The Three Insulin Response Sequences in the Glucose-6-phosphatase Catalytic Subunit Gene Promoter Are Functionally Distinct*

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Glucose-6-phosphatase catalyzes the terminal step in the gluconeogenic and glycogenolytic pathways. In HepG2 cells, the maximum repression of basal glucose-6-phosphatase catalytic subunit (G6Pase) gene transcription by insulin requires two distinct promoter regions, designated A (located between –231 and –199) and B (located between –198 and –159), that together form an insulin response unit. Region A binds hepatocyte nuclear factor-1, which acts as an accessory factor to enhance the effect of insulin, mediated through region B, on G6Pase gene transcription. We have previously shown that region B binds the transcriptional activator FKHR (FOXO1a) in vitro. Chromatin immunoprecipitation assays demonstrate that FKHR also binds the G6Pase promoter in situ and that insulin inhibits this binding. Region B contains three insulin response sequences (IRSs), designated IRS 1, 2, and 3, that share the core sequence T(G/A)TTTT. However, detailed analyses reveal that these three G6Pase IRSs are functionally distinct. Thus, FKHR binds IRS 1 with high affinity and IRS 2 with low affinity but it does not bind IRS 3. Moreover, in the context of the G6Pase promoter, IRS 1 and 2, but not IRS 3, are required for the insulin response. Surprisingly, IRS 3, as well as IRS 1 and IRS 2, can each confer an inhibitory effect of insulin on the expression of a heterologous fusion gene, indicating that, in this context, a transcription factor other than FKHR, or its orthologs, can also mediate an insulin response through the T(G/A)TTTT motif.

Glucose-6-phosphatase catalyzes the final step in the glycolytic and gluconeogenic pathways, the hydrolysis of glucose-6-phosphate (G6P) into glucose and inorganic phosphate (1–4). Glucose-6-phosphatase activity is predominantly detected in liver and kidney (1–4), but is also present in the small intestine (5), pancreatic islets (6), and brain (7). The enzyme is located in the endoplasmic reticulum (ER) membrane and is thought to exist as a multicomponent system consisting of a catalytic subunit in addition to specific transporters for G6P, glucose, and inorganic phosphate (1–4). To date the glucose-6-phosphatase catalytic subunit (G6Pase) (8) and the G6P transporter (9) are the only components of the system that have been identified. Recently, several novel genes encoding putative glucose transporters have been identified, but whether any of these transporters are localized to the ER is currently unknown (10). A promising candidate appears to be GLUT9, which, like G6Pase, is predominantly expressed in liver and kidney (11). Moreover, although GLUT9 does not contain a carboxyl-terminal di-lysine ER retention motif, it does contain a weak amino-terminal di-arginine ER retention motif (12).

In vivo studies in liver and in situ studies in liver-derived cell lines or in primary hepatocytes have shown that G6Pase gene expression is stimulated by glucose, glucocorticoids, cAMP, fatty acids, leptin, and β-adrenergic receptor agonists, whereas expression is inhibited by tumor necrosis factor α, interleukin-6, and insulin (see Ref. 13 for individual citations). Insulin inhibits basal as well as glucose-, glucocorticoid-, cAMP-, and fatty acid-stimulated G6Pase gene expression. In HepG2 cells, the effect of insulin on basal mouse and human G6Pase gene transcription is mediated through a multi-component insulin response unit, which consists of two regions, designated regions A and B (14–16). In the mouse G6Pase promoter, region A is located between –231 and –199, whereas region B is located between –198 and –158. Region B contains an insulin response sequence (IRS), because it can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene (14, 15). In contrast, region A acts as an accessory element to enhance the effect of insulin on G6Pase expression mediated through region B. The accessory factor that binds region A has been identified as hepatocyte nuclear factor-1 (HNF-1) (15).

Multiple distinct IRSs have been identified through which insulin can stimulate gene transcription, but only two inhibitory IRSs have been well characterized (17). One of these inhibitory IRSs was identified through studies on the glucagon promoter (18, 19), whereas the other was first identified through studies on the phosphoenolpyruvate carboxykinase insulin response factor; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; GST, glutathione S-transferase; HGP, hepatic glucose production; IPTG, isopropyl-1-thio-β-D-galactopyranoside; WT, wild-type; TM, triple mutant; FKR, protein kinase B; SDM, site-directed mutation.

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4 The abbreviations used are: G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase catalytic subunit; HNF, hepatocyte nuclear factor; IRS, insulin response sequence; ChIP, chromatin immunoprecipitation; ER, endoplasmic reticulum; PEPCK, phosphoenolpyruvate carboxykinase; IGFBP-1, insulin-like growth factor binding protein-1; IRF, interferon regulatory factor; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; GST, glutathione S-transferase; HGP, hepatic glucose production; IPTG, isopropyl-1-thio-β-D-galactopyranoside; WT, wild-type; TM, triple mutant; FKR, protein kinase B; SDM, site-directed mutation.

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PF (PEPCK) promoter (20, 21). This PEPCK IRS has the sequence TGTTTGG (21, 22); similar elements were subsequently identified that mediate inhibitory effects of insulin on transcription of the genes encoding insulin-like growth factor-binding protein-1 (IGFBP-1) (23), tyrosine aminotransferase (24), and G6Pase (14). The IGFBP-1 promoter has two of these motifs arranged as an inverted palindromic (23), whereas inspection of the region B sequence in the G6Pase promoter reveals that it has three of these motifs arranged in tandem (14). A comparison of the sequences of the IRS motifs from these genes suggests that the consensus sequence of this element is T(G/A)T(T/G)/(G/T), which we refer to as the PEPCK-like IRS (25). The identity of the insulin response factor (IRF) that binds this motif and mediates the action of insulin has been elusive. Candidates have included C/EBP (26, 27), HNF-3 (22, 28), and NF-kappaB (29). However, detailed mutagenesis studies revealed that the effect of insulin on gene transcription mediated through this element does not correlate with the binding of any of these factors. More recently, substantial attention has focused on the potential role of the winged helix/forkhead transcription factor FKHR (FOXO1a) and its orthologs, FKHR1 (FOXO3a) and AFX (FOXO4a), as the elusive IRF.

FKHR and its orthologs emerged as candidates for the IRF indirectly from genetic experiments in Caenorhabditis elegans that identified a homologous transcription factor, Daf-16, that identified a homologous transcription factor, Daf-16, as indirectly from genetic experiments in C. elegans (30, 31). Subsequently, it was shown that FKHR can bind the PEPCK, IGFBP-1, and G6Pase IRSs in vitro (16, 32–34). In addition, FKHR was shown to stimulate PEPCK, IGFBP-1, and G6Pase fusion gene expression (32, 34, 35). It has been proposed that insulin inhibits FKHR, FKHRL1, and AFX-mediated transcriptional activation through the phosphatidylinositol 3-kinase-dependent activation of PKB, which leads to the phosphorylation and nuclear exclusion of these factors (33, 36–42).

Although these studies support the hypothesis that FKHR and its orthologs are the insulin response factors that bind the PEPCK-like IRS motif and mediate the inhibitory effect of insulin on gene transcription through this element, other results are not consistent with this model. Thus, there are several studies that suggest PKB either is not required or is not sufficient for insulin-regulated PEPCK and G6Pase gene expression (43–45). In addition, a detailed base-by-base analysis of the PEPCK IRS indicated that the effect of insulin mediated through this element on heterologous gene transcription does not correlate with FKHRL1 binding (46). Because many of the reported studies on FKHR, FKHRL1, and AFX have involved overexpression of wild-type or mutated forms of these proteins, one concern is that these factors are simply displacing the endogenous IRF. Therefore, the experiments described in this report were specifically designed to assess what role, if any, endogenous transcriptional activators, such as FKHR, play in the repression of basal G6Pase gene transcription by insulin. Our results, derived from fusion gene analyses, are consistent with a model in which insulin inhibits basal G6Pase gene transcription by inhibiting the function of a transcriptional activator, such as FKHR. Moreover, chromatin immunoprecipitation (ChIP) assays demonstrate that FKHR does bind the G6Pase promoter in situ and that insulin inhibits this binding. However, our experiments also revealed that the three G6Pase IRSs are functionally distinct. Thus, detailed gel retardation analyses show that FKHR binds IRS 1 with high affinity and IRS 2 with low affinity but that it does not bind IRS 3. Moreover, mutational analyses demonstrate that, in the context of the G6Pase promoter, only IRS 1 and 2, but not IRS 3, are required for the insulin response. Surprisingly, IRS 1 and IRS 2 are equally important for the insulin response despite their very different affinities for FKHR. Moreover, IRS 3, as well as IRS 1 and IRS 2, can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene. These observations suggest that a transcription factor other than FKHR, or its orthologs, can also mediate an insulin response through the T/G/A/T/T/T motif.

EXPERIMENTAL PROCEDURES

Materials—[alpha-32P]dATP (>3000 Ci mmol−1) was obtained from PerkinElmer Life Sciences. Specific antisera to FKHR (sc-11350) and rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology, Inc. Plasmid Construction—The generation of more than 50 human insulin-responsive acetyltransferase (CAT) fusion genes, containing promoter sequences located between −231 and +66, −198 and +66, and −158 and +66, relative to the transcription start site, has been described (14, 15) as has the use of a three-step PCR strategy (47) to introduce single point mutations into all three of the region B IRS motifs (15). The resulting construct, designated −751 region B SDM, (15), was generated within the context of the −751 to +66 G6Pase promoter fragment. A truncated version of this construct, designated −231 TM, was generated within the context of the −231 to +66 G6Pase promoter fragment using PCR. The same three-step PCR strategy (47) was used to introduce various point mutations into the individual region B IRS motifs all within the context of the −231 to +66 G6Pase promoter fragment. The constructs generated contained either mutations in the individual IRS motifs (designated −231 IRS 1 SDM, −231 IRS 2 SDM, and −231 IRS 3 SDM), mutations in two of the three IRS motifs (designated −231 IRS 1 + 2 SDM), mutations that switched the order of IRS 3 to that of IRS 1 (designated −231 IRS 1:2:1), or mutations that switched the order of both IRS 1 and IRS 2 to that of IRS 3 (designated −231 IRS 3:3:3). All promoter fragments generated by PCR were completely sequenced, using the U. S. Biochemical Corp. Sequenase kit, to verify the absence of polymerase errors.

The plasmid TK-CVI contains the herpes simplex virus thymidine kinase (TK) promoter ligated to the CAT reporter gene (22, 48). The TK promoter extends from −480 to +51, and contains a BamHI site located between positions −40 and −35. Various double-stranded oligonucleotides representing the G6Pase promoter sequence between −197 and −159 or G6Pase IRS 1 (GATACCTGTGTGT), or G6Pase IRS 3 (GATACCACTTTTTTT) were synthesized with BamHI-compatible ends and cloned, as a single copy in same orientation as found in the endogenous TK-CVI by standard techniques (14, 49). The CAT reporter gene was then replaced with the more sensitive firefly luciferase reporter by re-isolating the various TK-CVI promoter constructs, as BamHI-Xhol fragments, from the plasmids described above and ligating them into the pGL3-Mod vector. This vector is based on the pGL2 Basic firefly luciferase vector (Promega, Madison, WI) but contains a modified polylinker (50).

The generation of human IGFBP-1 chloramphenicol acetyltransferase fusion genes, containing promoter sequences located between −1205 and +68, −1232 and +68, and −103 and +68, relative to the transcription start site, has been described (51), as has the generation of a site-directed mutation of both IGFBP-1 IRS motifs in the context of the −1205 to +68 promoter fragment (52). The construction of a human pcDNA3-FKHR expression vector has also been previously described (32). All plasmid constructs were purified by centrifugation through cesium chloride gradients (49).

Cell Culture and Transient Transfection—Rat H4IEI hepaticoma cells were grown in Dulbecco’s modified Eagle’s medium containing 2.5% fetal calf serum and 2.5% newborn calf serum. HepG2 Hep2 and Hep2EII cells were grown in the same media supplemented with 5% (v/v) Nu serum IV (Becton Dickinson). H4IEI and Hep2EII cells were transiently transfected in suspension with the plasmids indicated in the figure legend using the calcium phosphate-DNA co-precipitation method as previously described (22, 36, 50). In experiments using HepG2 cells, an expression vector encoding the insulin receptor, courtesy of Dr. Jonathan Whittaker, was co-transfected with the reporter gene construct.

CAT, β-Galactosidase, and Luciferase Assays—CAT and β-galactosidase assays were performed exactly as previously described (14). Luciferase assays were performed using the Promega Dual-Luciferase Reader System according to the instructions of the manufacturer. For comparisons of basal gene expression, basal CAT activity directed by the various fusion gene constructs was corrected for the β-galactosidase activity in the same samples. Because insulin stimulates Rous sarcoma virus-β-galactosidase expression in HepG2 cells and...
SV40-Renilla luciferase expression in H4IIE cells (data not shown), for comparisons of the effect of insulin on fusion gene expression, CAT or firefly luciferase activity from control and insulin-treated cells was corrected for the protein concentration in the cell lysate, as measured by the Pierce BCA assay. Each construct was analyzed in duplicate or in triplicate in multiple transfections, as specified in the figure legends, using several independent plasmid preparations.

Chromatin Immunoprecipitation Assays—A plasmid encoding a glutathione S-transferase (GST)-FKHR fusion protein (32) was transformed into Escherichia coli (XL1-Blue). This plasmid was derived from the pGEX-5X-3 vector (Amersham Biosciences) such that fusion gene expression can be induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG) (32). Bacteria were grown to an A600 of ~0.7 in LB supplemented with 200 μg/ml ampicillin, and GST-FKHR expression was induced by incubation with 1 mM IPTG for 2 h at 37 °C. Bacteria were pelleted by centrifugation; resuspended in 50 mM HEPES, pH 7.5, 200 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride; and lysed by sonication. Complementary oligonucleotides representing the wild type G6Pase region B sequence between –196 and –155 (Table I) were synthesized with BamHI-compatible ends, gel-purified, annealed, and then labeled with [α-32P]ATP by using the Klenow fragment of E. coli DNA polymerase I to a specific activity of ~2.5 μCi/pmol (49). Labeled oligonucleotide (~7 fmol) was incubated with bacterial lysate (~12 μg) in a reaction volume of 20 μl containing, at final concentrations, 25 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 5% glycerol (v/v), 1 mg/ml bovine serum albumin, and 20 ng of poly(dG-dC)poly(dG-dC). For competition experiments, unlabeled competitor DNA was mixed with the labeled oligomer at the indicated molar excess prior to the addition of bacterial extract. After incubation for 10 min on ice, the reagents were loaded onto a 6% polyacrylamide gel and electrophoresed at 4 °C for 120 min at 190 V in 0.5× TBE (49). Following electrophoresis the gels were dried and exposed to Kodak XAR5 film, and binding was analyzed by autoradiography. Data were quantitated through the use of a Packard Instant Imager.

RESULTS

The G6Pase Promoter Contains Three IRSs That Can Each Confer an Inhibitory Effect of Insulin on the Expression of a Heterologous Fusion Gene—We have previously shown that an oligonucleotide representing region B of the G6Pase promoter can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene (14, 15). Region B contains three elements, designated IRS 1, IRS 2, and IRS 3, that closely match the PEPCPK-like IRS consensus sequence (Fig. 1A; Ref. 14). However, these IRSs are not identical and only the se- quence representing IRS 2 had been shown to independently mediate an inhibitory effect of insulin on the expression of a heterologous fusion gene (22); it had not previously been determined whether the same was true for IRS 1 and IRS 3. This is an important caveat because the proposed consensus sequence for the PEPCPK-like IRS is only based on a comparison of IRS motifs in various insulin-regulated genes, rather than a precise base-by-base analysis of this element. Thus, we sought to determine whether IRS 1 and 3 could independently confer an effect of insulin on the expression of a heterologous herpes simplex virus TK-luciferase fusion gene (Fig. 1A). Double-stranded oligonucleotides representing IRS 1 and 3 were synthesized and ligated in triplicate into the TK promoter, and the effect of insulin on the expression of the resulting fusion genes was analyzed by transient transfection of the liver-derived rat H4IIE cell line (Fig. 1B).
We have previously shown that insulin has little effect on reporter gene expression directed by the native TK promoter, whereas ligations of an oligonucleotide representing the wild-type (WT) G6Pase region B sequence between −197 and −159 into the TK promoter confers an insulin-dependent inhibition of fusion gene expression (Fig. 1B; Ref. 14). In contrast, when an oligonucleotide representing this region B sequence, but containing point mutations in each putative IRS motif (Fig. 1A; TM), is ligated into the TK promoter, it does not confer an insulin-dependent inhibition of reporter gene expression (Fig. 1B; Ref. 14). Fig. 1B shows that, when three copies of IRS 1 or 3 were ligated into the TK promoter, they conferred an insulin-independent inhibition of luciferase expression (Fig. 1B). Single copies of IRS 1 and IRS 3 each mediated an inhibitory effect of insulin on luciferase expression as well, albeit to a smaller degree than three copies (data not shown). These results suggest that G6Pase IRS 1, IRS 2, and IRS 3 can individually be defined as IRSs.

Disruption of the IGFBP-1 IRSs Results in a Significant Decrease in Basal Fusion Gene Expression, but Disruption of the G6Pase IRSs Does Not—Results from a number of studies suggest that, when overexpressed, FKHR can mediate insulin repression of IGFBP-1 and G6Pase gene transcription through their PEPCK-like IRS motifs (32, 34, 38, 60). In addition, we have previously shown that recombinant FKHR can bind an oligonucleotide representing the wild-type G6Pase region B sequence, but not an oligonucleotide containing point mutations in each of the three region B IRS motifs (Fig. 1A; Ref. 16). Because the ability of FKHR to bind these oligonucleotides in vitro correlates with their ability to mediate an insulin effect in situ (Fig. 1B), this suggests that FKHR, even at endogenous levels, may function as an IRF. To further investigate whether endogenous levels of FKHR mediate the inhibitory effect of insulin on G6Pase, and also IGFBP-1 gene expression, the effects of deleting/mutating the G6Pase and IGFBP-1 IRSs on basal fusion gene expression were investigated. We reasoned that if insulin represses G6Pase and IGFBP-1 gene expression simply by inhibiting FKHR transactivation, as proposed (see Introduction), then we would predict that deletions/mutations that prevent FKHR binding should result in a decrease in basal gene expression.

We first sought to determine whether deletion of both IGFBP-1 IRSs results in a decrease in basal IGFBP-1 gene expression. To address this question, liver-derived human HepG2 cells were transiently transfected with an IGFBP-1-CAT fusion gene containing promoter sequence located between −132 and −68, which includes both IGFBP-1 IRS motifs, or an IGFBP-1-CAT fusion gene containing promoter sequence located between −103 and +68, in which both IRS motifs have been deleted (Fig. 2A). Deletion of the IGFBP-1 IRSs resulted in a significant decrease in basal fusion gene expression (Fig. 2A). Next, HepG2 cells were transiently transfected with an IGFBP-1-CAT fusion gene containing promoter sequence located between −1205 and +68, or a fusion gene, designated −1205 DM, containing the same promoter sequence but with both IRS motifs mutated (Fig. 2A). The mutations introduced into the IGFBP-1 promoter have been previously shown to disrupt FKHR binding to the IGFBP-1 IRSs in vitro (32). Fig. 2A shows that mutation of the two IGFBP-1 IRS motifs resulted in a significant decrease in basal fusion gene expression. The results of these experiments, in which the IGFBP-1 IRS motifs have been deleted or mutated, are consistent with the proposed model for the role of FKHR as an activator of basal IGFBP-1 gene transcription that is disabled by insulin signaling.

Similar experiments were then performed to investigate the effect of deleting/mutating the G6Pase IRSs on basal fusion gene expression. HepG2 cells were transiently transfected with a G6Pase-CAT fusion gene containing promoter sequence located between −198 and +66, which includes all three IRS motifs, or a G6Pase-CAT fusion gene containing promoter se-
quence located between −158 and +66, in which all three IRS motifs have been deleted (Fig. 2B). Surprisingly, deletion of the G6Pase IRSs resulted in a significant increase in basal fusion gene expression (Fig. 2B). Next, HepG2 cells were transiently transfected with a G6Pase-CAT fusion gene containing promoter sequence located between −231 and +66 or a fusion gene, designated −231 TM, containing the same promoter sequence but with all three IRS motifs mutated (Fig. 2B). The mutations introduced into the G6Pase promoter have been previously shown to disrupt FKHR binding to the G6Pase IRSs in vitro (16). Fig. 2B shows that mutation of the three G6Pase IRS motifs did not result in a significant change in basal fusion gene expression. The results of these experiments, in which the G6Pase IRS motifs have been deleted or mutated, did not appear to be consistent with the proposed model for the role of FKHR in the insulin-dependent inhibition of G6Pase gene transcription. Therefore, the molecular basis for these observations were investigated further.

**FKHR Activates G6Pase-CAT Fusion Gene Expression**—We hypothesized that one possible explanation for the unexpected absence of a decrease in basal G6Pase fusion gene expression upon deletion/mutation of the three IRSs in region B was that a transcriptional activator, such as FKHR, does not bind region B in situ. To address this possibility, we determined whether overexpression of FKHR could stimulate G6Pase fusion gene expression. Thus, although FKHR is expressed in HepG2 cells (34) and overexpression of FKHR stimulates both endogenous G6Pase (61) and G6Pase fusion gene (34) expression in H4IIE cells, FKHR has not previously been shown to stimulate G6Pase gene expression in HepG2 cells. This is an important distinction because the hormonal regulation of fusion gene expression can differ markedly in these two cell types (62).

To determine whether FKHR can stimulate G6Pase fusion gene expression in HepG2 cells, a G6Pase-CAT fusion gene, containing promoter sequence located between −751 and +66, was co-transfected with either a control expression vector or the same vector encoding FKHR. At the maximum amount assayed (1 μg), the control expression vector had no effect on basal G6Pase-CAT fusion gene expression (data not shown). In contrast, overexpression of FKHR in HepG2 cells stimulated G6Pase-CAT fusion gene expression in a concentration-dependent fashion (Fig. 3A), but insulin was able to repress both basal and FKHR-stimulated G6Pase-CAT fusion gene expression to the same degree (Fig. 3B). To determine whether the stimulation of G6Pase-CAT fusion gene expression by FKHR was mediated through region B, HepG2 cells were transiently transfected with a G6Pase-CAT fusion gene containing a promoter sequence located between −231 and +66 or the fusion gene described above, designated −231 TM, that contains the same promoter sequence but with all three IRS motifs mutated (Fig. 3C). This G6Pase promoter sequence between −231 and +66 is the shortest sequence that contains both regions A and B, and confers a maximal repression of basal G6Pase-CAT fusion gene transcription by insulin (15). Fig. 3C shows that mutation of the three G6Pase IRS motifs, which disrupts FKHR binding to the G6Pase IRSs in vitro (16), also abolished the stimulatory effect of FKHR on fusion gene expression. This result demonstrates that the stimulatory effect of FKHR on G6Pase fusion gene expression is mediated through region B and provides evidence that FKHR can bind region B in situ when overexpressed.

To determine whether endogenous FKHR is also bound to the endogenous G6Pase promoter in the absence of insulin, ChIP assays (53, 63) were performed. Fragmented chromatin from formaldehyde-cross-linked HepG2 or H4IIE cells was subjected to immunoprecipitation with an FKHR antibody, and the presence of the G6Pase promoter in the immunoprecipitates was then analyzed by PCR using primers representing the proximal G6Pase promoter sequence. As can be seen from Fig. 3D, in the basal state, the G6Pase promoter is enriched in the FKHR-immunoprecipitate compared with the IgG control. In contrast, this enrichment was abolished when this analysis was repeated using cells that were pretreated with insulin for 15 min (Fig. 3D). The same results were obtained when these analyses were performed using HepG2 or H4IIE cells (Fig. 3D). These data are consistent with the proposed model of insulin action on FKHR in that insulin stimulates the phosphorylation and nuclear exclusion of FKHR within 15 min of insulin treatment (36, 37, 40).

To test the specificity of the antibody-chromatin interactions, these immunoprecipitates were also analyzed for the presence of exon 5 of the G6Pase gene (8, 59) using PCR primers that represent G6Pase exon 5 coding sequence. Approximately 10.1 kbp of genomic DNA separates exon 5 and the human G6Pase promoter (8, 50) and FKHR would not be predicted to associate with exon 5. As expected, no enrichment of human G6Pase exon 5 was detected in the FKHR immunoprecipitate compared with the IgG control (Fig. 3E). The absence of a signal in the experimental lanes cannot be explained by the lack of chromatin containing human G6Pase exon 5 sequence in the starting material, as a signal of the expected size can be seen in the chromatin input prior to immunoprecipitation. The same result was obtained when this analysis was repeated using H4IIE cells (data not shown). Together, these results demonstrate that FKHR binds to the G6Pase promoter inside intact HepG2 and H4IIE cells and that insulin disrupts this association.

**FKHR Binds G6Pase IRS 1 and IRS 2, but It Does Not Bind IRS 3**—Based on these results, we sought to develop an alternate hypothesis to explain the unexpected absence of a decrease in basal G6Pase fusion gene expression upon deletion/mutation of the three IRSs in region B (see above). Although the three IRSs in region B can each mediate an inhibitory effect of insulin on fusion gene expression (Fig. 1B), slight sequence variations exist between the individual motifs (Fig. 1A) and these differences are conserved, with the exception of a single base pair, in the rat and human G6Pase promoters (14). This suggests that these sequence variations may have some functional significance. Interestingly, there are more than 100 members of the winged helix/forkhead transcription factor family (64), many of which, like FKHR, bind A/T-rich sequences (65). We therefore hypothesized that the different IRS motifs within region B may bind distinct members of the winged helix/forkhead transcription factor family. Furthermore, if correct, a model could be envisaged in which a transcriptional activator, potentially FKHR, and a transcriptional repressor bind the individual IRS motifs within region B, such that no net change in basal G6Pase fusion gene expression is detected upon deletion/mutation of these three motifs.

To begin to explore this alternate model, we first investigated the ability of FKHR to bind the individual region B IRS motifs using the gel retardation assay (Fig. 4). When a labeled double-stranded oligonucleotide, designated G6P IRS WT, representing the wild-type G6Pase region B promoter sequence from −196 to −155 (Table I), was incubated with a crude extract prepared from bacterial cells expressing a GST-FKHR fusion protein, a single IPTG-induced protein-DNA complex was detected (Fig. 4A, arrow). The G6P IRS WT oligonucleotide competed effectively for the binding of the induced protein-DNA complex (Fig. 4A–C), whereas an oligonucleotide designated G6P IRS TM, which contains point mutations in all three of the individual IRS motifs (Table I), failed to compete with the labeled probe for protein binding.
FKHR activates G6Pase-CAT fusion gene expression through the IRS motifs and binds the G6Pase promoter in situ. Panels A and B. HepG2 cells were transiently co-transfected, as described under "Experimental Procedures," with a G6Pase-CAT fusion gene (15 μg), containing promoter sequence located between −751 and +66, and expression vectors encoding either the insulin receptor (5 μg) or β-galactosidase (2.5 μg), and also various amounts of either a pcDNA3 expression vector encoding FKHR or the empty pcDNA3 vector control. Following transfection, cells were incubated for 18–20 h in serum-free medium in the absence (A) or presence (B) of 100 nM insulin. The cells were then harvested, and CAT activity, β-galactosidase activity, and protein concentration were assayed as described under "Experimental Procedures." In panel A, results are presented as the ratio of CAT:β-galactosidase activity relative to the value obtained in the absence of FKHR. Results represent the mean ± S.E. of three experiments, in which each point in the dose-response curve was assayed in quadruplicate. In panel B, results are presented as the ratio of CAT activities, corrected for the protein concentration in the cell lysate, in insulin-treated versus control cells, expressed as percent control, and represent the mean ± S.E. of three experiments, in which each point in the dose response curve was assayed in duplicate.

Panel C, HepG2 cells were transiently co-transfected with either the pcDNA3 expression vector encoding FKHR or the empty vector control (1.0 μg) and G6Pase-CAT fusion genes (15 μg), containing either the WT promoter sequence located between −231 and +66, or the same sequence but with point mutations (Fig. 1A) in each of the three IRS motifs (−231 TM). Expression vectors encoding either the insulin receptor (5 μg) or β-galactosidase (2.5 μg) were also included. Results are presented as the ratio of CAT:β-galactosidase activity relative to the value obtained with the −231 WT fusion gene in the absence of FKHR. Results represent the mean ± S.E. of three experiments, in which each experimental condition was assayed in quadruplicate. *, p < 0.05. Panels D and E, FKHR binding to the G6Pase promoter was analyzed in situ in control or insulin-treated HepG2 and H4IIE cells using the ChIP assay as described under "Experimental Procedures." Chromatin from formaldehyde-treated HepG2 or H4IIE cells was immunoprecipitated using anti-FKHR antibodies or, as a control, using IgG. The presence of the G6Pase promoter (D) and exon 5 (E) in the chromatin preparation prior to immunoprecipitation (input) and in the immunoprecipitates was then assayed using PCR as described under "Experimental Procedures.” MW, molecular weight. Representative experiments are shown. The data in panel E were obtained using chromatin isolated from formaldehyde-treated HepG2 cells incubated in the absence of insulin.

(Fig. 4, A–C). These results demonstrate that this is a specific protein-DNA interaction. Competition experiments, in which a variable molar excess of unlabeled DNA was incubated with the labeled probe, were used to determine which of the three IRS motifs bind FKHR. The unlabeled competitors contained point mutations in the
S.E. of at least three experiments.

Panel A formed as described in Experimental Procedures. Results represent the mean ± S.E. of at least three experiments.

IRS motifs, either individually or in combination (Table I). A representative autoradiograph is shown in Fig. 4A, whereas panels B and C show quantified data from multiple experiments. Fig. 4B shows the results of competition experiments using oligonucleotides in which only one IRS motif was left intact. Overall, these experiments indicate that FKHR binds IRS 1 with high affinity and IRS 2 with low affinity, but that it does not bind IRS 3. Thus, an oligonucleotide containing point mutations in IRS 2 and 3, designated G6P IRS 2 + 3 SDM, competed effectively against the G6P IRS WT probe for FKHR binding (Fig. 4B), indicating that FKHR can bind IRS 1. In contrast, an oligonucleotide containing point mutations in IRS 1 and 2, designated G6P IRS 1 + 2 SDM, did not compete for FKHR binding (Fig. 4B), indicating that FKHR cannot bind IRS 3. An oligonucleotide containing point mutations in IRS 1 and 3, designated G6P IRS 1 + 3 SDM, competed against the labeled probe for FKHR binding, but not as well as the unlabeled G6P IRS WT or G6P IRS 2 + 3 SDM oligonucleotides (Fig. 4B). This suggests that FKHR can bind IRS 2, but that it does so with a lower affinity than it binds IRS 1.

Fig. 4C shows the results of competition experiments using oligonucleotides in which only one IRS motif was mutated. The results of these experiments are consistent with the conclusion that FKHR binds IRS 1 with high affinity and IRS 2 with low affinity, but that it does not bind IRS 3 (Fig. 4C). Thus, an oligonucleotide containing a point mutation in IRS 1, designated G6P IRS 1 SDM, competed poorly against the G6P IRS WT probe for FKHR binding (Fig. 4C), indicating that FKHR does bind IRS 1. This result also reveals that FKHR can bind IRS 2 with a low affinity, given that it does not bind IRS 3 (see above). In contrast, an oligonucleotide containing a point mutation in IRS 3, designated G6P IRS 3 SDM, competed as well as the unlabeled G6P IRS WT oligonucleotide for FKHR binding (Fig. 4C), indicating that FKHR binds IRS 3. An oligonucleotide containing a point mutation in IRS 2, designated G6P IRS 2 SDM, competed against the labeled probe for FKHR binding but not as well as the unlabeled G6P IRS WT oligonucleotide (Fig. 4C), indicating that FKHR does bind IRS 2. The fact that the G6P IRS 2 SDM oligonucleotide competes much more effectively than the G6P IRS 1 SDM oligonucleotide for FKHR binding is again consistent with the conclusion that FKHR binds IRS 1 with higher affinity than IRS 2 (Fig. 4C).

The conclusions reached through these in vitro FKHR binding analyses are supported by experiments in which HepG2 cells were co-transfected with G6Pase-CAT fusion genes containing the same IRS mutations as described above (Table I), along with either a control expression vector or the same vector encoding FKHR. These mutations were all generated in the context of the −231 to +66 G6Pase-CAT fusion gene, which again contains both regions A and B, and therefore mediates a
maximal repression of basal G6Pase-CAT fusion gene expression by insulin (15). FKHR induced expression of the wild-type −231 G6Pase-CAT fusion gene, but it did not induce expression of the −231 TM fusion gene in which all three IRS motifs have been mutated (Figs. 3C and 5). Mutation of IRS 1 or IRS 2, or both together, reduced the stimulatory effect of FKHR on G6Pase-CAT fusion gene expression, whereas mutation of IRS 3 did not (Fig. 5). These results are consistent with the observation that FKHR can bind both IRS 1 and IRS 2, but that it cannot bind IRS 3 (Fig. 4). Mutation of all three IRSs together had a more deleterious effect on the induction of G6Pase-CAT fusion gene expression by FKHR than mutation of either IRS 1 or IRS 2 alone (Fig. 5).

The relative location of the individual IRS motifs does not appear to be significant with respect to transactivation by FKHR. Thus, a G6Pase-CAT fusion gene, designated −231 IRS 3:3:3, was generated in which the sequence of IRS 1 and 2 were switched to that of IRS 3. Although this fusion gene contains three consecutive IRS 3 elements (Table I), its expression was still not induced by FKHR overexpression (Fig. 5), suggesting that IRS 3 cannot bind FKHR even when multimerized. These results indicate that IRS 3 is neither necessary nor sufficient to mediate FKHR transactivation in the context of the G6Pase promoter. In contrast, when the sequence of IRS 3 is switched to that of IRS 1 (Table I), to create a construct designated −231 IRS 1:2:1, the induction of fusion gene expression by FKHR was enhanced (Fig. 5), consistent with enhanced FKHR binding. In summary, results from these FKHR overexpression studies are consistent with the in vitro FKHR binding studies (Fig. 4), because the ability of FKHR to bind IRS 1, 2, and 3 in vitro corresponds with its ability to activate G6Pase-CAT fusion gene expression through these motifs in situ (Fig. 5). Overall, these results indicate that the single base pair variations between the three region B IRS motifs (Fig. 1A) affect FKHR binding affinity. Most significantly, the fact that the region B IRS 3 motif can mediate an inhibitory effect of insulin on the expression of a heterologous fusion gene (Fig. 1), but cannot bind FKHR (Fig. 4), indicates the existence of an insulin response factor other than FKHR that can regulate gene expression through the PECK-like IRS motif.

One possible caveat to this conclusion is that the configuration of the triple IRS 3 element that was used in the heterologous fusion gene experiment (Fig. 1) might bind FKHR even though, in the context of the region B sequence, IRS 3 does not. This would then explain the ability of IRS 3 to mediate an insulin response in a heterologous context. To address this possibility, a labeled double-stranded oligonucleotide, designated TK IRS 3:3:3, representing the sequence of the triple IRS 3 motifs in the IRS 3 TK-pG3 fusion gene (Fig. 1), was incubated with a crude extract prepared from bacterial cells expressing a GST-FKHR fusion protein. No IPTG-induced protein-DNA complex was detected (Fig. 6). In contrast, a single IPTG-induced protein-DNA complex was detected when the G6P IRS WT oligonucleotide (Table I) was used as the labeled probe (Fig. 6, arrow). The unlabeled G6P IRS WT oligonucleotide competed effectively for the binding of the induced protein-DNA complex (Fig. 6), whereas the TK IRS 3:3:3 oligonucleotide did not. These results demonstrate that FKHR cannot bind the multimerized IRS 3 element, which mediates an insulin effect on the TK-pG3 heterologous fusion gene (Fig. 1).

G6Pase IRS 1, IRS 2, and IRS 3 Are Functionally Distinct Elements in the Context of the G6Pase Promoter—The observation that the different IRS motifs within region B bind FKHR with distinct affinities is consistent with the proposed hypothesis that the different IRS motifs within region B bind distinct members of the winged helix/forkhead transcription factor family. Unfortunately, the identification of PECK-like IRS-binding proteins using liver/hepatoma nuclear extracts and gel retardation analyses has been largely unsuccessful (17); even FKHR binding cannot be detected using this approach. Indeed, the potential significance of FKHR in insulin-regulated gene expression only became apparent through genetic studies in C. elegans. Gel retardation experiments using G6Pase region B also fail to identify candidate factors binding the individual IRS motifs.
motifs (data not shown). Nevertheless, the observation that the different IRS motifs within region B bind FKHR with distinct affinities suggests that these motifs may be functionally distinct.

To explore this possibility, the same fusion gene constructs described above were transiently transfected into HepG2 cells and the effect of mutating the individual IRS motifs on basal G6Pase-CAT fusion gene expression was investigated (Fig. 7). As shown in Figs. 2B and 7, basal expression of the wild-type −231 G6Pase-CAT fusion gene is similar to that of the −231 TM fusion gene in which all three IRS motifs have been mutated. Similarly, mutation of IRS 2 had little effect on basal G6Pase-CAT fusion gene expression (Fig. 7). However, when IRS 1 was mutated, there was a statistically significant decrease in basal fusion gene expression, indicating that a transcriptional activator may bind this site (Fig. 7). No further reduction in basal fusion gene expression was detected if IRS 2 was mutated in combination with mutation of IRS 1 (Fig. 7). In contrast, when IRS 3 was mutated, there was a significant increase in basal −231 G6Pase-CAT fusion gene expression, suggesting that a transcriptional repressor may bind this site (Fig. 7).

To further this analysis we examined the effect of switching the sequence of IRS 1 and 2 to that of IRS 3 (Table I). The G6Pase-CAT fusion gene containing this mutation, designated −231 IRS 3:3:3, would be predicted to bind three repressor molecules. Fig. 7 shows that basal CAT expression directed by this fusion gene was decreased relative to that directed by the wild type G6Pase promoter. Moreover, the magnitude of this decrease in basal fusion gene expression was greater than that observed upon mutation of IRS 1 (Fig. 7). This observation is consistent with a gain of repressor binding to IRS 1 and IRS 2 in the −231 IRS 3:3:3 construct, in conjunction with loss of activator binding to IRS 1 (Fig. 7). We also examined the effect of switching the sequence of IRS 3 to that of IRS 1 (Table I). The G6Pase-CAT fusion gene containing this mutation, designated −231 IRS 1:2:1, would be predicted to bind two activator molecules. Fig. 7 shows that basal CAT expression directed by this fusion gene was increased relative to that directed by the wild type G6Pase promoter. However, this increase in basal fusion gene expression was no different from that observed upon mutation of IRS 3, suggesting that the loss of repressor binding to IRS 3, rather than the gain of activator binding, can explain this result (Fig. 7). In contrast, this same mutation did enhance the stimulation of G6Pase-CAT fusion gene expression by overexpressed levels of FKHR (Fig. 5), suggesting that the effects of these mutations are dependent upon the relative abundance of activator/repressor proteins within the cell.

In summary, these observations suggest that the three IRS motifs within region B are functionally distinct, consistent with the observation that they bind FKHR with differing affinities. Furthermore, the observations that IRS 1 binds a transcriptional activator, potentially FKHR, and that IRS 3 binds an (unidentified) transcriptional repressor can explain why no net change in basal G6Pase fusion gene expression is detected when these motifs are deleted/mutated in combination (Fig. 2).

G6Pase IRS 3 Is Neither Necessary nor Sufficient for the Repression of G6Pase Fusion Gene Expression by Insulin—Because the three IRS motifs within region B are functionally distinct with respect to the regulation of basal G6Pase-CAT fusion gene expression, we next investigated the relative contribution of these motifs to the inhibition of basal G6Pase-CAT fusion gene expression by insulin. The same fusion gene constructs described above were transiently transfected into HepG2 cells and the effect of mutating the individual IRS motifs on the repression of basal G6Pase-CAT fusion gene expression by insulin was investigated (Fig. 8). As shown in Fig. 8, insulin repressed basal expression of the wild-type −231 G6Pase-CAT fusion gene, but had almost no effect on the expression of the −231 TM G6Pase-CAT fusion gene in which all three IRS motifs have been mutated. Mutation of IRS 1 or IRS 2 partially impaired the repression of basal G6Pase-CAT fusion gene expression by insulin, whereas mutation of IRS 1 and IRS 2 together almost completely abolished the insulin response (Fig. 8). This suggests that both IRS 1 and IRS 2 are required for the full effect of insulin on basal G6Pase-CAT fusion gene expression and that IRS 3 is not sufficient for an insulin response. Surprisingly, when IRS 3 was mutated, the repression of basal −231 G6Pase-CAT fusion gene expression by insulin was not impaired, suggesting that IRS 3 is also not required for the insulin response (Fig. 8). To further investigate this observation, we examined the effect of switching the sequence of IRS
1 and 2 to that of IRS 3 (Table I). Basal expression of the G6Pase-CAT fusion gene containing this mutation, designated −231 IRS 3:3:3, was not repressed by insulin (Fig. 8). This suggests that, even when multimerized, IRS 3 is not able to mediate an effect of insulin on G6Pase-CAT fusion gene expression (Fig. 8), although it can mediate an effect of insulin on the expression of a heterologous fusion gene (Fig. 1B). Finally, we also examined the effect of switching the sequence of IRS 3 to that of IRS 1 (Table I). Insulin repressed the basal expression of the G6Pase-CAT fusion gene containing this mutation, designated −231 IRS 1:2:1, although the effect of insulin was not enhanced relative to that seen with the wild-type −231 G6Pase-CAT fusion gene. In summary, these observations suggest that the three IRS motifs within region B are functionally distinct with respect to their effect on both basal (Fig. 7) and insulin-regulated (Fig. 8) G6Pase-CAT fusion gene expression.

DISCUSSION

In both poorly controlled type I diabetics and in type II diabetics, the ability of insulin to stimulate peripheral glucose utilization and to repress hepatic glucose production (HGP) is reduced as a consequence of insulin resistance. The elevated HGP results from an increased rate of gluconeogenesis (66, 67), and evidence suggests that this can be explained in part by increased expression of key gluconeogenic enzymes, such as G6Pase (68, 69). Clearly a better understanding of the mechanism by which insulin inhibits G6Pase gene transcription has the potential to reveal novel therapeutic targets for treatment of this increased HGP. In particular, identification of the insulin response factor(s) that mediates the inhibitory effect of insulin on G6Pase and PEPCK gene transcription through the PEPCK-like IRS motifs is of major interest.

Substantial attention has recently focused on the potential role of the winged helix/forkhead transcription factor FKHR and its orthologs, FKHRL1 and AFX, as the elusive IRF. Moreover, a signaling pathway has been proposed in which insulin inhibits G6Pase gene transcription has the potential to reveal novel therapeutic targets for treatment of this increased HGP. In particular, identification of the insulin response factor(s) that mediates the inhibitory effect of insulin on G6Pase and PEPCK gene transcription through the PEPCK-like IRS motifs is of major interest.

There are several studies that support the involvement of this pathway in the regulation of G6Pase gene expression by insulin. Thus, FKHR binds region B in the G6Pase promoter, which contains three PEPCK-like IRS motifs (16, 34), and overexpression of FKHR stimulates G6Pase fusion gene expression through these elements in both H4IE (34) and HepG2 (Fig. 3, A–C) cells. Moreover, overexpression of constitutively active PKB mimics the inhibitory effect of insulin on G6Pase gene fusion expression, an effect that is mediated through the G6Pase PEPCK-like IRS motifs and is blocked by overexpression of a PKB-insensitive FKHR mutant (34). Similarly, disruption of FKHR expression or overexpression of a non-insulin-sensitive FKHR mutant alters G6Pase gene expression in vivo in a manner consistent with the hypothesis that FKHR directly regulates expression of this gene (70). Finally, expression of FKHR in kidney cells, in which G6Pase gene expression is normally refractory to insulin action, confers insulin-regulated G6Pase gene expression (71). This latter study demonstrates that FKHR can function as an IRF through the PEPCK-like IRS motif. However, a concern with the interpretation of studies in which wild-type or mutated forms of FKHR have been overexpressed in liver cells, in which insulin regulates endogenous G6Pase gene expression, is that FKHR is simply displacing the endogenous IRF. To avoid this complication, the experiments described in this report were specifically designed to assess what role, if any, endogenous transcriptional activators, such as FKHR, play in the repression of basal G6Pase and IGFBP-1 gene transcription by insulin. ChIP assays demonstrate that FKHR does bind the G6Pase promoter in situ and that insulin inhibits this binding (Fig. 3D). Moreover, mutations in G6Pase IRS 1 and IRS 2, which prevent FKHR binding (Fig. 4), also block the inhibitory effect of insulin (Fig. 8). However, this correlation is not perfect because mutations in IRS 1 and IRS 2 are equally deleterious to the insulin response (Fig. 8), whereas binding analyses indicate that FKHR binds IRS 1 with much higher affinity than it binds IRS 2 (Fig. 4). This suggests that either (i) other factors bound to the G6Pase promoter influence the binding affinity of FKHR to IRS 1 and IRS 2 in situ or (ii) another factor(s) exists that functions as an IRF, besides FKHR and its orthologs, which have almost identical binding specificities (72). The former idea is not consistent with the observation that mutation of IRS 1, although not IRS 2, reduces basal G6Pase fusion gene expression (Fig. 7). In contrast, the existence of IRFs, other than FKHR and its orthologs, is supported by the studies of Hall and colleagues (46), who demonstrated that the binding of FKHRL1 to the PEPCK IRS only correlates with the effect of insulin mediated through that element when FKHRL1 is overexpressed. Thus, when FKHRL1 is overexpressed, there is a direct correlation between the ability of mutant IRSs to bind FKHRL1 and their ability to mediate an inhibitory effect of insulin on heterologous fusion gene transcription. However, when FKHRL1 is not overexpressed, there is a dissociation between FKHRL1 binding and insulin action through this element (46). These studies suggest that endogenous levels of FKHR family members likely do not function as the true IRF in this context, but when FKHRL1 is overexpressed, it can disrupt the activity of the true IRF and mediate an effect of insulin through the PEPCK-like IRS. Similarly, the fact that the G6Pase IRS 3 sequence can mediate an insulin response in a heterologous context (Fig. 1), but does not bind FKHR in vitro (Fig. 4), strongly suggests that another IRF exists. This result also implies that the PEPCK-like IRS motif actually represents a group of related IRFs. Despite the fact that studies in C. elegans led to a major breakthrough in the understanding of insulin-regulated gene expression in mammals (30, 31), our conclusion that FKHR and its orthologs are not the only IRFs that act through the PEPCK-like IRS motif suggests a limitation in extending the interpretation of studies in C. elegans to mammals. Thus, in C. elegans Ruvkun and colleagues (73) demonstrated that Daf-16 is the major output of insulin signaling in C. elegans and suggested that the same might apply to FKHR and its orthologs in mammals, a conclusion that our data and that of Hall et al. (46) do not support.

Although several studies support the hypothesis that PKB is sufficient for the repression of PEPCK, IGFBP-1, and G6Pase gene expression (34, 60, 74), there are several other studies that suggest PKB is either not required or is not sufficient (43–45). The existence of PKB-independent mechanisms for regulating FKHR would help explain some of the controversy in the literature regarding the role of this kinase in insulin-regulated gene expression. Indeed, Kenyon and colleagues (75) have recently suggested that insulin also regulates Daf-16 through non-AKT (PKB) consensus phosphorylation sites in C. elegans, an observation that is consistent with several studies demonstrating the existence of additional protein kinases that phosphorylate and regulate FKHR function in mammalian cells (76–78). The results of Kenyon and colleagues (75) are in disagreement with those of Ruvkun and colleagues (73), who suggested that Daf-16 is regulated by insulin-like signaling in C. elegans entirely through AKT (PKB) consensus phosphorylation sites. Although the reason for this difference is unclear, an additional complication in the study of Daf-16/ FKHR phosphorylation arises from the fact that that different
kinases may phosphorylate the same sites as PKB on these proteins, as pointed out by Kops and Burge (79). This possibility might reconcile the observation by Ruvkun and colleagues (73) that Daf-16 is regulated by insulin-like signaling in C. elegans entirely through AKT (PKB) consensus phosphorylation sites with their earlier conclusion, based on genetic data, that AKT is not the only signaling pathway leading to Daf-16 that is activated downstream of the insulin receptor in C. elegans (80).

The conclusion that FKHR and its orthologs are not the only IRFs that act through the PEPPCK-like motifs is potentially consistent with the results of studies investigating the signal transduction pathways through which insulin regulates PEPPCK, IGFBP-1, and G6Pase gene expression. Thus, several such studies have concluded that insulin regulates the expression of these genes through distinct pathways. For example, insulin regulation of IGFBP-1 gene expression (81, 82), although not PEPPCK (83) or G6Pase (43) gene expression, is dependent on the mammalian target of rapamycin. Moreover, it is likely that the regulation of these genes is highly complex, given the large number of kinase inhibitors/activators that can affect their expression (84–86). In addition to these differences in the signaling pathways that regulate PEPPCK, IGFBP-1, and G6Pase gene expression, the function of the PEPPCK-like IRS motif in their promoters also varies. In the PEPPCK promoter, the single copy of this motif appears to be important for the repression of glucocorticoid-stimulated, but not basal, gene transcription by insulin (17). In contrast, the IGFBP-1 and G6Pase promoters contain multiple copies of this motif that function to mediate the repression of both glucocorticoid-stimulated and basal gene transcription by insulin (15, 23, 52).

Taken together, our studies support the hypothesis that insulin inhibits G6Pase gene expression by inactivating the transcriptional activator FKHR. We also show that G6Pase region B is a complex element containing three functionally distinct PEPPCK-like IRS motifs that potentially bind distinct members of the forkhead family of transcription factors. Importantly, we also provide evidence that strongly suggests another IRF, other than FKHR or its orthologs, can regulate gene transcription through the PEPPCK-like IRS motif.

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