberty (61), in which the induction of the *CYP2C12* gene is indispensable (62). The transcriptional activity of the promoter of this gene, belonging to the cytochrome P450 superfamily, is strongly induced by HNF3β (62). Likewise, expression of other cytochromes is stimulated by FoxA transcription factors (43, 63). We are tempted to speculate that the expression of cytochromes, holoproteins using heme as prosthetic group, and the expression of ALAS, the step limiting enzyme of heme biosynthesis, might share at least some common regulatory elements.

The data reported in this paper demonstrate that transcription factors HNF3β and NF1 are involved in the basal ALAS gene transcription. Furthermore, the paper provides a potential mechanism for the reported ability of insulin to downregulate the ALAS mRNA in murine hepatocytes (18) and hepatoma human cells (19). Because the HNF3-binding site in the ALAS promoter overlaps the IRE core motif, we propose a model in which insulin is postulated to exert its negative effect through the disturbance of HNF3β binding or transactivation potential, probably due to specific phosphorylation of this transcription factor by Akt. Because of this event, NF1 would lose accessibility to the promoter. The posttranslational modification of HNF3 would allow the binding of a protein complex that recognizes the core IRE. It is possible that such a factor may bind only after changing its phosphorylated status. In this regard, results obtained from transfection experiments using kinase inhibitors support this hypothesis. We detected two polypeptides between 20 and 24 kDa that were able to interact specifically with the ALAS-IRE. Studies designed to determine the identity of these proteins that could be part of a putative insulin response factor are in progress.

REFERENCES

1. Hall, R., and Grannner, D. (1999) *J. Basic Clin. Physiol. Pharmacol.* **10,** 119–133
2. O'Brien, R., Streeper, R., Ayala, J., Stradelmaier, B., and Hornbuckle, L. (2001) *Biochem. Soc. Trans.* **29,** 552–558
3. Yeagley, D., Moll, J., Vinson, C. A., and Quinn, P. G. (2000) *J. Biol. Chem.* **275,** 17814–17820
4. Suwanichkul, A., Morris, S., and Powell, D. (1993) *J. Biol. Chem.* **268,** 17063–17068
5. Ganss, R., Weih, F., and Schutz, G. (1994) *Mol. Endocrinol.* **8,** 895–903
6. Streeper, R. S., Svitek, C. A., Chapman, S., Greenbaum, L. E., Taub, R., and O'Brien, R. M. (1997) *J. Biol. Chem.* **272,** 11698–11701
7. Li, W. W., Dammerman, M. M., Smith, J. D., Metzger, S., Breslow, J. L., and Leff, T. (1995) *J. Clin. Investig.* **96,** 2601–2605
8. Beurton, F., Bandyopadhyay, U., Dieumegard, B., Barouki, R., and Aggerbeck, M. (1999) *Biochem. J.* **343,** 687–695
9. O'Brien, R. M., Lucas, P. C., Yamasaki, T., Noisin, E. L., and Granner, D. K. (1994) *J. Biol. Chem.* **269,** 30419–30428
10. Hall, R. K., Yamasaki, T., Kucera, T., Waltner-Law, M., O'Brien, R., and Granner, D. K. (2000) *J. Biol. Chem.* **275,** 30169–30175
11. Cooke, D. W., and Lane, M. D. (1999) *J. Biol. Chem.* **274,** 12917–12924
12. Kappas, A., Sassa, S., Galbraith, R. A., and Nordmann, Y. (1995) in *The Metabolic and Molecular Basis of Inherited Diseases* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2103–2159, 7th Ed., McGraw-Hill Inc., New York
13. Sadlon, T. J., Dell'Orso, T., Surinya, K. H., and May, B. K. (1999) *Int. J. Biochem. Cell Biol.* **31,** 1153–1167
14. Ponka, P. (1999) *Am. J. Med. Sci.* **318,** 241–256
15. Giono, L. E., Varone, C. L., and Cánepa, E. T. (2001) *Biochem. J.* **353,** 307–315
16. Li, B., Holloszy, J. O., and Semenkovich, C. F. (1999) *J. Biol. Chem.* **274,** 17534–17540
17. Guberman, A. S., Scassa, M. E., Giono, L. E., Varone, C. L., and Cánepa, E. T. (2003) *J. Biol. Chem.* **278,** 2317–2326
18. Scassa, M. E., Varone, C. L., Montero, L. E., and Cánepa, E. T. (1998) *Exp. Cell Res.* **244,** 460–469
19. Scassa, M. E., Guberman, A. S., Varone, C. L., and Cánepa, E. T. (2001) *Exp. Cell Res.* **271,** 201–213
20. Olive, M., Krylov, D., Echlin, D. R., Gardner, K., Taparowsky, E., and Vinson, C. (1997) *J. Biol. Chem.* **272,** 18586–18594
21. Chaya, D., Hayamizu, T., Bustin, M., and Zaret, K. S. (2001) *J. Biol. Chem.* **276,** 44385–44389
22. Rausa, F., Tan, Y., Zhou, H., Yoo, K., Stolz, D., Watkins, S., Franks, R., Unterman, T., and Costa, R. (2000) *Mol. Cell. Biol.* **20,** 8264–8282
23. Ortiz, L., Aza-Blanc, P., Zannini, M., Cato, A., and Santisteban, P. (1998) *J. Biol. Chem.* **274,** 15213–15221
24. Seed, B., and Sheen, J. Y. (1988) *Gene* (*Amst.*) **67,** 271–277
25. Navas, M. A., Vaisse, C., Boger, S., Heimesaat, M., Kollee, L. A., and Stoffel, M. (2000) *Hum. Hered.* **50,** 370–381
26. Sumner, C., Shinohara, T., Durham, L., Traub, R., Major, E. O., and Amemiya, K. (1996) *J. Neurovirol.* **2,** 87–100
27. Wang, J., Stafford, J., Scott, D., Sutherland, C., and Granner D. (2002) *J. Biol. Chem.* **275,** 14717–14721
28. Chomczinsky, P., and Sacchi, N. (1987) *Anal. Biochem.* **71,** 341–350
29. Bishop, D. F. (1990) *Nucleic Acids Res.* **18,** 7187–7188
30. Varone, C. L., Giono, L. E., Ochoa, A., Zakin, M. M., and Cánepa, E. T. (1999) *Arch. Biochem. Biophys.* **372,** 261–270
31. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., pp. 7.42–7.45 and 17.48–17.51, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32. Andrews, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 496–503
33. Shimizu, S., Miyamoto, Y., Hayashi, M. (2002) *Biochim. Biophys. Acta* **1574,** 337–344
34. Dong, Y., Asch, H., Ying, A., and Asch, B. (2002) *Exp. Cell Res.* **276,** 328–336
35. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) *Nucleic Acids Res.* **23,** 4878–4884
36. Guo, S., Cichy, S. B., He, X., Yang, Q., Regland, M., Chosh, A. K., Johnson, P. P., and Unterman, T. (2001) *J. Biol. Chem.* **276,** 8516–8523
37. Wang, J. C., Strömstedt, P. E., Sugiyama, T., and Granner, D. K. (1999) *Mol. Endocrinol.* **13,** 604–618
38. Cooke, D. W., and Lane, M. D. (1999) *Biochem. Biophys. Res. Commun.* **260,** 600–604
39. Cha, J., Kim, H., Im, S., Li, T., and Ahn, Y. (2001) *Exp. Mol. Med.* **30,** 59–63
40. Gronostajski, R. (2000) *Gene* (*Amst.*) **249,** 31–45
41. Yeagley, D., Agati, J. M., and Quinn, P. G. (1998) *J. Biol. Chem.* **273,** 18743–18750
42. Carlsson, P., and Mahlapuu, M. (2002) *Dev. Biol.* **250,** 1–23
43. Wang, D. P., Stroup, D., Marrapodi, M., Crestani, M., Galli, G., and Chang, J. Y. (1996) *J. Lipid Res.* **37,** 1831–1841
44. Duncan, S. A., Navas, M. A., Dufort, D., Rossant, J., and Stoffel, M. (1998) *Science* **281,** 692–695
45. Hoodless, P. A., Pye, M., Chazaud, C., Labbe, E., Attisano, L., Rossant, J., and Wrana, J. L. (2001) *Genes Dev.* **15,** 1257–1271
46. Wang, J. C., Strömstedt, P. E., O'Brien, R. M., and Granner, D. K. (1996) *Mol. Endocrinol.* **10,** 794–800
47. Roux, J., Pictet, R., and Grange, T. (1995) *DNA Cell Biol.* **14,** 385–396
48. Patel, S., Lochlead, P. A., Rena, G., Sutherland, C. (2001) *Biochem. J.* **359,** 611–619
49. Cockell, M., Stolarczyk, D., Frutiger, S., Hughes, G. J., Hagenbüchle, O., and Wellauer, P. K. (1995) *Mol. Cell. Biol.* **15,** 1933–1941
50. Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M., and Zaret, K. S. (2002) *Mol. Cell* **9,** 279–289
51. Johnson, T. M., Rosenberg, M. P., and Meisler, M. H. (1993) *J. Biol. Chem.* **268,** 464–468
52. Wolfrum, C., Besser, D., Luca, E., and Stoffel, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11624–11629
53. Czech, M. P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11198–11200
54. Shim, E. Y., Woodcock, C., and Zaret, K. S. (1998) *Genes Dev.* **12,** 5–10
55. May, B. K., Dogra, S. C., Sadlon, T. J., Bhasker, C. R., Cox, T. C., and Bottomley, S. S. (1995) *Prog. Nucleic Acids Res. Mol. Biol.* **51,** 1–51
56. Anderson, C., Bylesjö, I., and Lithner, F. (1999) *J. Intern. Med.* **245,** 193–197
57. Brodie, M. J., Moore, M. R., Thompson, G. G., Goldberg, A. (1977) *Clin. Sci. Mol. Med.* **53,** 365–371
58. Varone, C. L., Cánepa, E. T., Llambías, E. B. C., and Grinstein, M. (1996)

Biochem. Cell Biol. **74,** 271–281
59. Sund, N. J., Ang, S. L., Sackett, S. D., Shen, W., Daigle, N., Magnuson, M. A., and Kaestner, K. H. (2000) *Mol. Cell. Biol.* **20,** 5175–5183
60. Sund, N. J., Vatamaniuk, M. Z., Casey, M., Ang, S. L., Magnuson, M. A., Stoffers, D. A., Matschinsky, F. M., and Kaestner, K. H. (2001) *Genes Dev.* **15,** 1706–1715
61. Waxman, D. J., and Chang, T. K. H. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed) 2nd Ed., pp. 391–417, Plenum Publishing Corp., New York
62. Delesque-Touchard, N., Park, S. H., and Waxman, D. J. (2000) *J. Biol. Chem.* **275,** 34173–34182
63. Park, S. H., and Waxman, D. J. (2001) *J. Biol. Chem.* **276,** 43031–43039