

Conservation of an Insulin Response Unit Between Mouse and Human Glucose-6-Phosphatase Catalytic Subunit Gene Promoters

Transcription Factor FKHR Binds the Insulin Response Sequence

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Because overexpression of the glucose-6-phosphatase catalytic subunit (G-6-Pase) in both type 1 and type 2 diabetes may contribute to the characteristic increased rate of hepatic glucose production, we have investigated whether the insulin response unit (IRU) identified in the mouse G-6-Pase promoter is conserved in the human promoter. A series of human G-6-Pase-chloramphenicol acetyltransferase (CAT) fusion genes was transiently transfected into human HepG2 hepatoma cells, and the effect of insulin on basal CAT expression was analyzed. The results suggest that the IRU identified in the mouse promoter is conserved in the human promoter, but that an upstream multimerized insulin response sequence (IRS) motif that is only found in the human promoter appears to be functionally inactive. The G-6-Pase IRU comprises two distinct promoter regions, designated A and B. Region B contains an IRS, whereas region A acts as an accessory element to enhance the effect of insulin, mediated through region B, on basal G-6-Pase gene transcription. We have previously shown that the accessory factor binding region A is hepatocyte nuclear factor-1, and we show here that the forkhead protein FKHR is a candidate for the insulin-responsive transcription factor binding region B. *Diabetes* 48:1885-1889, 1999

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CAT, chloramphenicol acetyltransferase; G-6-Pase, glucose-6-phosphatase catalytic subunit; GST, glutathione-S-transferase; HNF-1, hepatocyte nuclear factor-1; IGFBP-1, insulin-like growth factor binding protein-1; IPTG, isopropylthio- β -D-galactosidase; IRF, insulin-responsive transcription factor; IRS, insulin response sequence; IRU, insulin response unit; PCR, polymerase chain reaction; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase.

Two regions of the mouse glucose-6-phosphatase catalytic subunit (G-6-Pase) promoter, designated A (from -231 to -199) and B (from -198 to -159), are required for the maximal repression of basal G-6-Pase gene transcription by insulin (1,2). Region B contains an insulin response sequence (IRS), since it can confer an inhibitory effect of insulin on the expression of a heterologous fusion gene (1,2). Within region B, there are three copies of the consensus inhibitory IRS, T(G/A)TTT(T/G)(G/T), which is found in several insulin-regulated genes (1,3). Thus, the IRSs identified in the phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT), and apolipoprotein CIII promoters all contain this same motif (4-6), whereas the IRS in the insulin-like growth factor binding protein-1 (IGFBP-1) promoter has two copies of this motif arranged as an inverted palindrome (7,8). Region A in the G-6-Pase promoter is not an IRS, but rather acts as an accessory element that enhances the action of the IRS located in region B (2). Such an arrangement is referred to as an insulin response unit (IRU) (9). The accessory factor binding region A is hepatocyte nuclear factor-1 (HNF-1) (2), but the identity of the insulin-responsive transcription factor (IRF) binding region B in the G-6-Pase promoter, and the related IRSs described above, has proven to be elusive (3). However, Durham et al. (10) have recently shown that the IRS in the IGFBP-1 promoter binds the forkhead protein FKHR (11). Given the possible involvement of G-6-Pase overexpression in the increased hepatic glucose production associated with both type 1 and type 2 diabetes (12-14), the experiments described in this study were designed to address the questions of whether the IRU identified in the mouse G-6-Pase promoter is also conserved in the human promoter and whether the region B G-6-Pase IRS binds FKHR.

An alignment of the mouse (1), rat (15), and human (16) G-6-Pase promoter sequences reveals that the HNF-1 site (2,17) and the three copies of the consensus inhibitory IRS in region B (1,2) are highly conserved in all three species (see

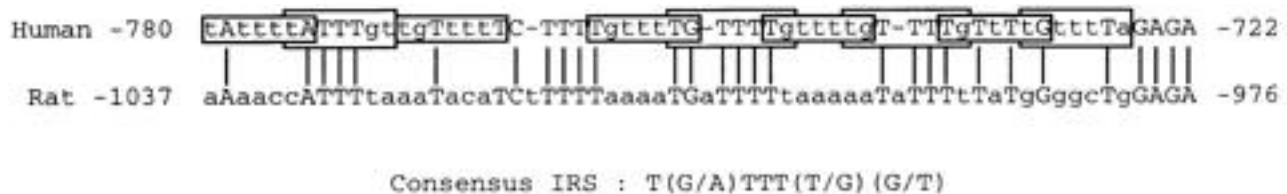


FIG. 1. The human G-6-Pase promoter contains an upstream multimerized IRS motif. The human and rat G-6-Pase promoter sequences were aligned using the Needleman-Wunsch pairwise method in conjunction with software from IntelliGenetics. The sequences are numbered relative to the experimentally determined transcription start sites (15,16). The nine homologies in the human promoter with the consensus IRS shown are in boxes.

Fig. 2A in Ref. 2 and Fig. 3A in Ref. 1 for sequence comparisons). However, an alignment of upstream promoter sequences reveals that a region of the human promoter between -780 and -722 contains an additional nine copies of this consensus IRS motif, whereas the equivalent region of the rat promoter, located between -1037 and -976, does not (Fig. 1). Our longest fragment of the mouse promoter extends to -751 and contains no additional upstream IRS motifs.

To investigate the functional significance of the upstream multimerized IRS motif in the human G-6-Pase promoter and to determine whether the more proximal IRU identified in the mouse promoter is functionally conserved in the human promoter, a series of truncated human G-6-Pase-chloramphenicol acetyltransferase (CAT) fusion genes were constructed with 5' end points equivalent to those used to define the two components of the mouse IRU, namely region A and region B. The basal CAT expression directed by these constructs and the ability of insulin to repress this basal expression was analyzed by transient transfection of HepG2 cells (Fig. 2).

Figure 2A shows that basal CAT expression directed by the full-length mouse G-6-Pase-CAT fusion gene, which contains the promoter sequence between -751 and +66, is approximately threefold higher than that obtained with the full-length human G-6-Pase-CAT fusion gene, which contains the promoter sequence between -826 and +7. This difference is mainly explained by cis-acting elements located between -751 and -231 in the mouse promoter, since, upon deletion of this region, the basal expression of the truncated fusion gene is similar to that of the equivalent human G-6-Pase-CAT fusion gene (Fig. 2B). In contrast, deletion of the human promoter sequence between -826 and -235 had little effect on basal CAT expression (Fig. 2B).

As previously reported by Lin et al. (17), deletion of the HNF-1 site in the human promoter, located between -235 and -196, results in a reduction in basal fusion gene expression that is partially restored by further deletion of the region between -196 and -156 containing the three IRS motifs (Fig. 2B). We had previously shown that deletion of the mouse promoter sequence between -271 and -158, which contains the IRU, results in little change in basal fusion gene expression (1) and that similar basal CAT expression is obtained with mouse G-6-Pase-CAT fusion gene constructs with end points of -231 and -198 (2). However, in the current series of transfections, the basal CAT expression directed by these constructs varied with a pattern similar to that seen with the equivalent human G-6-Pase-CAT fusion genes (Fig. 2B). Thus, in these experiments, deletion of the HNF-1 site in the mouse promoter, located between -231 and -198, did result in a reduction in basal G-6-Pase-CAT fusion gene expression, whereas in earlier experiments (1,2), deletion of

this HNF-1 site had no such effect. The explanation for this variable effect of deleting the HNF-1 site on basal mouse G-6-Pase gene expression is unknown.

We next sought to analyze the effect of insulin on the basal expression of the human G-6-Pase-CAT fusion genes. Figure 2C shows that deletion of the human promoter sequence between -826 and -235, which contains the upstream multimerized IRS motif located between -780 and -722 (Fig. 1), does not reduce the inhibitory effect of insulin on basal human G-6-Pase-CAT expression. In addition, Fig. 2C also shows that the proximal IRU identified in the mouse promoter is functionally conserved in the human promoter. Thus, deletion of the HNF-1 site located between -235 and -196, designated region A in the mouse promoter, partially reduces the inhibitory effect of insulin, and deletion of the three IRS motifs located between -196 and -156, designated region B in the mouse promoter, almost abolishes the remaining inhibitory effect of insulin (Fig. 2C).

Although the upstream multimerized IRS motif was functionally inactive in the presence of the proximal IRU (Fig. 2C), it was possible that this multimerized IRS motif represented a redundant element through which insulin could repress human G-6-Pase gene expression. To directly address this hypothesis, region B was mutated in the context of the -826 to +7 G-6-Pase promoter fragment by introducing mutations into all three copies of the T(G/A)TTT(T/G)(G/T) IRS motif at positions that are known to abolish the effect of insulin mediated through this sequence (1,2). The ability of insulin to suppress basal human G-6-Pase-CAT expression directed by this construct was substantially reduced (data not shown), indicating that the upstream multimerized IRS motif cannot act in a redundant fashion to compensate for the mutated proximal IRU.

Finally, when HepG2 cells were transiently transfected with the full-length human or mouse G-6-Pase-CAT fusion genes, which contain the promoter sequence between -826 and +7 or -751 and +66, respectively, insulin inhibited the basal expression of both fusion genes with the same concentration dependence (Fig. 3). Since only the human G-6-Pase-CAT fusion gene contains the upstream multimerized IRS motif, this indicates that this sequence does not increase the sensitivity of human G-6-Pase-CAT fusion gene expression to the action of insulin.

The sequence of the region B IRS is highly conserved between the mouse and human promoters (Fig. 4A), suggesting that the same IRF will mediate the effect of insulin through both elements. Durham et al. (10) have recently shown that the related IRS in the IGFBP-1 promoter binds the forkhead protein FKHR (11). To investigate whether FKHR can also bind the G-6-Pase IRS, a labeled double-stranded

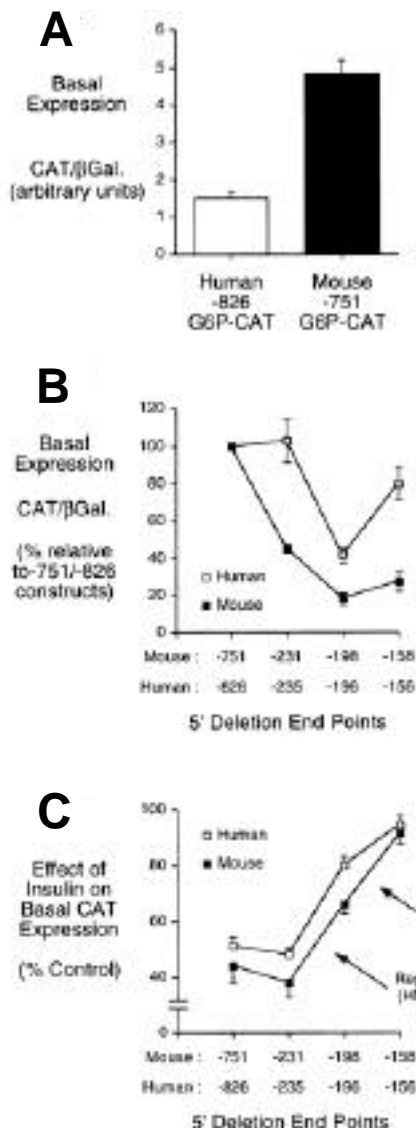


FIG. 2. The mouse G-6-Pase IRU is conserved in the human promoter. HepG2 cells were transiently cotransfected, as previously described (1,2), with various G-6-Pase-CAT (G6P-CAT) fusion genes (15 μ g) containing distinct lengths of promoter sequence, as indicated by the 5' deletion end point, and expression vectors encoding the insulin receptor (5 μ g) and β -galactosidase (β Gal.) (2.5 μ g). After transfection, cells were incubated for 18–20 h in serum-free medium in the presence or absence of 10 nmol/l insulin. The cells were then harvested, and CAT activity, β -galactosidase activity, and protein concentration were all assayed as described in METHODS. In *A* and *B*, results are presented as the ratio of CAT-to- β -galactosidase activity in control cells and are expressed in arbitrary units or as a percentage relative to the values obtained with the full-length constructs, respectively. In *C*, results are presented as the ratio of CAT activities, corrected for protein concentration in the cell lysate, in insulin-treated versus control cells (expressed as percent control). Results represent the mean \pm SE of 3–9 (*A*) and 5–15 (*B* and *C*) experiments, in which each construct was assayed in duplicate.

oligonucleotide, designated G-6-P WT, representing the wild-type G-6-Pase region B promoter sequence from -197 to -159 (Fig. 4A), was incubated with a crude extract prepared from bacterial cells expressing a glutathione-S-transferase (GST)-FKHR fusion protein. When protein binding was analyzed using the gel retardation assay, a single isopropylthio- β -D-galactosidase (IPTG)-induced protein-DNA complex was

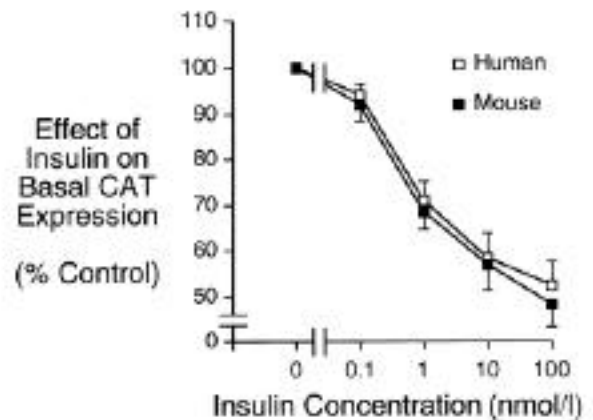


FIG. 3. Insulin inhibits basal human and mouse G-6-Pase-CAT fusion gene expression with a similar concentration dependence. HepG2 cells were transiently cotransfected, as previously described (1,2), with either a mouse or human G-6-Pase-CAT fusion gene (15 μ g) containing the promoter sequence from -751 to +66 or -826 to +7, respectively, and an expression vector (5 μ g) encoding the insulin receptor. After transfection, cells were incubated for 18–20 h in serum-free medium in the absence or presence of various concentrations of insulin. The cells were then harvested, and CAT activity and protein concentration were assayed as described in METHODS. Results are presented as the ratio of CAT activities, corrected for protein concentration in the cell lysate, in insulin-treated versus control cells (expressed as percent of control) and represent the mean \pm SE of four experiments.

detected (Fig. 4B, arrow). A competition experiment, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, was used to correlate protein binding with the insulin response. The G-6-P WT oligonucleotide competed effectively for the binding of the induced protein-DNA complex (Fig. 4B), indicating that the other, non-IPTG-induced bands detected in the assay represent nonspecific binding (Fig. 4B). In contrast, an oligonucleotide, designated G-6-P MUT (Fig. 4A), that contains mutations that in functional assays abolish the effect of insulin (1, 2) failed to compete with the labeled probe for protein binding (Fig. 4B). Thus, the binding of FKHR to the region B G-6-Pase IRS correlates with the insulin response. This result and the recent observations that insulin and IGF-1 stimulate the phosphorylation of FKHR (18,19) suggest that FKHR is a candidate to mediate the action of insulin on G-6-Pase gene transcription.

RESEARCH DESIGN AND METHODS

Plasmid construction. Generation of a full-length mouse G-6-Pase-CAT fusion gene containing promoter sequence spanning nucleotides -751 to +66 relative to the transcription start site has previously been described (1). A human G-6-Pase promoter fragment spanning nucleotides -826 to +7 (16) was isolated using the polymerase chain reaction (PCR) in conjunction with Vent DNA polymerase (New England Biolabs, Beverly, MA), human genomic DNA (Clontech, Palo Alto, CA), and the following 5' and 3' oligonucleotide primers: 5'-GAAGATCTGGAAAGACCAGCAAGATGATAGTCCC-3'; 5'-GAAGATCTGCTATGAGTCTGTGCTTGTGCCCC-3' (*Bgl*II sites used for cloning are underlined). The PCR product was digested with *Bgl*II and ligated into *Bgl*II-digested pSP72 (Promega, Madison, WI) for sequencing. After confirming the absence of polymerase errors, by comparison with the published human G-6-Pase promoter sequence (16), the human promoter was re-isolated from pSP72 and ligated into the pCAT(An) expression vector (20). A series of truncated human G-6-Pase-CAT fusion genes, with 5' end points as shown in Fig. 2, was then constructed by PCR using the full-length human G-6-Pase promoter fragment as the template. A previously described three-step PCR strategy (2) was used to create a site-directed mutant of the three region B IRS motifs within the context of the -826 to +7 human G-6-Pase promoter fragment. All promoter fragments

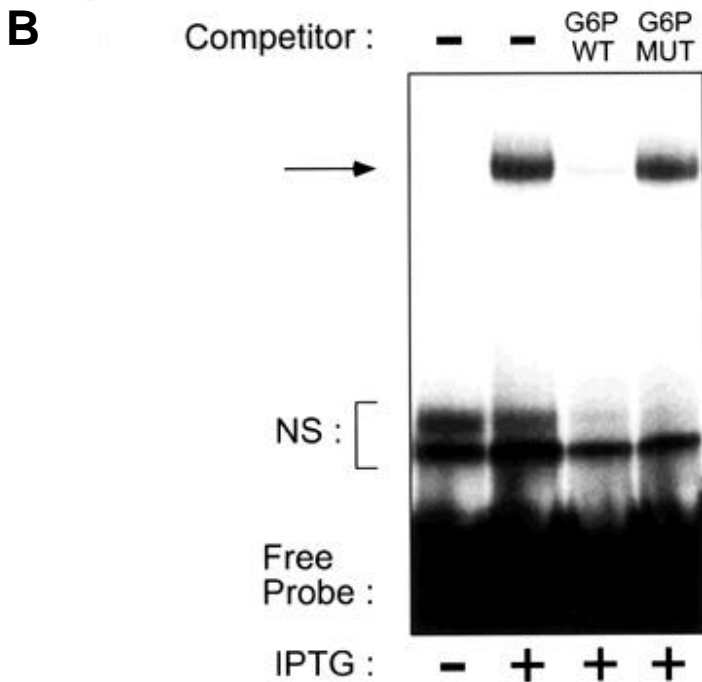
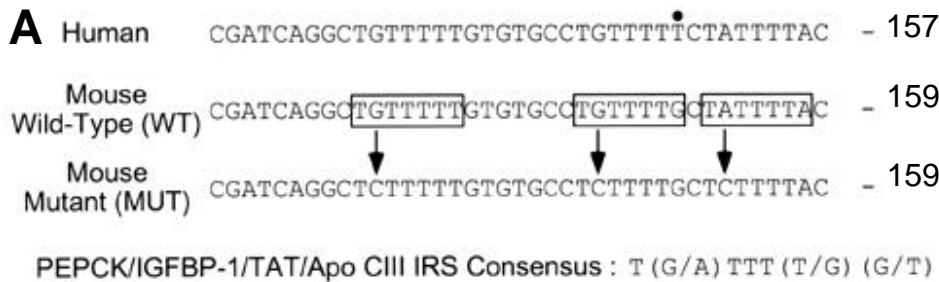


FIG. 4. The transcription factor FKHR binds the G-6-Pase IRS. **A:** Comparison of the mouse G-6-Pase region B promoter sequence between -197 and -159 with the equivalent sequence in the human G-6-Pase gene. The single base-pair change in the human sequence is indicated with a black dot. The homologies with the PEPCK/IGFBP-1/TAT/apolipoprotein CIII IRSs are boxed. **B:** The labeled wild-type mouse G-6-Pase region B IRS, designated G-6-P WT, was incubated in the absence (-) or presence of a 100-fold molar excess of the unlabeled competitor DNAs representing the wild-type (G-6-P WT) or mutated (G-6-P MUT) G-6-Pase IRS. Bacterial extract from either control (-) or IPTG-treated (+) cells transformed with a plasmid encoding a GST-FKHR fusion protein (10) was then added, and protein binding was analyzed using the gel retardation assay, as described in METHODS. The specific IPTG-induced protein-DNA complex is indicated by the arrow. A representative autoradiograph is shown. NS, nonspecific binding.

generated by PCR were completely sequenced, using the U.S. Biochemical Sequenase kit (Cleveland, OH), to verify the absence of polymerase errors. Plasmid constructs were purified by centrifugation twice through cesium chloride gradients.

Cell culture and transient transfection. Human HepG2 hepatoma cells were grown and transiently transfected as previously described (1,2). Each construct was analyzed in duplicate in multiple transfections, as specified in the figures, using at least three independent plasmid preparations.

CAT and β -galactosidase assays. CAT and β -galactosidase assays were performed exactly as previously described (1).

Gel retardation assay. A plasmid encoding a GST-FKHR fusion protein (10) was transformed into *Escherichia coli* (XL1-Blue). This plasmid was derived from the pGEX-5X-3 vector (Pharmacia, Uppsala, Sweden) such that fusion gene expression can be induced by IPTG (10). Bacteria were grown to an optical density (OD)₆₀₀ of ~0.7 in Luria-Bertani media supplemented with 200 μ g/ml ampicillin, and GST-FKHR expression was induced by incubation with 1 mmol/l IPTG for 2 h at 37°C. Bacteria were pelleted by centrifugation, resuspended in 50 mmol/l HEPES (pH 7.5), 200 mmol/l NaCl, and 1 mmol/l phenylmethylsulfonyl fluoride and lysed by sonication.

Complementary oligonucleotides representing the G-6-Pase region B sequence between -197 and -159 (Fig. 4A) were synthesized with *Bam*HI-compatible ends, gel purified, annealed, and then labeled with [α -³²P]dATP using the Klenow fragment of *E. coli* DNA polymerase I to a specific activity of ~2.5 mCi/pmol. Labeled oligonucleotide (~7 fmol) was incubated with bacterial lysate (12 μ g) in a reaction volume of 20 μ l containing, at final concentrations, 25 mmol/l HEPES (pH 7.5), 100 mmol/l NaCl, 2 mmol/l dithiothreitol, 5% glycerol (vol/vol), 1 mg/ml bovine serum albumin, and 20 ng poly(dG-dC)•poly(dG-dC). For competition experiments, a 100-fold molar excess of various unlabeled double-stranded oligonucleotides, as shown in Fig. 4A, were mixed with the labeled oligomer before the addition of bacterial extract. After incubation for 10 min on ice, the reactants were loaded onto a 6% polyacrylamide gel and electrophoresed at 4°C for 120 min at 190 V in 0.5 \times TBE (1 \times TBE = 90 mmol/l Tris, 90 mmol/l

boric acid, and 2 mmol/l EDTA). After electrophoresis, the gels were dried and exposed to Kodak XAR5 film, and binding was analyzed by autoradiography.

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