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Hepatic Nuclear Factor 3- and Hormone-Regulated Expression of the Phosphoenolpyruvate Carboxykinase and Insulin-Like Growth Factor-Binding Protein 1 Genes

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The rate of transcription of the hepatic phosphoenolpyruvate carboxykinase (PEPCK) and insulin-like growth factor-binding protein 1 (IGFBP-1) genes is stimulated by glucocorticoids and inhibited by insulin. In both cases, the effect of insulin is dominant, since it suppresses both basal and glucocorticoid-stimulated PEPCK or IGFBP-1 gene transcription. Analyses of both promoters by transfection of PEPCK or IGFBP-1–chloramphenicol acetyltransferase fusion genes into rat hepatoma cells has led to the identification of insulin response sequences (IRSs) in both genes. The core IRS, T(G/A)TTTTG, is the same in both genes, but the PEPCK promoter has a single copy of this element whereas the IGFBP-1 promoter has two copies arranged as an inverted palindrome. The IGFBP-1 IRS and PEPCK IRS both bind the α and β forms of hepatic nuclear factor 3 (HNF-3), although the latter does so with a sixfold-lower relative affinity. Both the PEPCK and the IGFBP-1 IRSs also function as accessory factor binding sites required for the full induction of gene transcription by glucocorticoids. A combination of transient transfection and DNA binding studies suggests that HNF-3 is the accessory factor that supports glucocorticoid-induced gene transcription. In both genes, the HNF-3 binding site overlaps the IRS core motif(s). A model in which insulin is postulated to mediate its negative effect on glucocorticoid-induced PEPCK and IGFBP-1 gene transcription indirectly by inhibiting HNF-3 action is proposed.

It is now well established that insulin can directly and specifically stimulate or inhibit the transcription of many genes in a variety of tissues (39). Although several *cis*-acting insulin response sequences, also called insulin response elements (IRSs/IREs), have now been identified (2, 6, 18, 23, 32, 35, 40, 47, 48, 57, 58, 64, 65), a consensus IRS/IRE has not been established. It is possible that, as with phorbol esters, multiple *cis*-acting IRSs/IREs exist (51).

Two of the best-characterized insulin-regulated genes in liver encode phosphoenolpyruvate carboxykinase (PEPCK) and insulin-like growth factor-binding protein 1 (IGFBP-1) (for a review, see references 29, 39, and 52). PEPCK catalyzes the rate-limiting step in gluconeogenesis, whereas IGFBP-1 is one of a family of six secreted proteins that bind insulin-like growth factor peptides with high affinity (29, 39, 52). In hepatoma cells, the expression of both genes is stimulated by glucocorticoids, whereas insulin acts in a dominant fashion to suppress both basal and glucocorticoid-stimulated transcription (20, 44, 49, 54, 70). Insulin primarily inhibits the initiation of PEPCK gene transcription, but it also has a significant but smaller effect on transcript elongation (55). Whether insulin inhibits both initiation and elongation of IGFBP-1 gene transcription is unknown.

A complex glucocorticoid response unit mediates the stimulatory action of glucocorticoids on the PEPCK gene (22). This glucocorticoid response unit consists of a tandem array (5′ to 3′) of two accessory factor binding sites (AF1, located from positions −455 to −431, and AF2, located from positions −420 to −403) and two glucocorticoid receptor binding sites (GR1 and GR2, from positions −395 to −349). AF1 and AF2 do not function as glucocorticoid response elements themselves, but when both are mutated the promoter is no longer responsive to glucocorticoids. Thus, GR1 and GR2 are also inert by themselves (22).

At least two *cis*-acting elements mediate insulin's action on PEPCK gene transcription (17, 40). One element, designated the distal IRS, has been located between positions −416 and −407 (38, 40). Since this distal IRS coincides with AF2, it is ideally positioned to interfere with AF2 function, and this could provide a mechanism for the dominant negative effect insulin has on glucocorticoid-stimulated PEPCK gene transcription.

A glucocorticoid response element overlaps an IRS in both the rat and the human IGFBP-1 gene promoters (18, 60, 63, 64). The human IGFBP-1 promoter contains a second glucocorticoid response element that is not present in the rat promoter; however, both promoters contain only a single IRS (18, 60, 63, 64). The IGFBP-1 IRS is perfectly conserved between rat and human species (18, 64), as is the PEPCK IRS (43). As with PEPCK, the human IGFBP-1 IRS also acts as an accessory factor binding site required for the glucocorticoid response (the rat IGFBP-1 IRS apparently does not; compare references 18, 60, and 63). The studies reported in this paper demonstrate that the core IRSs in the PEPCK and IGFBP-1 promoters are identical and that both elements bind hepatic nuclear factor 3 (HNF-3). The potential role of HNF-3 in insulin action and/or as an accessory factor for the glucocorticoid response is investigated, and the significance of the observation that the PEPCK IRS also binds C/EBP but that the IGFBP-1 IRS does not is discussed.

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TABLE 1. Sequences of the sense strands of oligonucleotides used[a]

Oligonucleotide	Sequence	Gene
PC433	−433 GTGACACCTCACAGCTGTGGTGTTTTGACAACCAGCAG −396	PEPCK
M2	−433 GTGACACCTCACAGCTGTGGTGGGGGTACAACCAGCAG −396	
M4	−433 GTGACACCTCACAGCTGGTCCCTTTTGACAACCAGCAG −396	
PC425	−416 TGGTGTTTTGACAAC −402	
PC42	−416 TGGTGTTTTG −407	
PCTG2	−416 ACCTGTTTTG −407	
CRE	−99 GGCCCCTTACGTCAGAGGCGAG −78	
mCRE	−99 GGCCCGGGTCGACCCAGGCGAG −78	
IGFBP-WT	−124 CACTAGCAAAACAAACTTATTTTGAACAC −96	IGFBP-1
AmBm	−124 CACTAGCCCCGGGAACTTAGGGGTAACAC −96	
IGFBP-LM	−124 CACTAGCAAAACACCGGTATTTTGAACAC −96	
IGFBP-CM	−124 CACTAGCAAAAGAAACTTCTTTTGAACAC −96	
TTR	−111 GTTGACTAAGTCAATAATCAGAATCAG −85	TTR

[a] Sequences are numbered relative to the start sites of transcription from their respective genes. Underlined bases are mutated. All oligonucleotides have *Bam*HI-compatible ends (GATC) except for the CRE oligonucleotides, which have *Hin*dIII (AGCT)-compatible ends and additional linker sequence (50).

MATERIALS AND METHODS

Materials. [α-^{32}P]dATP (>3,000 Ci mmol^{-1}) and [^{3}H]acetic acid, sodium salt (>10 Ci mmol^{-1}), were obtained from Amersham and ICN, respectively. Insulin was purchased from Collaborative Bioproducts. All other chemicals were of the highest grade available.

Plasmid construction. DNA manipulations were accomplished by standard techniques (53), and the orientations of all subcloned DNA fragments were determined by restriction enzyme analysis and confirmed by DNA sequencing using the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio). Plasmid TKC-VI (kindly provided by T. Sudhof, Southwestern Medical School, Dallas, Tex.) contains the herpes simplex virus thymidine kinase (TK) promoter ligated to the chloramphenicol acetyltransferase (CAT) gene (59). The TK promoter sequence extends from positions −480 to +51 and contains a *Bam*HI linker between positions −48 and −32 (59). Various complementary oligonucleotides representing the PEPCK IRS (Table 1) were synthesized with *Bam*HI-compatible ends by using an Applied Biosystems 380A DNA synthesizer and were cloned in either orientation into *Bam*HI-cleaved TKC-VI by standard techniques (53). The complementary oligonucleotides representing the wild-type (IGFBP-WT) and mutated (AmBm) IGFBP-1 IRSs are identical to those described by Suwanickul et al. (64), except that they were synthesized with *Bam*HI- instead of *Xba*I-compatible ends (Table 1). These oligonucleotides and others representing novel IGFBP-1 IRS mutations (IGFBP-LM and IGFBP-CM; Table 1) were also cloned into *Bam*HI-cleaved TKC-VI. A 1.3-kb fragment of human genomic DNA which contains sequence spanning bp −1205 to +68 relative to the IGFBP-1 transcription start site was inserted into the promoterless pCAT (An) vector to create p1205CAT as described previously (62). Plasmid pRShGRα, which expresses human glucocorticoid receptor under the control of the Rous sarcoma virus (RSV) long terminal repeat, was kindly provided by Ronald M. Evans (The Salk Institute for Biological Studies, La Jolla, Calif.). All plasmid constructs were purified by centrifugation twice through cesium chloride gradients (53).

Site-directed mutagenesis. The 1.3-kb IGFBP-1 promoter fragment present in the M13-based cloning vector M13mp18 was mutated by the Kunkel method, using synthetic oligonucleotides and the Muta-Gene kit (Bio-Rad) as described previously (62). In all cases, the sequences of mutations and orientations of constructs were confirmed by DNA sequence analysis using Sequenase (U.S. Biochemicals) and the dideoxy chain termination method as described previously (62).

Oligonucleotide 5′-ACTAGCAAAACACCGGTATTTTGAACAC-3′ has the AACT motif, spanning bp −111 to −108 of the IGFBP-1 promoter, changed to CCGG, a mutation equivalent to that in IGFBP-LM (Table 1). Oligonucleotide 5′-GCACTAGCAAAAGAAACTTCTTTTGAACACTC-3′ has the C and A nucleotides located at bp −113 and −106 of the IGFBP-1 promoter changed to G and C, respectively, a mutation equivalent to that in IGFBP-CM (Table 1). The construction of pAmBm, in which the AAAACA sequence spanning bp −117 to −112 of IRE A is mutated to CCCGGG and the TTTTG sequence spanning bp −105 to −101 of IRE B is mutated to GGGGT, has been described previously (64).

Transient transfection and CAT assays. (i) H4IIE cells. In the initial experiments (see Fig. 1) cells were transfected while attached to plates, while in subsequent experiments (see Fig. 3 and Tables 2 and 3) cells were transfected in solution. Transfection in solution using a smaller volume of the calcium phosphate-DNA coprecipitate results in a higher level of basal CAT expression. For the data shown in Fig. 1, H4IIE rat hepatoma cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 2.5% (vol/vol) newborn calf serum and 2.5% (vol/vol) fetal calf serum, diluted the day before transfection, and replated in 75-cm^2 culture dishes (2×10^6 to 3×10^6 cells per dish). Attached cells were then transfected, after removal of medium, by incubation for 30 min at room temperature with 2 ml of a calcium phosphate-DNA coprecipitate containing 30 μg of PEPCK-TK-CAT plasmid DNA and 20 μM chloroquine. This solution was then diluted by addition of 8 ml of serum-containing medium supplemented with 5% (vol/vol) concentrations of both the 0.25 M calcium chloride–40 μM chloroquine solution and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (pH 7.10), which were used to prepare precipitates (40). The cells were then returned to a humidified 5% CO_2–95% air atmosphere in a Queue cell culture incubator. For the data shown in Fig. 3 and Table 2, H4IIE cells were grown to 40 to 70% confluence in T150 flasks in alpha modified Eagle's medium containing 2% (vol/vol) fetal calf serum, 3% (vol/vol) newborn calf serum, and 5% (vol/vol) calf serum. Cells from individual T150 flasks were harvested by trypsinization, pelleted by centrifugation, and resuspended in 2 ml of a calcium phosphate-DNA coprecipitate containing 60 μg of PEPCK-TK-CAT or IGFBP-TK-CAT plasmid DNA and 20 μM chloroquine. After a 20-min incubation at room temperature, the cells were replated into four 75-cm^2 culture dishes (approximately 3×10^6 cells per dish) containing 10 ml of DMEM supplemented with 2.5% (vol/vol) newborn calf serum and 2.5% (vol/vol) fetal calf serum. The cells were then returned to a humidified 5% CO_2–95% air atmosphere in a Queue cell culture incubator.

Four to six hours after transfection (see Fig. 1 and 3 and Table 2), cells were treated with 20% (vol/vol) dimethyl sulfoxide in serum-containing DMEM for 5 min, washed in serum-containing and then serum-free DMEM, and then incubated for 18 h in serum-free DMEM in the presence or absence of 10 nM insulin. Cells were then harvested, and CAT activity was assayed as described below. Since β-galactosidase is very poorly expressed, and because RSV-luciferase expression is stimulated by insulin (approximately fourfold) in H4IIE cells (43), CAT activity was corrected for the protein concentration in the cell lysate, as measured by the Pierce bicinchoninic acid assay, and each plasmid construct was transfected multiple (4 to 14) times. The effects of IRS mutations on basal CAT activity directed by the TKC-VI vector were analyzed by cotransfection with an RSV-luciferase plasmid. There was no correlation between the presence of mutant or wild-type IRS constructs and basal activity (43). However, as reported by Sudhof et al. (59), the size of the TKC-VI insert does affect basal CAT activity.

Cells harvested by trypsin digestion were sonicated in 300 μl of 250 mM Tris · HCl at pH 7.8. After sonication, the extracts were heated for 10 min at 65°C and cellular debris was removed by centrifugation. CAT assays were performed on the supernatant as follows. A 100-μl aliquot of supernatant was incubated in a final volume of 250 μl containing (at final concentrations) 200 mM Tris · HCl (pH 7.8), 6 mM $MgCl_2$, 75 mM KCl, 0.4 mM coenzyme A, 3 mM ATP, 1 mM chloramphenicol, 0.03 U of acetyl coenzyme A synthetase, and 0.34 mM [^{3}H]acetate (0.5 Ci/mmol). This mixture was incubated for 3 h at 37°C, extracted with benzene, and transferred into scintillation vials. After removal of the benzene by evaporation, the [^{3}H]acetate incorporated into chloramphenicol was quantified by scintillation spectroscopy. The counts per minute in a lysate-free blank were subtracted, and CAT activity was corrected for variations in protein concentration as described above.

(ii) HepG2 cells. The maintenance and transfection of HepG2 human hepatoma cells have been described previously (62, 64). Cells were transfected with 5 μg of CAT plasmid containing IGFBP-1 promoter fragments and with 1 μg of the human glucocorticoid receptor expression vector pRShGRα. One microgram of pRSVL plasmid, which contains the RSV long terminal repeat upstream to the luciferase reporter gene, was cotransfected to control for transfection efficiency (62, 64). Transfected cells were washed three times in phosphate-buffered saline and then incubated for 18 h with serum-free medium (DMEM supplemented with 5 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml) in the presence or absence of 100 nM dexamethasone (Sigma). CAT and luciferase assays were performed as described previously (62, 64).

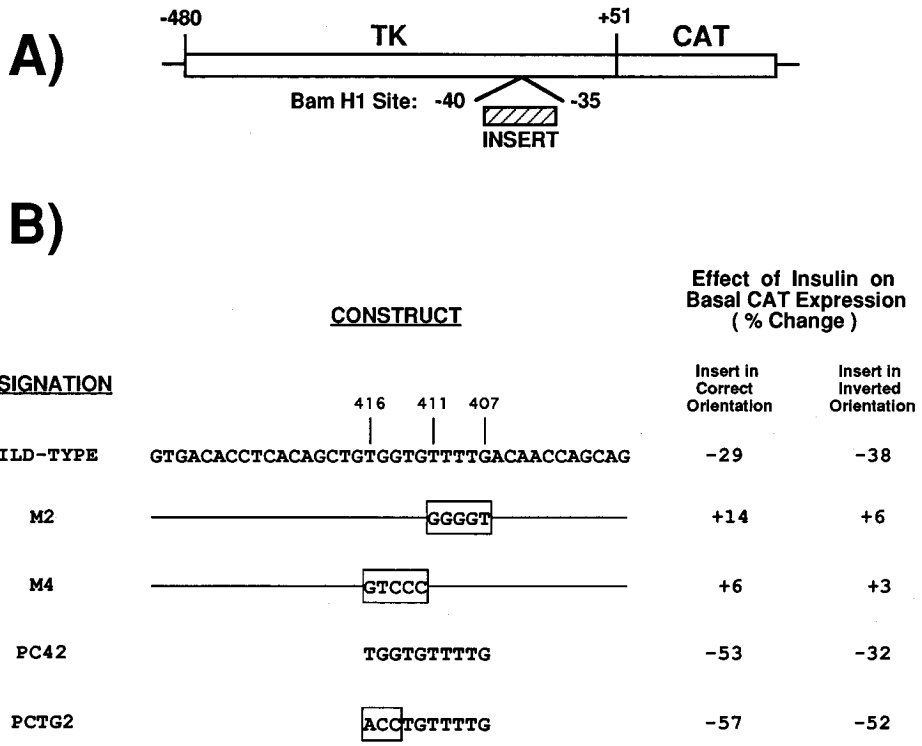

FIG. 1. Use of the herpes simplex virus TK promoter to delineate a PEPCK gene IRS. Various mutants of the PEPCK gene promoter sequence between positions −433 and −396 were synthesized with *Bam*HI (GATC) ends (B) and cloned, in either orientation, into the TKC-VI vector (A). The mutated sequences are boxed. The effect of insulin was analyzed by transient transfection of rat hepatoma H4IIE cells. Transfected cells were incubated in serum-free medium in the presence or absence of insulin (10 nM) and harvested after 18 h; CAT activity was assayed as described in Materials and Methods. Results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent change) and are the means of 8 to 14 transfections for each construct. Maximum error (± the standard error of the mean [SEM]) was 7%. The vector without an insert showed a slight stimulation in response to insulin [+(19 ± 6)%; n = 12].

Gel retardation assay. (i) Labeled probes. Complementary oligonucleotides representing the PEPCK IRS (either bp −433 to −396 [PC433] or −416 to −402 [PC425]; Table 1) or the IGFBP-1 IRS (bp −124 to −96 [IGFBP-WT]; Table 1) were gel purified, annealed, and then labeled with [α-^{32}P]dATP by using the Klenow fragment of *Escherichia coli* DNA polymerase I to a specific activity of approximately 2.5 μCi/pmol.

(ii) Nuclear extract preparation. H4IIE rat hepatoma cells were grown to approximately 80% confluence in T150 flasks as described above. Nuclear extracts were prepared by the method of Shapiro et al. (56) except that (i) all buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride, (ii) cells were broken by using 20 strokes of the Dounce homogenizer, and (iii) the nuclear extract was dialyzed without ammonium sulfate concentration. The protein concentration of the nuclear extract was determined by the Bio-Rad assay and was typically ~1 μg μl^{-1}. In some experiments, cells were treated with insulin (10 nM) for 1 h prior to extract preparation, and sodium orthovanadate (0.1 mM) was included in all buffers.

(iii) Standard binding assay. Labeled oligonucleotides (7 to 8 fmol; 25,000 cpm) were incubated with H4IIE rat hepatoma nuclear extract (4 μg) for 10 min at room temperature in the presence of either 20 mM HEPES (pH 7.8), 20 mM KCl, 1 mM spermidine, 10 mM dithiothreitol, 2 μg of poly(dI-dC) · poly(dI-dC), 10% (vol/vol) glycerol, and 0.1% (vol/vol) Nonidet P-40 in a final volume of 20 μl or 20 mM HEPES (pH 7.8), 20 mM KCl, 50 mM NaCl, 1 mM dithiothreitol, 1 μg of poly(dG-dC) · poly(dG-dC), and 10% (vol/vol) glycerol in a final volume of 20 μl. After incubation, the reactants were loaded onto a 6% acrylamide gel and electrophoresed at room temperature for 90 min at 150 V in a buffer containing 25 mM Tris base, 190 mM glycine, and 1 mM EDTA (40). Following electrophoresis, the gels were dried and exposed to Kodak XAR5 film, and binding was analyzed by autoradiography. Where appropriate, the ^{32}P associated with retarded complexes was quantified by liquid scintillation spectrometry.

(iv) Competition experiments. For competition experiments (see Fig. 4, 6, and 9) or assessment of the relative affinity of binding (see Fig. 8 and 11), unlabeled competitor DNA (5- to 100-fold molar excess) was mixed with the radiolabeled oligomer prior to addition of nuclear extract. Binding was analyzed by polyacrylamide gel electrophoresis as described above. The various wild-type and mutated PEPCK and IGFBP-1 IRS oligonucleotides are described in Table 1. Oligonucleotides representing the HNF-3 binding site (positions −111 to −85) in the transthyretin (TTR) gene (26) (Table 1) were synthesized with *Bam*HI-compatible ends by using a Cyclone Plus DNA synthesizer (Millipore), whereas oligonucleotides representing the PEPCK cyclic AMP (cAMP) response element (CRE and mCRE; Table 1) were synthesized with *Hin*dIII-compatible ends and have been previously described (50).

The possibility that mutations may not simply abolish binding sites but may introduce new binding motifs is a common concern. The M2, AmBm, IGFBP-LM, and IGFBP-CM mutant oligonucleotides (Table 1) have all been labeled, and no additional binding species were apparent (43).

(v) Antibody experiments. Preimmune serum (1 μl) or specific antiserum (1 μl) raised against HNF-3α, -β, or -γ was preincubated with H4IIE nuclear extract (4 μg) for 15 min at room temperature before analysis of protein-DNA binding by the standard binding assay using poly(dG-dC) · poly(dG-dC) as the nonspecific competitor (see above). The antibodies were a generous gift from J. Darnell and V. Prezioso, Rockefeller University.

In vitro transcription and translation. cDNAs encoding various C/EBP isoforms were inserted into the pGEM-7 vector (Promega) (41). Coupled in vitro transcription and translation of pGEM-C/EBPα, pGEM-C/EBPβ (LAP), and pGEM-C/EBPβ (LIP) were performed by using the TNT T7 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The final reaction volume was 25 μl, of which 1 μl was used in a gel retardation assay (see Fig. 5).

RESULTS

Insulin inhibits PEPCK gene transcription through a 7-bp sequence. The expression vector TKC-VI, which contains the herpes simplex virus TK promoter ligated to the CAT reporter gene, has previously been used to identify an IRS in the PEPCK promoter (38, 40). A 42-bp double-stranded oligomer consisting of the wild-type PEPCK sequence from positions −433 to −396 ligated into the *Bam*HI site of TKC-VI conferred an insulin-dependent, orientation-independent inhibition of CAT expression following transient transfection of rat H4IIE hepatoma cells (Fig. 1) (40). Two separate 5-bp mutations within this region were constructed by altering the se-

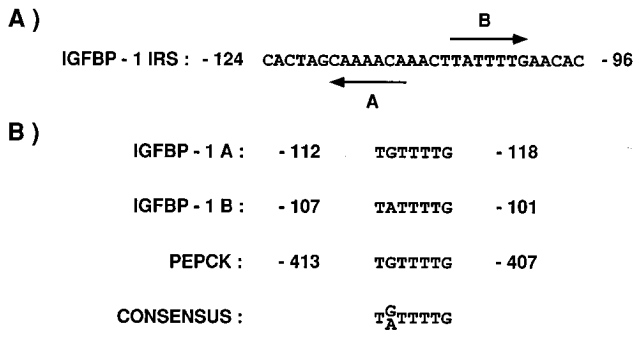

FIG. 2. Sequence similarities between the PEPCK and IGFBP-1 IRSs. The sequence of the human IGFBP-1 IRS functionally defined by transient transfection studies with human HepG2 cells in the context of a heterologous TK-CAT vector is shown (64). The inverted palindrome (arrows) is indicated. The two half sites, designated A and B, are compared with the 7-bp PEPCK IRS core to establish a putative consensus IRS.

quence between positions −411 and −407 and between positions −416 and −412, and they were designated M2 and M4, respectively. Both mutations abolished the insulin effect (Fig. 1). A smaller oligomer consisting of only the 10-bp wild-type PEPCK sequence from positions −416 to −407 (PC42) conferred insulin-dependent inhibition of CAT expression in the H4IIE cell transient transfection assay (Fig. 1) (38). During the construction of PC42-containing TKC-VI plasmids, a particular clone in which the initial 3 bp of PC42 had somehow been mutated from TGG to ACC was identified. Analysis of this mutation by transient transfection showed that it still conferred an insulin-dependent inhibition of CAT expression (PCTG2 in Fig. 1). In addition, ligation of a 10-bp double-stranded oligomer representing this mutated sequence into the *Bam*HI site of TKC-VI demonstrated that the insulin-dependent inhibition of CAT expression mediated by this sequence was also orientation independent (Fig. 1).

A comparison of the previously described 10-bp PEPCK core IRS, TGGTGTTTTG, with the recently described IGFBP-1 IRS suggested that the two elements are similar (18, 64). A comparison of the 7-bp sequence TGTTTTG with the IGFBP-1 IRS reveals a more striking homology, since the IGFBP-1 IRS contains two such elements arranged as an inverted palindrome (Fig. 2). Moreover, a base-pair-by-base-pair mutational analysis of the PEPCK IRS reveals that the first guanine in the 7-bp TGTTTTG sequence can be replaced by adenine but not by cytosine (43). This suggests that T(G/A)TTTTG may be the functional core sequence of both the PEPCK and the IGFBP-1 IRSs.

The PEPCK and IGFBP-1 IRSs are functionally similar. The IGFBP-1 IRS was originally identified by transient transfection of IGFBP-1–CAT fusion genes in human HepG2 hepatoma cells (64). A 29-bp oligomer (Fig. 2) representing the IGFBP-1 IRS was subsequently shown to mediate a negative effect of insulin on gene expression when ligated 5′ to a 107-bp TK promoter located upstream of a CAT reporter gene (64). To determine whether this IGFBP-1 sequence could function as an IRS in the same heterologous context as the PEPCK IRS, the 29-bp oligomer was ligated into the *Bam*HI site of TKC-VI. As a control, an oligomer (AmBm) in which the sequence of both IRS core elements had been mutated (see reference 64 and Table 1) was also ligated into TKC-VI. Only the wild-type oligomer conferred an insulin-dependent, orientation-independent inhibition of CAT expression following transient transfection of H4IIE cells (Fig. 3A).

Elucidation of the mechanism through which insulin regulates PEPCK and IGFBP-1 gene expression is complicated by the potential existence of multiple pathways of insulin action (15, 73). For example, stimulation of gene 33 transcription in H4IIE cells requires an insulin concentration 100 times greater

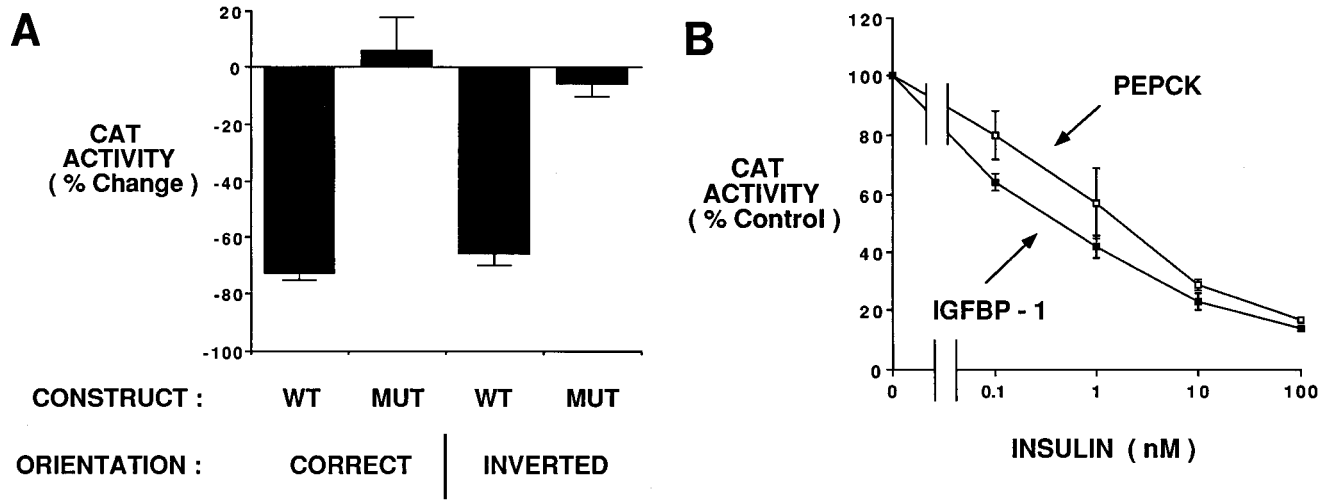

FIG. 3. Functional analysis of the IGFBP-1 IRS in the context of the TKC-VI vector. (A) Previously described oligonucleotides representing the wild-type (WT) and mutated (MUT) IGFBP-1 IRS between positions −124 and −96 (IGFBP-WT and AmBm; Table 1) were synthesized with *Bam*HI-compatible ends and cloned in either the correct or the inverted orientation, relative to that in the IGFBP-1 promoter, into the TKC-VI vector. The effect of insulin was analyzed by transient transfection of rat hepatoma H4IIE cells. Transfected cells were incubated in serum-free medium in the presence or absence of insulin (10 nM) and harvested after 18 h; CAT activity was assayed as described in Materials and Methods. Results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent change) and are means (± SEM) of 7 to 14 transfections for each construct. (B) H4IIE cells were transiently transfected with TKC-VI plasmids containing a single copy of either the 10-bp PEPCK IRS (PC42; Table 1) or the 29-bp IGFBP-1 IRS (IGFBP-WT; Table 1). Transfected cells were harvested after an 18-h incubation in serum-free medium in the presence or absence of various concentrations of insulin, and CAT activity was assayed. The results are expressed as percentages of the CAT activity in control cells and are the means (± SEM) of three transfections at each insulin concentration. The actual basal CAT activities, calculated as previously described (17), were approximately 1,500 and 220 pmol h^{-1} mg^{-1} for PC42 and IGFBP-WT, respectively.

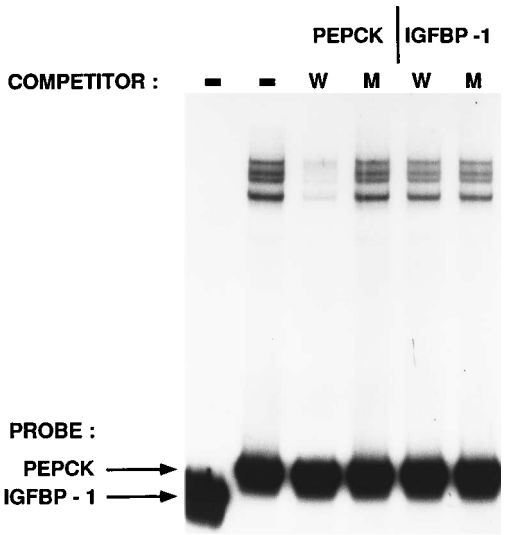

FIG. 4. Analysis of protein binding to the PEPCK and IGFBP-1 IRSs in the presence of poly(dI-dC) · poly(dI-dC). A gel retardation assay using poly(dI-dC) · poly(dI-dC) as the nonspecific competitor was performed as described in Materials and Methods. Double-stranded labeled oligonucleotide probes representing either the PEPCK IRS (PC433) or the IGFBP-1 IRS (IGFBP-WT) were analyzed. Four major bands are detected with the PEPCK IRS, but no protein binding to the IGFBP-1 IRS is seen under these conditions. DNAs representing the wild-type (lanes W) or mutated (lanes M) PEPCK and IGFBP-1 IRSs (PC433, M2, IGFBP-WT, and AmBm; Table 1) were added in a 100-fold molar excess in the competition assays. The autoradiograph shown is representative of three experiments.

than that needed for inhibition of PEPCK gene transcription assessed at the same time in the same cells (10). This observation could be explained by the presence of different pathways of insulin signal transduction. A comparison of the insulin concentration dependence for the inhibition of CAT expression directed by the TKC-VI vector containing either the PEPCK or the IGFBP-1 IRS revealed no such difference (Fig. 3B), a result that is consistent with insulin inhibiting the transcription of both genes through the same pathway. The PEPCK IRS was almost as potent as the IGFBP-1 IRS with respect to the inhibition of CAT expression even though the IGFBP-1 IRS contains two copies of the putative consensus IRS (Fig. 3B).

The PEPCK IRS but not the IGFBP-1 IRS binds C/EBP. Our laboratory and that of Richard Hanson have previously shown that a CRE located between positions −93 and −86 relative to the transcription start site plays a critical role in conferring the stimulatory effect of cAMP on PEPCK gene transcription (30, 50). It was surprising to find that, although the PEPCK IRS and CRE are functionally distinct, the two interact with a common set of liver nuclear proteins (41). These proteins interact with oligonucleotides representing the wild-type IRS (PC433) and CRE but not with mutated oligonucleotides (M2 and mCRE; Table 1) that in functional assays abolish insulin and cAMP responsiveness, respectively. We have recently identified three proteins in crude rat liver nuclear extracts that bind to both the PEPCK IRS and the CRE in gel retardation assays (41). These are 42-kDa C/EBPα, 30-kDa C/EBPα, and an unidentified 20-kDa factor termed p20-C/IBP (p20-CRE/IRS-binding protein). All three proteins can homodimerize and heterodimerize with each other to form all six possible combinations (41). These interactions are detected as four major bands (Fig. 4; see also reference 40) and two minor bands (not visible in Fig. 4) in the gel retardation assay. Several biochemical properties of the purified 20-kDa protein, including its heterodimerization capacity, profound heat stability, DNA-binding specificity, and lack of reactivity with antibodies raised against α, β, and δ C/EBP proteins, are consistent with the notion that p20-C/IBP represents a novel member of the C/EBP family of transcription factors (41). These three factors bind both the originally identified 38-bp PEPCK IRS (PC433; Table 1) and a 15-bp oligonucleotide that encompasses the IRS core (PC425; Table 1) (40). Recombinant C/EBP footprints the IRS region from positions −413 to −402 (43).

An experiment was conducted to determine whether the PEPCK and IGFBP-1 IRSs formed similar complexes with proteins found in H4IIE cell nuclear extracts. Oligonucleotides representing the wild-type PEPCK and IGFBP-1 IRSs were compared with oligonucleotides containing mutated sequences (that, in functional assays, abolish insulin responsiveness) for their ability to compete for protein binding to the labeled PEPCK IRS probe (Fig. 4). Only the unlabeled wild-type IRS oligonucleotide competed effectively for binding of all the protein-DNA complexes; the wild-type IGFBP-1 and mutated oligonucleotides were relatively ineffective (Fig. 4). In addition, when the IGFBP-1 IRS was used as the labeled probe, no protein binding was detected (Fig. 4). Goswami et al. (18) previously demonstrated protein binding to an IGFBP-1 IRS labeled probe in the presence of poly(dI-dC) · poly(dI-dC), as used in the experiment whose results are shown in Fig. 4, but since the oligonucleotide used contained extensive non-IGFBP-1 flanking sequence, their result is not directly comparable to that shown in Fig. 4. To confirm that C/EBPα does not bind the IGFBP-1 IRS, we synthesized full-length C/EBPα (27) and the full-length and truncated forms of C/EBPβ (called LAP and LIP, respectively [13]), using an in vitro transcription-translation system. The synthesis of full-length protein was confirmed by incorporation of [^{35}S]methionine and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while the nature of the product was confirmed by interaction with C/EBPα- and -β-specific antisera (data not shown). All three proteins bound to the PEPCK IRS but did not interact with the IGFBP-1 IRS (Fig. 5).

The PEPCK and IGFBP-1 IRSs both bind HNF-3. Assay conditions used to demonstrate protein binding to the PEPCK IRS did not favor binding of any protein to the IGFBP-1 IRS (Fig. 4) (40). However, by changing the so-called nonspecific competitor in the gel retardation assay from poly(dI-dC) · poly(dI-dC) to poly(dG-dC) · poly(dG-dC) and by optimizing binding by altering salt concentrations, etc. (see Materials and Methods), we were able to detect an interaction between the labeled IGFBP-1 IRS probe and proteins present in H4IIE nuclear extract (Fig. 6). The pattern of protein binding detected is distinct from that seen in the presence of poly(dI-dC) · poly(dI-dC) and when the PEPCK IRS is used as the labeled probe (Fig. 4). A competition experiment, in which a 100-fold excess of unlabeled DNA was included with the labeled IGFBP-1 IRS probe in a gel retardation assay, was performed to test whether under these modified assay conditions identical factors could be shown to bind both the IGFBP-1 and the PEPCK IRSs. Again, oligonucleotides representing the wild-type IGFBP-1 and PEPCK IRSs were compared with oligonucleotides containing mutated sequences for their ability to compete for protein binding. The unlabeled wild-type IGFBP-1 and PEPCK IRS oligonucleotides both competed effectively for the DNA-protein complex present in the band indicated by the arrow in Fig. 6; the mutated oligonucleotides were ineffective. The ability of this protein to recognize both the PEPCK IRS and the IGFBP-1 IRS is in contrast to that of C/EBPα and p20-C/IBP, which recognize only the PEPCK IRS

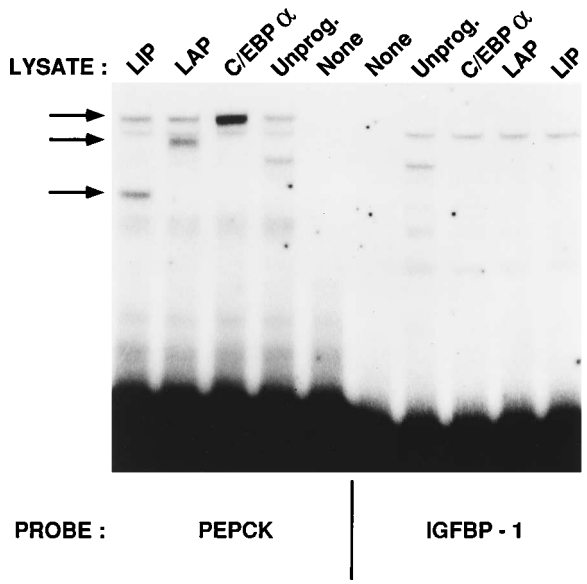

FIG. 5. Analysis of C/EBPα and C/EBPβ binding to the PEPCK and IGFBP-1 IRSs. Full-length C/EBPα and full-length and truncated C/EBPβ (LAP and LIP, respectively) were synthesized by using the Promega TNT T7 coupled reticulocyte lysate system. A gel retardation assay using poly(dI-dC) · poly(dI-dC) in conjunction with both the labeled PEPCK IRS (PC433) and IGFBP-1 IRS (IGFBP-WT) probes was performed as described in Materials and Methods. DNA binding activity in lysates programmed with expression vectors for C/EBPα, LAP, or LIP was compared with that in an unprogrammed lysate (lanes Unprog.). As a further control, the labeled probes were analyzed in the absence of lysate (lanes None). Positions of novel DNA-binding species detected in lysates programmed with the LAP and LIP expression vectors (arrows) are indicated, as is that of C/EBPα, which comigrates with a nonspecific DNA-protein interaction detected in unprogrammed lysate.

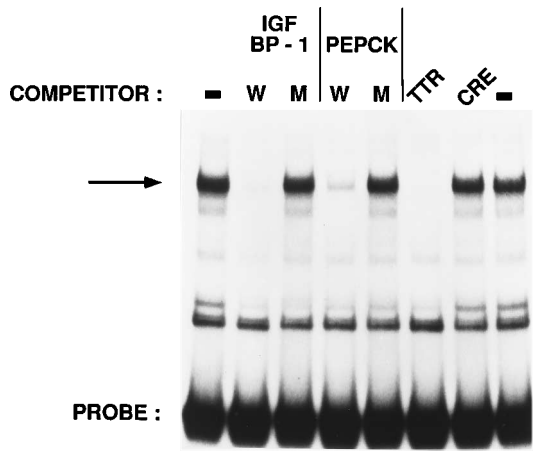

FIG. 6. Analysis of protein binding to the IGFBP-1 IRS in the presence of poly(dG-dC) · poly(dG-dC). A gel retardation assay using poly(dG-dC) · poly(dG-dC) as the nonspecific competitor with the IGFBP-1 IRS (IGFBP-WT) as the labeled probe was performed as described in Materials and Methods. Competitor DNAs representing the wild-type (lanes W) or mutated (lanes M) PEPCK and IGFBP-1 IRSs (PC433, M2, IGFBP-WT, and AmBm; Table 1), the PEPCK CRE (lane CRE), and the transthyretin HNF-3 binding site (lane TTR) were added in a 100-fold molar excess. The specific protein-DNA interaction detected by the IGFBP-1 IRS probe (arrow) is indicated. The autoradiograph shown is representative of three experiments.

(see above). The prominent lower doublet shown in Fig. 6 probably represents nonspecific protein-DNA interactions, since the intensity of these complexes was not reduced by a 100-fold excess of any of the oligonucleotides. A similar excess of an unlabeled oligonucleotide representing the PEPCK CRE also failed to compete for complex formation. Thus, unlike C/EBPα and p20-C/IBP, the specific protein detected under these modified gel retardation conditions does not bind to the PEPCK CRE.

The PEPCK IRS and the IGFBP-1 IRS are similar to the sequences noted for certain HNF-3 binding sites (originally called HNF-5 [19]) (25, 36, 45). To investigate whether the protein that binds to these elements is HNF-3, we performed a competition experiment in which a 100-fold excess of an unlabeled oligonucleotide representing the TTR HNF-3 binding site (positions −111 to −85; Table 1) (26) was included with the labeled IGFBP-1 IRS probe in a gel retardation assay (Fig. 6). The transthyretin oligomer competed effectively for specific protein binding (Fig. 6). Next, specific antisera raised against HNF-3α, HNF-3β, and HNF-3γ were tested for their ability to disrupt DNA-protein complex formation in the gel retardation assay (Fig. 7). By increasing the duration of gel electrophoresis, the specific protein-DNA interaction detected with the labeled IGFBP-1 probe (Fig. 6) can be separated into what appears to be two overlapping protein-DNA interactions (Fig. 7). The HNF-3α antiserum specifically prevents formation of the less prominent, more slowly migrating protein-DNA complex, and a weak supershifted band is also detected (Fig. 7). By contrast, the HNF-3β antiserum prevents formation of the more prominent, faster-migrating protein-DNA complex and, again, a weak supershifted band is also detected (Fig. 7). The close migration of HNF-3α and -β when bound to the IGFBP-1 IRS labeled probe is similar to that seen when other HNF-3 binding sites are used as probes in the gel retardation assay (36, 37). The HNF-3γ antiserum had no effect on DNA binding, nor did any of the HNF-3 preimmune antisera (Fig. 7). As a control, the oligonucleotide representing the TTR HNF-3 binding site (Table 1) was labeled and used as the probe in a gel retardation assay. On the basis of cross-reaction with the specific HNF-3 antiserum, the TTR oligonucleotide binds HNF-3α and HNF-3β from H4IIE nuclear extract but in addition binds HNF-3γ from liver nuclear extract (43). The unlabeled, wild-type IGFBP-1 and PEPCK IRS oligonucleotides competed effectively for the binding of all three HNF-3 isoforms, whereas the mutated oligonucleotides were ineffective (43).

To estimate the relative affinity of HNF-3α and -β binding to the IGFBP-1 IRS and the PEPCK IRS, the labeled IGFBP-1

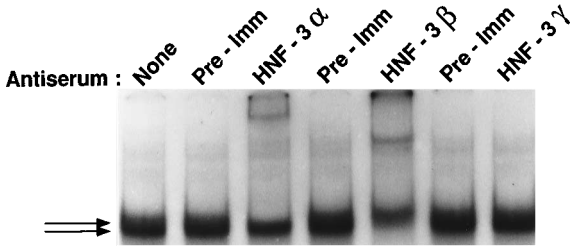

FIG. 7. Effect of HNF-3 antibodies on protein binding to the IGFBP-1 IRS. H4IIE nuclear extract was preincubated for 15 min at room temperature in the absence (lane None) or presence (lanes Pre-Imm) of preimmune serum or antisera specific for HNF-3α, -β, or -γ. A gel retardation assay was then performed with poly(dG-dC) · poly(dG-dC) as the nonspecific competitor and the IGFBP-1 IRS (IGFBP-WT) as the labeled probe. The relative positions of HNF-3α (upper arrow) and HNF-3β (lower arrow) in the polyacrylamide gel are indicated. Each preimmune serum was isolated from the same animal from which the specific antiserum was subsequently obtained. For clarity, the free probe is not shown. The autoradiograph shown is representative of three experiments.

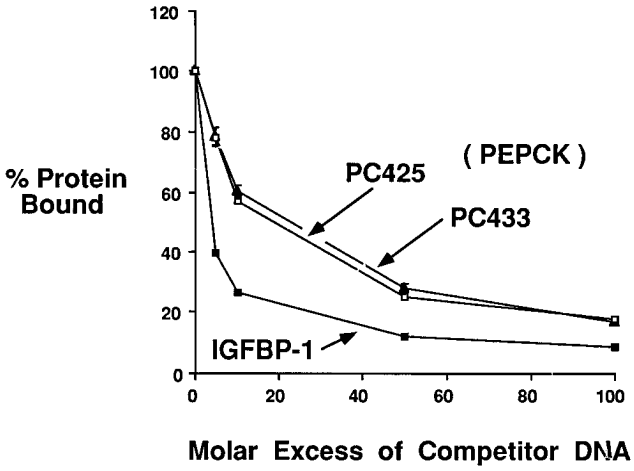

FIG. 8. Estimation of the relative affinity of HNF-3α and -β binding to the PEPCK and IGFBP-1 IRSs. Various concentrations of double-stranded oligonucleotides representative of the wild-type IGFBP-1 IRS (IGFBP-WT) and PEPCK IRS (either PC425 or PC433; Table 1) were incubated with a labeled IGFBP-1 IRS probe prior to addition of nuclear extract and analysis of protein binding by the gel retardation assay using poly(dG-dC) · poly(dG-dC) as the nonspecific competitor as described in Materials and Methods. Protein binding was quantified by liquid scintillation counting of the ^{32}P associated with retarded complexes. The data illustrated are the means ± standard deviations of three experiments. Where error bars are not visible, they are smaller than the plot symbol.

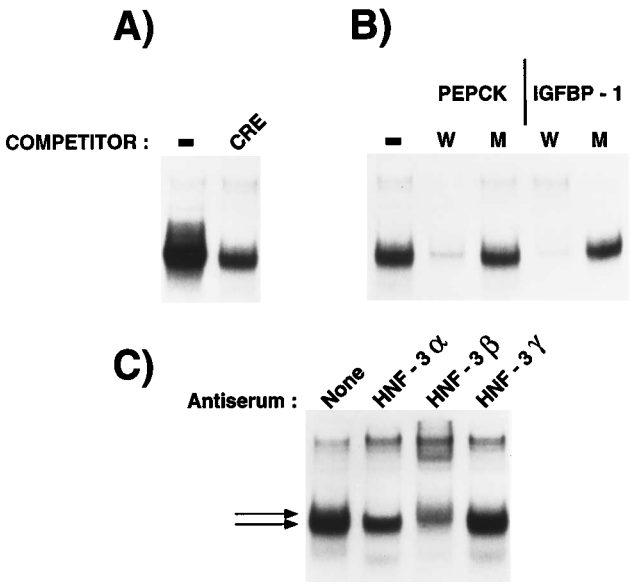

FIG. 9. Analysis of protein binding to the PEPCK IRS in the presence of poly(dG-dC) · poly(dG-dC). A gel retardation assay using poly(dG-dC) · poly (dG-dC) as the nonspecific competitor and PEPCK IRS (PC425) as the labeled probe was performed as described in Materials and Methods. Competitor DNA representing the PEPCK CRE (lane CRE) was added in a 100-fold molar excess. (A) In the presence of poly(dI-dC) · poly(dI-dC) the PEPCK CRE oligonucleotide completely blocks protein binding to the PEPCK IRS labeled probe (43), but in the presence of poly(dG-dC) · poly(dG-dC) partial competition is detected. (B) Competitor DNAs representing the wild-type (lanes W) or mutated (lanes M) PEPCK IRS (PC433 and M2) and IGFBP-1 IRS (IGFBP-WT and AmBm) were added in a 100-fold molar excess. Inclusion of a 100-fold excess of the PEPCK CRE oligonucleotide in each lane reveals a protein-DNA interaction that is specific to the wild-type but not mutated PEPCK and IGFBP-1 IRSs. (C) H4IIE nuclear extract was preincubated for 15 min at room temperature in the absence or presence of antisera specific for HNF-3α, -β or -γ. A gel retardation assay was then performed. A 100-fold molar excess of the PEPCK CRE oligomer was included in the assay to prevent C/EBP binding, as explained in the text. The relative positions of HNF-3α (upper arrow) and HNF-3β (lower arrow) in the polyacrylamide gel are indicated. For clarity, the free probe is not shown. The autoradiographs shown are representative of two experiments.

IRS oligonucleotide was preincubated with various concentrations of unlabeled wild-type IGFBP-1 or PEPCK IRS oligonucleotides prior to addition of H4IIE nuclear extract. Protein binding to the labeled IGFBP-1 IRS probe was then quantified by the gel retardation assay. On the basis of the molar excess of unlabeled oligonucleotide required to reduce binding to the labeled probe by 50%, the relative affinity of HNF-3α and -β binding was estimated to be approximately sixfold higher for the IGFBP-1 IRS than for the PEPCK IRS (Fig. 8). A short, 15-bp oligonucleotide representing the core PEPCK IRS (PC425; Table 1) was as effective as the longer 38-bp oligonucleotide with additional flanking sequence (PC433; Table 1) in competing for HNF-3 binding (Fig. 8).

In a complementary experiment, the PEPCK IRS (PC425; Table 1) was labeled and the gel retardation assay included poly(dG-dC) · poly(dG-dC) rather than poly(dI-dC) · poly(dI-dC). Under these conditions, a 100-fold molar excess of the unlabeled PEPCK CRE oligonucleotide competes partially for protein binding (Fig. 9A). By contrast, in the presence of poly(dI-dC) · poly(dI-dC), the unlabeled PEPCK CRE prevents detectable protein binding (43). On the basis of this observation, we speculate that C/EBPα and p20-C/IBP bind the labeled PEPCK IRS probe when either of the nonspecific competitors is included in the assay but that additional proteins that are not detected in the presence of poly(dI-dC) · poly (dI-dC) bind in the presence of poly(dG-dC) · poly(dG-dC). A 100-fold excess of the unlabeled IGFBP-1 IRS oligonucleotide competes partially for protein binding to the labeled PEPCK IRS in the presence of poly(dG-dC) · poly(dG-dC) (43) and hardly at all in the presence of poly(dI-dC) · poly(dI-dC) (Fig. 4). However, in the presence of poly(dG-dC) · poly(dG-dC) and a 100-fold excess of the PEPCK CRE oligonucleotide to prevent C/EBP binding, specific competition for a single band by a 100-fold excess of the unlabeled wild-type IGFBP-1 IRS oligonucleotide can be detected, whereas the mutated sequence has no effect (Fig. 9B). The band that is specifically competed for actually represents two overlapping protein-DNA interactions (see below). A 100-fold excess of the wild-type PEPCK IRS oligonucleotide also competes specifically for formation of this complex, and the mutated sequence is ineffective (Fig. 9B). Qualitatively similar results are obtained with PC425 and PC433 as the labeled PEPCK IRS oligonucleotide (43).

Having directly demonstrated, through the use of a specific antiserum, that HNF-3α and -β bind the labeled IGFBP-1 IRS, we next determined whether these proteins bound to the PEPCK IRS. The HNF-3α, -β, and -γ antisera were tested for their ability to inhibit the formation of the specific band detected in Fig. 9B. This specific protein-DNA interaction detected with the labeled PEPCK IRS probe (Fig. 9B) can be separated into what appears to be two overlapping protein-DNA interactions (Fig. 9C, arrows). The HNF-3α antiserum specifically prevents formation of the less prominent, more slowly migrating protein-DNA complex (Fig. 9C). By contrast, the HNF-3β antiserum prevents formation of the more prominent, faster-migrating protein-DNA complex, and, in addition, a weak supershifted band is also detected (Fig. 9C). The HNF-3γ antiserum had no effect on DNA binding. The low affinity of HNF-3 binding to the PEPCK IRS in conjunction with the comigrating C/EBPα and p20-C/IBP protein-DNA interactions explains why, even when using poly(dG-dC) · poly

A)

Gene		Sequence		Match
HNF - 3 Consensus :		A/C/G A A/T T G/A T T G T/A C/T T C/T		
IGFBP - 1 (anti - sense)	- 105	A T A A G T T T G T T T	- 116	10 / 12
IGFBP - 1 (sense)	- 114	A C A A A C T T A T T T	- 103	9 / 12
PEPCK	- 420	G C T G T G G T G T T T	- 409	7 / 12

B)

Oligonucleotide		Sequence		Match (sense)	Match (anti - sense)
IGFBP - 1 (wild - type)	- 124	CACTAGCAAAACAAACTTATTTTGAACAC	- 96	9 / 12	10 / 12
IGFBP - LM	- 124	——————CCGG——————	- 96	7 / 12	7 / 12
IGFBP - CM	- 124	——————G——C——————	- 96	8 / 12	9 / 12

FIG. 10. Relative locations of the HNF-3 binding site and IRS core motif in the PEPCK and IGFBP-1 IRSs. (A) The sequences of the IGFBP-1 and PEPCK IRSs are compared with the HNF-3 consensus binding sequence (45). Two potential HNF-3 binding sites are present, one on each strand, in the IGFBP-1 IRS, compared with a single site in the PEPCK IRS. The parts of the IRS core motif that overlap the HNF-3 recognition sequence are included in boxes. In the PC425 oligonucleotide (Table 1), part of the HNF-3 binding site in the PEPCK IRS may be fortuitously conserved by the *Bam*HI (GATC) linker. However, since PC425, in which the GATC ends are single stranded, competes for HNF-3 binding as well as PC433, in which these bases are double stranded (Fig. 8), this may not be significant. (B) Two mutants (IGFBP-LM and IGFBP-CM) were found to distinguish HNF-3 binding and insulin action (see text, Fig. 11, and Tables 2 and 3). The IRS core motifs are boxed.

(dG-dC), we were unable in earlier experiments (43) to detect HNF-3 binding to the PEPCK IRS, despite its obvious homology with certain HNF-3 binding sites (25, 36, 45) (originally called HNF-5 [19]).

Role of HNF-3 binding to the IGFBP-1 IRS. With the demonstration that HNF-3 binds to both the PEPCK and the IGFBP-1 IRSs, the question of the precise location of the HNF-3 recognition sequence arises, especially as this relates to the putative IRS core motifs. On the basis of the recently elucidated HNF-3 consensus binding sequence (45), the IGFBP-1 IRS appears to contain two potential HNF-3 binding sites. One is located on each strand, and both partially overlap the two IRS core motifs (Fig. 10A). Footprinting studies using recombinant HNF-3β show that this protein protects a region from positions −121 to −99 that includes both potential HNF-3 binding sites (61). The PEPCK IRS core motif overlaps a single potential HNF-3 binding site (Fig. 10A). On the basis of a comparison with the consensus HNF-3 binding sequence, the IGFBP-1 IRS would be predicted to be a stronger HNF-3 binding site than is the PEPCK IRS. This is consistent with the relative affinity data presented above (Fig. 8).

Two mutated oligonucleotides, designated IGFBP-LM and IGFBP-CM, were constructed to determine whether HNF-3 binding directly correlates with repression of transcription by insulin. In the IGFBP-LM oligonucleotide, the 4 bp between the two IRS core motifs were mutated to reduce the affinity of HNF-3 binding (Fig. 10B). In the IGFBP-CM oligonucleotide, the critical base pair identified as essential for a functional PEPCK IRS (43) was mutated in each IRS motif (Fig. 10B). The two oligonucleotides show similarly reduced affinities for HNF-3 (Fig. 11). However, when analyzed by transient transfection in the context of the TKC-VI vector, only the IGFBP-LM oligonucleotide was able to confer a repression of CAT expression in response to insulin (Table 2). Thus, HNF-3 binding does not directly correlate with the action of insulin in this system.

Since an HNF-3 binding site is required for the full response of the tyrosine aminotransferase gene to glucocorticoids (36, 37), the same mutations represented by the IGFBP-LM and IGFBP-CM oligonucleotides were introduced into the context of a IGFBP-1–CAT fusion gene by site-directed mutagenesis. Both mutations reduced the magnitude of glucocorticoid-induced CAT expression but did not have as drastic an effect as did the AmBm mutation (Table 1) that abolishes HNF-3 binding (Table 3). Thus, HNF-3 binding is correlated with the ability of glucocorticoids to induce transcription of the IGFBP-1 gene.

DISCUSSION

The multihormonal regulation of the genes for PEPCK and IGFBP-1 is similar in that cAMP, thyroid hormone, and glucocorticoids stimulate the hepatic expression of both genes whereas insulin has a dominant inhibitory effect (5, 29, 30, 39, 52). An IRS that mediates the dominant negative effect of insulin on glucocorticoid-stimulated gene transcription has been identified in the promoter of both genes (18, 40, 64). In this paper, we propose that the motif T(G/A)TTTTG, common to the PEPCK and the IGFBP-1 IRSs, is a consensus sequence. Whether such a sequence is important in other hepatic genes that show a similar regulation of expression (stimulation by glucocorticoids and inhibition by insulin), such as the genes for

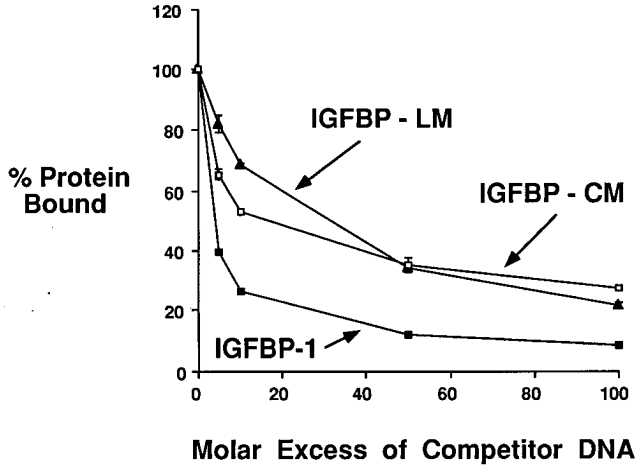

FIG. 11. Estimation of the relative affinity of HNF-3α and -β binding to the wild-type and mutated IGFBP-1 IRSs. Various concentrations of the double-stranded IGFBP-WT, IGFBP-LM, and IGFBP-CM oligonucleotides were incubated with the labeled IGFBP-1 IRS (IGFBP-WT) probe prior to addition of nuclear extract and analysis of protein binding by the gel retardation assay using poly(dG-dC) · poly(dG-dC) as the nonspecific competitor as described in Materials and Methods. Protein binding was quantified by liquid scintillation counting of the ^{32}P associated with retarded complexes. The data illustrated are the means ± standard deviations of three experiments. Where error bars are not visible, they are smaller than the plot symbol.

aspartate aminotransferase (1), tyrosine aminotransferase (8, 36, 37), and argininosuccinate lyase (21), remains to be determined. Of broader interest is whether this sequence will be found to mediate the effect of insulin on the expression of genes in other tissues (39). An IRS in the α-amylase promoter which contains a sequence similar to that of the PEPCK and IGFBP-1 IRSs has been identified (23). However, the α-amylase IRS does not contain the core T(G/A)TTTTG sequence, so it is unclear whether insulin mediates its effect via the same *trans*-acting factor as is used in the PEPCK and IGFBP-1 genes.

Although the PEPCK and IGFBP-1 IRSs have a common sequence motif, there are differences in the pattern of protein

TABLE 2. Functional analysis of IGFBP-1 oligonucleotides in the context of the TKC-VI vector[a]

TKC-VI insert	HNF-3 binding	Effect of insulin on basal CAT expression (% change)[b]
IGFBP-WT	++	−73 ± 2
IGFBP-LM	+	−65 ± 6
IGFBP-CM	+	+2 ± 8
AmBm	−	+6 ± 12

[a] Oligonucleotides representing the wild-type and mutated IGFBP-1 IRSs between positions −124 and −96 (Table 1) were synthesized with *Bam*HI-compatible ends and cloned into the TKC-VI vector in either the correct or inverted orientation relative to that in the IGFBP-1 promoter. The effect of insulin was analyzed by transient transfection of rat hepatoma H4IIE cells. Transfected cells were incubated in serum-free medium in the presence or absence of insulin (10 nM) and harvested after 18 h; CAT activity was assayed as described in Materials and Methods.

[b] Results are presented as the ratio of CAT activities in insulin-treated versus control cells (expressed as percent change) and are the means (± SEM) of 4 to 10 transfections for each construct. The data obtained with the inserts in the correct orientation are shown. With the inserts in the inverted orientation, the effects of insulin on basal CAT expression, expressed as percent change, were −(66 ± 4)% (IGFBP-WT), −(60 ± 7)% (IGFBP-LM), +(6 ± 7)% (IGFBP-CM), and −(6 ± 4)% (AmBm).

TABLE 3. Analysis of IGFBP1- IRS mutations in the context of the IGFBP-1 promoter[a]

p1205CAT mutation	HNF-3 binding	Fold induction of CAT activity by dexamethasone[b]
None (IGFBP-WT)	++	5.5 ± 2.2
IGFBP-LM	+	2.9 ± 0.6
IGFBP-CM	+	2.8 ± 0.5
AmBm	−	1.5 ± 0.5

[a] Mutations equivalent to those represented by the AmBm, IGFBP-LM and IGFBP-CM oligonucleotides (Table 1) were introduced into an IGFBP-1–CAT fusion gene (p1205CAT) (62) by site-directed mutagenesis as described in Materials and Methods. The effect of dexamethasone (100 nM) on CAT expression was analyzed by transient transfection of human HepG2 hepatoma cells as described in Materials and Methods.

[b] Means ± standard deviations of four transfections for each construct.

binding to the two elements (Fig. 4 to 6). Presumably, this reflects differences in the flanking sequence surrounding the core IRS. The PEPCK IRS binds C/EBPα and an unknown C/EBP-related protein that we have designated p20-C/IBP; the IGFBP-1 IRS does not bind these proteins (Fig. 4) (41). The PEPCK and IGFBP-1 IRSs both bind HNF-3α and -β, although the latter does so with a sixfold-higher affinity (Fig. 8). The PEPCK IRS also serves as an accessory factor binding site (AF2) necessary for full induction of PEPCK gene expression by glucocorticoids, and the human IGFBP-1 IRS plays a similar role (see the introduction). C/EBP and HNF-3 binding sites are both required for the stimulation of tyrosine aminotransferase gene transcription by glucocorticoids (19, 36, 37), and the data presented in Table 3 support a role for HNF-3 as the accessory factor required for full glucocorticoid induction of IGFBP-1 gene transcription. Unterman et al. have recently confirmed some of these observations (68). They have demonstrated HNF-3 binding to the rat IGFBP-1 IRS and shown that this element serves as an accessory factor binding site required for glucocorticoid-stimulated IGFBP-1 gene transcription. Interestingly, earlier studies had shown that only the human and not the rat IGFBP-1 IRS was an accessory element (compare references 18, 60, and 63). The explanation for this variability is unclear.

Since the HNF-3 binding site in the IGFBP-1 IRS overlaps the two IRS core motifs, we propose a model in which insulin is postulated to exert its negative effect on glucocorticoid-stimulated IGFBP-1 gene transcription through the inhibition of HNF-3 binding or transactivation potential (Fig. 12). At present, no candidate factor that recognizes the core IRS has been detected. However, given the well-documented involvement of protein phosphorylation in insulin action (15, 73), it is possible that such a factor may bind only in its phosphorylated form, a modification that may not survive the lengthy nuclear extract isolation procedure. A comparison of H4IIE nuclear extracts isolated from control or insulin-treated cells reveals little change in the protein-DNA binding interactions in the presence of either poly(dI-dC) · poly(dI-dC) or poly(dG-dC) · poly(dG-dC) (Fig. 13). Nevertheless, we have recently shown that the phosphatase inhibitor okadaic acid mimics the effect of insulin on PEPCK gene transcription through the IRS (42).

Since the 5-bp mutation (M2; Table 1) that abolishes the accessory factor activity of the PEPCK IRS (31) inhibits both C/EBP and HNF-3 binding (Fig. 4, 6, and 9), further studies are required to determine whether either or both of these factors are the accessory factor in vivo. The binding of the same putative insulin response factor described above to the

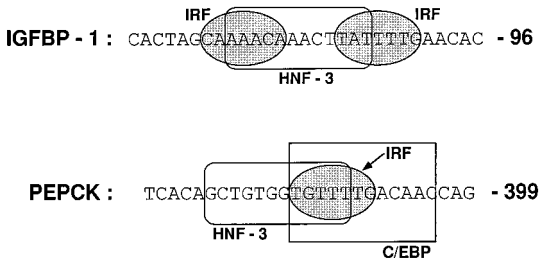

FIG. 12. Proposed mechanism of insulin action through the PEPCK and IGFBP-1 IRSs. On the basis of the data presented in this paper, we postulate that insulin may inhibit glucocorticoid-induced IGFBP-1 and PEPCK gene transcription by inhibiting the binding and/or transactivation potential of HNF-3 (IGFBP-1) or HNF-3 and/or C/EBP (PEPCK). An insulin response factor (IRF) is postulated to bind the IRS core motif(s) in each gene in response to insulin treatment, perhaps through a phosphorylation-dephosphorylation-dependent mechanism (see text). The identification of the putative IRS core motif is described in the text. The locations of the HNF-3 binding sites are based on gel retardation studies and on comparisons with the HNF-3 consensus sequence (45), whereas the C/EBP binding site was identified by a DNase I footprint analysis (43).

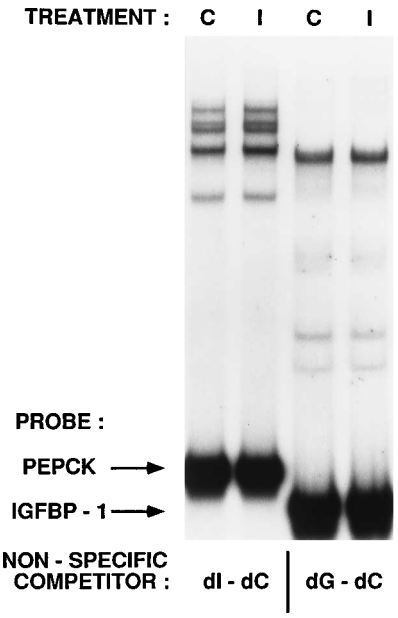

FIG. 13. Comparison of nuclear extracts prepared from control and insulin-treated H4IIE cells. H4IIE cells were incubated for 1 h in serum-free media in the presence or absence of 10 nM insulin. Nuclear extracts were then prepared as described in Materials and Methods. A gel retardation assay was performed to analyze DNA binding by proteins in nuclear extracts obtained from control (lanes C) or insulin-treated (lanes I) cells. Protein binding to the labeled PEPCK IRS and IGFBP-1 IRS was analyzed in the presence of poly(dI-dC) · poly(dI-dC) or poly(dG-dC) · poly(dG-dC) as the nonspecific competitor, respectively.

core PEPCK IRS could interfere with both C/EBP and HNF-3 binding or transactivation potential (Fig. 12).

Nakajima et al. (33) recently demonstrated that NF-IL6, the human homolog of C/EBPβ, is phosphorylated and activated by *ras*-dependent mitogen-activated protein (MAP) kinases. The phosphorylation occurs at threonine 235, which corresponds to threonine 189 in rat C/EBPβ (33). Interestingly, this threonine is conserved at position 226 in C/EBPα, along with a surrounding amino acid sequence that matches the consensus site for MAP kinase substrates (-Pro-Xaa-Ser/Thr-Pro-) (4). It is therefore possible that C/EBPα is also phosphorylated by MAP kinases in vivo. Because insulin is known to activate the MAP kinase cascade in a *ras*-dependent manner (for a review, see reference 73), it is intriguing to speculate that such a phosphorylation event could be a critical step in the inhibition of PEPCK gene transcription by insulin (in addition to the induced binding of a factor to the core IRS motif). Similarly, although there are currently no published reports documenting the phosphorylation of HNF-3 by specific protein kinases, experiments with two-dimensional gels suggest that multiple phosphorylated HNF-3β forms exist in liver nuclear extract (46).

Although the multihormonal regulation of PEPCK gene expression is similar to that of the IGFBP-1 gene in many respects (29, 30, 39, 52), an exception concerns the effect of phorbol esters. Phorbol esters, like insulin, inhibit both basal and glucocorticoid-stimulated PEPCK gene transcription (11). By contrast, in cultured cell lines phorbol esters stimulate IGFBP-1 gene expression (28, 60, 69), but it is not clear whether this effect is dominant over the negative action of insulin (compare references 60 and 69). Although there is considerable controversy as to the role of protein kinase C in insulin action (see references 7 and 16 for a review), it is clear that a prolonged (16-h) treatment of H4IIE cells with phorbol esters abolishes the inhibitory effect of a subsequent phorbol ester treatment on PEPCK gene expression, whereas the effect of insulin is unaltered (12). Even though such a pretreatment with phorbol esters is now known to down-regulate only some protein kinase C family members (7, 16), our previous conclusion that insulin and phorbol esters act through different pathways with regard to PEPCK gene expression is still valid. Surprisingly, we have found that the PEPCK IRS also mediates the negative effect of phorbol esters on PEPCK gene transcription, so, even though insulin and phorbol esters initially activate different pathways, their actions converge at this point (38). Thus, the PEPCK IRS is actually a combined IRS-phorbol ester response sequence. We speculate that C/EBPα and/or p20-C/IBP might also mediate the negative effect of phorbol esters on the PEPCK gene. Interestingly, in vivo stimulation of the protein kinase C pathway by phorbol esters increases the phosphorylation of serine 105 within the C/EBPβ activation domain (67). This phosphorylation is not directly catalyzed by protein kinase C itself but appears to be mediated by an unidentified kinase. The best candidate substrate for such a phosphorylation may be the p20-C/IBP protein, since the relevant serine is not conserved in C/EBPα. It should be noted that the effect of phorbol esters on IGFBP-1 gene expression is somewhat unusual; Lee et al. (28) speculated that protein kinase C activity tonically inhibits IGFBP-1 gene expression and that the mechanism of phorbol ester stimulation is through protein kinase C down-regulation.

IRSs/IREs that have sequences apparently distinct from that of the PEPCK and IGFBP-1 IRSs have recently been identified in a number of other genes (2, 6, 23, 32, 35, 47, 48, 57, 58, 65). In most cases, relatively little is known about the proteins that bind them. Exceptions are the IRSs/IREs identified in the promoters of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L-type pyruvate kinase (6, 35, 65). Nasrin et al. (34) used an expression screening approach to clone a protein that binds to the 3′ domain of the GAPDH IRE. The sequence specificity of this factor overlaps that of an adipocyte nuclear protein, IRP-A, that binds the core IRE defined in the GAPDH promoter (3). In contrast with the GAPDH IRE, the pattern of binding to the labeled PEPCK IRS does not change when nuclear extracts are prepared from H4IIE cells treated with or without insulin (35) (Fig. 13).

Moreover, since the GAPDH IRE differs markedly from the PEPCK IRS, the nuclear factors that bind the PEPCK IRS and the GAPDH IRE are probably not identical. The regulation of transcription of the L-type pyruvate kinase gene by insulin differs from that of PEPCK in that the stimulatory action of insulin on the former is dependent on the presence of glucose (6, 65). A combined IRE-glucose response element in the pyruvate kinase promoter binds the transcription factor MLTF/USF (14, 71); however, whether insulin alters the binding or transactivation capability of MLTF/USF is unknown (14, 71). The studies of Wakao and colleagues on the regulation of β-casein gene expression are also of considerable interest with regard to insulin-regulated gene expression (see reference 72 and references therein). It had been previously shown that a combination of insulin, prolactin, and glucocorticoids is required for the stimulation of casein gene transcription in mammary gland explants; omission of insulin reduces casein gene transcription to undetectable levels (9). Wakao et al. purified and cloned a protein called mammary gland factor (MGF) that is essential for β-casein gene expression and showed that prolactin stimulates MGF binding to DNA, probably by inducing the phosphorylation of MGF on tyrosine residues (72). Although insulin also stimulates the tyrosyl phosphorylation of nuclear proteins (24), the connection between MGF and the mechanism of insulin action on β-casein gene expression is as yet unknown.

From the data reported in this paper, it is apparent that insulin could interfere with HNF-3α and -β binding or transactivation potential to mediate its negative effect on glucocorticoid-induced PEPCK and IGFBP-1 gene transcription. The putative insulin response factor that binds the IRS core motif in both genes remains to be identified. However, it is becoming increasingly apparent that the high degree of specificity of gene activation through the use of a relatively small number of transcription factors is achieved through the assembly of stereospecific nucleoprotein complexes (see reference 66 for a review). Thus, we hope that ongoing studies analyzing the binding of proteins to the IRS in the context of larger promoter fragments may ultimately lead to the identification of the insulin response factor.

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