Differential Role of Hepatocyte Nuclear Factor-1 in the Regulation of Glucose-6-phosphatase Catalytic Subunit Gene Transcription by cAMP in Liver- and Kidney-derived Cell Lines*

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In liver and kidney, the terminal step in gluconeogenesis is catalyzed by glucose-6-phosphatase. To examine the effect of the cAMP signal transduction pathway on transcription of the gene encoding the catalytic subunit of glucose-6-phosphatase (G6Pase), G6Pase-chloramphenicol acetyltransferase (CAT) fusion genes were transiently transfected into either the liver-derived HepG2 or kidney-derived LLC-PK cell line. Co-transfection of an expression vector encoding the catalytic subunit of cAMP-dependent protein kinase (PKA) markedly stimulated G6Pase-CAT fusion gene expression, and mutational analysis of the G6Pase promoter revealed that multiple regions are required for this PKA response in both the HepG2 and LLC-PK cell lines. A sequence in the G6Pase promoter that resembles a cAMP response element is required for the full PKA response in both HepG2 and LLC-PK cells. However, in LLC-PK cells, but not in HepG2 cells, a hepatocyte nuclear factor-1 (HNF-1) binding site was critical for the full induction of G6Pase-CAT expression by PKA. Changing this HNF-1 motif to that for the yeast transcription factor GAL4 reduces the PKA response in LLC-PK cells to the same degree as deleting the HNF-1 site. However, co-transfection of this mutated construct with chimeric proteins comprising the GAL4-DNA binding domain ligated to the coding sequence for HNF-1α, HNF-1β, HNF-3, or HNF-4 completely restored the PKA response. Thus, we hypothesize that, in LLC-PK cells, HNF-1 is acting as an accessory factor to enhance PKA signaling through the cAMP response element by altering G6Pase promoter conformation or accessibility rather than specifically affecting some component of the PKA signal transduction pathway.

Glucose-6-phosphatase is a multicomponent system located in the endoplasmic reticulum (ER) that catalyzes the terminal step in gluconeogenesis and hepatic glycogenolysis (1–4). The active site of the catalytic subunit of glucose-6-phosphatase (G6Pase) is located within the lumen of the ER (1–4). The other components of the glucose-6-phosphatase system act as transport proteins to shuttle both substrate and product across the ER membrane (1–4). These include a glucose-6-phosphate transporter, an inorganic phosphate transporter, and a glucose transporter, termed T1, T2, and T3, respectively (1–5). T1 and T2 may be separate activities manifested by a single protein (6–8). The functional importance of each component of the glucose-6-phosphatase system is apparent from the pathophysiology of glycogen storage disease (GSD) types 1a–1d. GSD types 1a–1d are caused by mutations within the catalytic, T1, T2, and T3 subunits, respectively, and result in little or no glucose-6-phosphatase activity (5, 6, 9, 10). Type 1 GSD is characterized by severe hypoglycemia in the postabsorptive state, hyperlipidemia, hyperuricemia, and lactic acidemia (9, 11, 12). In addition, patients are prone to such complications as growth retardation, hepatic steatosis and cirrhosis, hepatic adenoma, and renal failure (9, 11–13).

Type II, non-insulin-dependent diabetes mellitus is characterized by defects in insulin secretion, insulin-dependent peripheral glucose utilization and hepatic glucose production (14). The elevated hepatic glucose production is a consequence of an increased rate of gluconeogenesis, rather than glycogenolysis (15). It has been suggested that abnormal expression of key gluconeogenic enzymes, such as G6Pase, may contribute to the increase in hepatic glucose production (16–18). Consistent with this hypothesis, G6Pase is overexpressed in animal models of diabetes (19–23). Furthermore, Newgard and colleagues (24) demonstrated that a modest overexpression of G6Pase in rat liver results in glucose intolerance and hyperinsulinemia.

G6Pase is predominantly expressed in the liver and the proximal tubule of the kidney with lower levels in the β-cells of pancreatic islets (1–4). Multiple hormones and metabolites are known to regulate hepatic G6Pase gene expression. Thus, cAMP, glucocorticoids, glucose, and fatty acids all stimulate (19, 22, 25–29), whereas insulin, tumor necrosis factor-α, and interleukin-6 all inhibit G6Pase gene expression (19, 25, 30–34). Moreover, insulin is able to override the stimulatory effects of cAMP, glucocorticoids, glucose, and fatty acids (19, 22, 25, 27, 29, 33, 34). Little, however, is known about the regulation of G6Pase in the kidney. Mitieux and co-workers (23, 35) have demonstrated that renal G6Pase expression and activity both increase during a prolonged fast, a condition associated with alterations in the concentration of multiple metabolites including elevated renal cAMP concentrations (36). In addition,
parathormone, acting via cAMP, stimulates renal glucose-6-phosphatase activity (37).

Recently, Chou and colleagues (38) reported that a region of the human G6Pase promoter encompassing the sequence between −136 and −134 was required for the stimulatory effect of cAMP on G6Pase fusion gene expression in HepG2 cells. By contrast, Burchell and co-workers (39) found that in H4IIE hepatoma cells the human G6Pase promoter sequence located between −161 and −152 was critical for the combined stimulatory effects of cAMP and glucocorticoids. This region was shown to contain a cAMP response element (CRE) based on the ability of this sequence to confer cAMP responsiveness on the expression of a heterologous fusion gene (39). The explanation for this discrepancy is unclear, but, in the present study, we examined the effect of the cAMP signal transduction pathway on the regulation of G6Pase gene transcription in both the liver-derived HepG2 and kidney-derived LLC-PK1 cell lines. We provide evidence that the regulation of G6Pase gene transcription by cAMP is more complex than originally reported (38, 39). Specifically, we find that multiple promoter elements are required for the stimulatory effect of cAMP on G6Pase fusion gene transcription in both HepG2 and LLC-PK1 cells. However, the qualitative importance of specific elements differs in the two cell types. Most notably, hepatocyte nuclear factor-1β (HNF-1β) plays an essential role in the induction of G6Pase fusion gene transcription by cAMP in LLC-PK1 cells, but not in HepG2 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (>5000 Ci mmol−1), [α-32P]dATP (>3000 Ci mmol−1), and [γ-32P]UTP (>3000 Ci mmol−1) were obtained from Amersham Pharma Biotech, whereas [3H] acetic acid, sodium salt (>1.0 Ci mmol−1) and [14C] sodium chloride (>800 Ci mmol−1) were obtained from ICN. B-saturated monooxygenase-3′-5′-monophosphate, cyclic (8-Br-cAMP) and 8-(4-chlorophenylthio)adenosine-3′-5′-monophosphate, cyclic (8-CPT-cAMP) were purchased from Sigma and Roche Molecular Biochemicals, respectively. Insulin was purchased from Collaborative Bioproducts. Antiserum to HNF-1α (sc-6547 X) and HNF-1α (sc-7411 X) were obtained from Santa Cruz Biotechnology, Inc.

Isolation of Purine G6Pase and Cyclophilin A Genomic DNA Fragments—Porcine genomic DNA was isolated by the DNAzol genomic DNA isolation reagent (Molecular Research Center and Roche Molecular Biochemicals, respectively. Insulin was purchased by using PCR and the following primers: 5′-GCAATCTGGATGAGGCTGG-3′ and 5′-TCCTCGAG-3′, complementary to exon 1 of the human G6Pase gene (42) and a 30-bp primer (5′-ATGTCGAAGAACACGGTGTTGGAGGTAG-3′), complementary to exon 1 of the human cyclophilin A gene (41) and used as a non-hormone-responsive internal control, were synthesized. For the determination of the porcine kidney G6Pase gene transcription start site, a 27-bp primer (5′-GAGTCTCGTGAATATCTGCTGGAGGTTGAG-3′), complementary to exon 1 of the porcine G6Pase gene (see above) was synthesized. Following gel purification (45), these primers were then 5′-end labeled with [γ-32P]ATP to a specific activity of ~2 Ci mmol−1 (45). The labeled primers (~3 × 10^6 cpm) were annealed to 50 μl of either total HepG2 RNA or total porcine kidney RNA for 1 h at 60 °C, and then primer extension was performed as described previously. Products were then visualized by electrophoresis on polyacrylamide/urea/TBE gels (46). The human G6Pase promoter was ligated into the predicted extension product of 168 bp (42), whereas the porcine G6Pase primer gave an extension product of 153 bp, indicative of a transcription start site identical to that of the mouse G6Pase gene (Fig. 1B, Ref. 43). The human cyclophilin A primer gave a cluster of extension products between 73 and 75 bp (Fig. 1C), the published transcription start site predicts a product of 73 bp with this primer (41).

Ribonuclease Protection Assay—[α-32P]UTP-labeled antisense porcine G6Pase and cyclophilin A probes were generated using the plasmids described above and the MAXIscript T7 kit (Ambion) according to the manufacturer’s instructions. Ribonuclease protection assays were performed using 10 μg of total LLC-PK1 RNA and the RPA III kit (Ambion), again according to the manufacturer’s instructions, except that the combined RNA and probe precipitate was dissolved in 1 μl of water prior to the addition of 10 μl of hybridization buffer. Following RNase A/T1 digestion, RNA products were resolved on 5% polyacrylamide/urea/TBE gels and sizes estimated by comparison with co-electrophoresed DNA sequencing reactions. The calculated sizes of the G6Pase and cyclophilin A probes were 234 and 162 bp, respectively, whereas the sizes of the LLC-PK1 cell G6Pase and cyclophilin A products were close to the calculated sizes of 185 and 113 bp, respectively.

**Plasmid Construction**—The generation of the full-length mouse G6Pase-CAT fusion gene, containing promoter sequence spanning nucleotides −751 to +66 relative to the transcription start site, the series of 5′-truncated G6Pase-CAT fusion genes and the site-directed mutant of the G6Pase HNF-1 site in the context of the −231 G6Pase-CAT fusion gene have all been described previously (32, 33). A similar strategy as used to generate the −231 HNF-1 SDM construct was used to replace the G6Pase HNF-1 motif with that of the yeast transcription factor GAL4. Thus, the HNF-1 binding site in the G6Pase SDM promoter was changed to a GAL4 binding site by site-directed mutagenesis within the context of the −231 to +66 promoter fragment by using PCR and the following oligonucleotide as the 5′ primer: 5′-TGCCTCGAG-3′, complementary to exon 1 of the porcine G6Pase gene and the CAT reporter gene to be the same as that in all other G6Pase-CAT fusion gene constructs.

A previously described three-step PCR strategy (32, 47) was used to create site-directed mutants of the CRE1 and CRE2 motifs (see “Results”). The mRNAs transcribed were shown in the adult rat liver to contain the 5′-truncated G6Pase-CAT fusion gene construct, designated −231 CRE1 SDM and −231 CRE2 SDM (Fig. 7), were generated within the context of the −231 to +66 G6Pase promoter fragment. All promoter fragments generated by PCR were completely sequenced, using the United States Biochemical Corp. Sequenase kit, to verify the absence of polymerase errors.

The generation of the heterologous XMB vector that contains a minimal Xenopus 68-kDa albumin promoter ligated to the CAT reporter...
gene has previously been described (48). Double-stranded complementary oligonucleotides, representing the wild-type or mutated CRE1 and CRE2 motifs (Table I), were synthesized with HindIII-compatible ends and ligated into HindIII-cleaved XMB in multiple (3–4) copies. The number of inserts was determined by restriction enzyme analysis and confirmed by DNA sequencing.

Expression vectors encoding the α and β forms of the catalytic subunit of PKA were a generous gift from Dr. Richard Maurer (49). An empty vector control was generated by digesting the PKAβ plasmid with XhoI and HindIII to remove the open reading frame, filling in the non-compatible ends with the Klenow fragment of Escherichia coli DNA polymerase I, and then religating.

Expression vectors encoding the chimeric GAL4 DNA binding domain and GAL4 DBD-HNF-1β proteins were constructed by cloning the open reading frames of mouse HNF-1α and HNF-1β, isolated from the plasmids pBJ5-HNF-1α (50) and pBJ5-HNF-1β (51), a generous gift from Dr. Gerald Crabtree, into the pSG424 vector (52) such that the HNF-1α and HNF-1β coding sequence was in frame with that of the GAL4 DBD. Expression vectors encoding chimeric GAL4 DBD-HNF-3 and GAL4 DBD-HNF-4 proteins were a generous gift from Dr. Daryl Granner (53). All plasmid constructs were purified by centrifugation twice through cesium chloride gradients (45).

Cell Culture and Transient Transfection—1 Human HepG2 hepato-23oma cells were grown in Dulbecco’s modified Eagle’s medium containing 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal calf serum, and were then purified by centrifugation twice through cesium chloride gradients (45).

Cell Culture and Transient Transfection—1 Human HepG2 hepato-23oma cells were grown in Dulbecco’s modified Eagle’s medium containing 2.5% (v/v) newborn calf serum and 2.5% (v/v) fetal calf serum and were then transiently transfected in solution using the calcium phosphate-DNA co-precipitation method exactly as described for HepG2 cells (32, 33). Where indicated in the figure legends, the reporter gene construct (15 μg) was co-transfected with expression vectors encoding β-galactosidase (2.5 μg) and the indicated amount of plasmids encoding the catalytic subunit of protein kinase A, courtesy of Dr. Richard Maurer (49), or the empty PKA vector, as described previously (32, 33). Where indicated in the figure legends, the reporter gene construct (15 μg) was co-transfected with expression vectors encoding β-galactosidase (2.5 μg) and the indicated amount of plasmids encoding the catalytic subunit of protein kinase A, courtesy of Dr. Richard Maurer (49), or the empty PKA vector, as described previously (32, 33).

CAT and β-Galactosidase Assays—CAT and β-galactosidase assays were performed exactly as described previously (32, 33). CAT activity directed by the various fusion gene constructs was corrected for the β-galactosidase activity in the same samples and each construct was analyzed in duplicate in multiple transfections, as specified in the figure legends, using at least three independent plasmid preparations.

Gel Retardation Assay—HepG2 or LLC-PK nuclear extracts were prepared previously (47). Where indicated in the figure legends, the CAT reporter gene construct (15 μg) was co-transfected with expression vectors encoding β-galactosidase (2.5 μg) and the indicated amount of plasmids encoding the catalytic subunit of protein kinase A, and then co-transfected with expression vectors encoding β-galactosidase (2.5 μg) and the indicated amount of plasmids encoding the catalytic subunit of protein kinase A, and then transiently transfected in solution using the calcium phosphate-DNA co-precipitation method exactly as described for HepG2 cells (32, 33). Where indicated in the figure legends, the reporter gene construct (15 μg) was co-transfected with expression vectors encoding β-galactosidase (2.5 μg) and the indicated amount of plasmids encoding the catalytic subunit of protein kinase A, courtesy of Dr. Richard Maurer (49), or the empty PKA vector, as described previously (32, 33).

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treating transfected cells with 8-Br-cAMP or (ii) co-transfecting the catalytic subunit of PKA along with the G6Pase-CAT fusion gene. In both cell lines, 8-Br-cAMP treatment resulted in a dose-dependent stimulation of G6Pase-CAT gene expression (Fig. 2A). The maximally effective concentration of 8-Br-cAMP was 1 mM for LLC-PK cells and 100 μM for HepG2 cells (Fig. 2A). A concentration of 1 mM 8-Br-cAMP was toxic to the HepG2 cell line (data not shown). Co-transfection with PKA also resulted in a concentration-dependent stimulation of G6Pase-CAT gene expression in both cell lines (Fig. 2B). However, the magnitude of the stimulation of G6Pase-CAT gene expression was much greater when co-transfecting PKA as compared with treating the cells with a maximally effective concentration of 8-Br-cAMP (Fig. 2C). In LLC-PK cells, basal G6Pase-CAT gene expression was not detected, whereas in HepG2 cells basal expression was considerable. Therefore, in Fig. 2C the results from the LLC-PK studies are expressed as a percentage of the maximal induction by cAMP/PKA, whereas the results from the HepG2 studies are expressed as -fold induction.

**A Hepatocyte Nuclear Factor-1 Binding Site in the G6Pase Promoter Is Critical for the Regulation of G6Pase Gene Transcription by PKA in LLC-PK Cells but Not in HepG2 Cells**—We utilized the observation that PKA co-transfection was more potent than cAMP treatment to identify the regions of the G6Pase promoter mediating the stimulatory effect of the cAMP signal transduction pathway on G6Pase gene transcription. The ability of PKA to stimulate the expression of a series of 5′-truncated G6Pase-CAT fusion genes was analyzed by transient transfection into both HepG2 and LLC-PK cells (Figs. 3 and 4). In both HepG2 and LLC-PK cells, deletion of the G6Pase promoter sequence between −751 and −231 had no effect on the ability of PKA to induce G6Pase fusion gene expression (data not shown). In HepG2 cells, deletion of the G6Pase promoter sequence from −198 to −158 resulted in a substantial reduction in the ability of PKA to stimulate G6Pase-CAT fusion gene expression (Fig. 3A). These data are consistent with that of Burchell and colleagues (39), who have previously demonstrated that the equivalent region of the human G6Pase promoter contains a CRE. Subsequent deletions of the G6Pase promoter sequence between −158 and −35 resulted in a progressive reduction in the stimulation of G6Pase-CAT gene expression by PKA (Fig. 3A). These data suggest that, in HepG2 cells, multiple elements within the G6Pase promoter are required for the full stimulatory effect of the cAMP signal transduction pathway on G6Pase gene transcription.

In LLC-PK cells, truncation of the G6Pase promoter sequence between −198 and −158 also resulted in a reduced ability of PKA to stimulate G6Pase-CAT gene expression (Fig. 4A). However, in contrast to the results obtained in HepG2 cells (Fig. 3), deletion of the G6Pase promoter sequence from −231 to −198 resulted in an approximately 95% reduction in the ability of PKA to induce G6Pase-CAT expression in LLC-PK cells (Fig. 4A). This region has previously been shown to contain a binding site for the transcription factor HNF-1 (32). In order to determine whether the HNF-1 binding site was required for the stimulatory effect of PKA on G6Pase-CAT gene expression in LLC-PK cells, this element was altered by site-directed mutagenesis within the context of an otherwise intact −231 to −66 G6Pase promoter fragment (i.e., the shortest sequence that confers a maximal stimulatory effect of PKA on G6Pase-CAT gene expression). This fusion gene construct, termed −231 HNF-1 SDM, was analyzed by transient transfection of LLC-PK cells in the presence and absence of PKA (Fig. 4B). Compared with the wild-type −231 G6Pase-CAT fusion gene construct, mutation of the HNF-1 site resulted in approximately a 95% reduction in the ability of PKA to stimulate G6Pase-CAT gene expression (Fig. 4B). This is equivalent to the result obtained with the −198 G6Pase-CAT fusion gene, in which the HNF-1 site is completely deleted (Fig. 4B).
In HepG2 cells, HNF-1 plays a less critical role in the stimulation of G6Pase gene transcription by the cAMP signal transduction pathway. When the data are expressed in terms of -fold induction of G6Pase-CAT gene expression by PKA (Fig. 3A), it appears that deletion of the G6Pase promoter sequence from -231 to -198, that contains the HNF-1 binding site, did not affect the ability of PKA to stimulate G6Pase-CAT gene expression. By contrast, when the data are expressed as a percentage of the maximal induction (i.e. the induction of -231 G6Pase-CAT gene expression by PKA is set to 100%), on average, an approximately 45% decrease in the stimulation of G6Pase-CAT expression is observed upon deletion of the HNF-1 binding site (Fig. 3B). This observation is explained by an equivalent decrease in basal G6Pase-CAT expression upon deletion of the HNF-1 binding site (Fig. 3C). However, it is apparent from the data shown in Fig. 3C, which represents the mean ± standard deviation of 16 experiments, that deletion of the HNF-1 binding site has a variable effect on basal G6Pase-CAT fusion gene expression. Thus, we initially reported that deletion of the HNF-1 site had little effect on basal G6Pase-CAT gene expression (32, 33), but we have subsequently found that on occasion this deletion does result in a reduction in basal G6Pase-CAT gene expression (61) and Fig. 3C. The explanation for this variable role of HNF-1 in regulating basal G6Pase-CAT fusion gene expression is unknown. However, regardless of how the data are presented, it is clear that, in LLC-PK cells, the stimulation of G6Pase-CAT expression by the cAMP pathway is considerably more dependent on the HNF-1 site than in HepG2 cells. As reviewed under “Discussion” and supported by experiments described below, the available literature and data strongly suggest that HNF-1 is acting as an accessory factor to enhance the effect of cAMP on G6Pase gene transcription mediated through a proximal promoter region, rather than acting as a CRE itself.

### Fig. 2.
The cAMP signal transduction pathway stimulates G6Pase-CAT fusion gene expression in both the LLC-PK and HepG2 cell lines. LLC-PK and HepG2 cells were transiently co-transfected, as described under “Experimental Procedures,” with a G6Pase-CAT fusion gene (15 μg), containing G6Pase promoter sequence from -751 to +66, and an expression vector encoding β-galactosidase (2.5 μg), and also, where indicated, with various amounts of an expression vector encoding the catalytic subunit of PKA. Following transfection, cells were incubated for 18–20 h in serum-free medium, in the presence or absence of various concentrations of 8-Br-cAMP. The cells were then harvested, and both CAT and β-galactosidase activity were assayed as described previously (32, 33). Results are expressed as a percentage of the maximum induction of CAT activity by cAMP (panel A) or PKA (panel B), corrected for β-galactosidase activity in the cell lysate. The results shown in panel C compare the induction of CAT activity by cAMP (100 and 1000 μM cAMP for HepG2 and LLC-PK cells, respectively) versus the induction of CAT activity by PKA (5 μg for both HepG2 and LLC-PK cells). Since basal G6Pase-CAT fusion gene expression is not detected in LLC-PK cells, in these experiments the CAT activity, corrected for β-galactosidase activity in the cell lysate, is expressed in arbitrary units. For the HepG2 experiments, results are presented as the ratio of CAT activity, corrected for β-galactosidase activity in the cell lysate, in PKA transfected or 8-Br-cAMP-treated versus untreated cells (expressed as -fold induction). Results represent the mean ± S.E. of 3–9 experiments in which each treatment was assayed in duplicate.

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### HNF-1α and HNF-1β Selectively Bind the HNF-1 Site in the G6Pase Promoter in HepG2 and LLC-PK Cells, Respectively—To determine whether both HNF-1α and β bind to the G6Pase promoter in LLC-PK cells, protein binding to the HNF-1 site was analyzed by using the gel retardation assay. When a labeled, double-stranded oligonucleotide representing the G6Pase promoter sequence from -231 to -199 (Table I), which contains the HNF-1 site (-221 to -209), was incubated

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**Fig. 2.** The cAMP signal transduction pathway stimulates G6Pase-CAT fusion gene expression in both the LLC-PK and HepG2 cell lines. LLC-PK and HepG2 cells were transiently co-transfected, as described under “Experimental Procedures,” with a G6Pase-CAT fusion gene (15 μg), containing G6Pase promoter sequence from -751 to +66, and an expression vector encoding β-galactosidase (2.5 μg), and also, where indicated, with various amounts of an expression vector encoding the catalytic subunit of PKA. Following transfection, cells were incubated for 18–20 h in serum-free medium, in the presence or absence of various concentrations of 8-Br-cAMP. The cells were then harvested, and both CAT and β-galactosidase activity were assayed as described previously (32, 33). Results are expressed as a percentage of the maximum induction of CAT activity by cAMP (panel A) or PKA (panel B), corrected for β-galactosidase activity in the cell lysate. The results shown in panel C compare the induction of CAT activity by cAMP (100 and 1000 μM cAMP for HepG2 and LLC-PK cells, respectively) versus the induction of CAT activity by PKA (5 μg for both HepG2 and LLC-PK cells). Since basal G6Pase-CAT fusion gene expression is not detected in LLC-PK cells, in these experiments the CAT activity, corrected for β-galactosidase activity in the cell lysate, is expressed in arbitrary units. For the HepG2 experiments, results are presented as the ratio of CAT activity, corrected for β-galactosidase activity in the cell lysate, in PKA transfected or 8-Br-cAMP-treated versus untreated cells (expressed as -fold induction). Results represent the mean ± S.E. of 3–9 experiments in which each treatment was assayed in duplicate.

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Multiple regions of the G6Pase promoter are required for the full induction of G6Pase-CAT fusion gene transcription by PKA in HepG2 cells. HepG2 cells were transiently co-transfected, as described under “Experimental Procedures,” with various G6Pase-CAT fusion genes (15 μg), containing either distinct lengths of wild-type (WT) promoter sequence, assayable as described previously (32, 33). In panel A, results are presented as the ratio of CAT activity, corrected for β-galactosidase activity in the cell lysate, in PKA transfected versus empty vector transfected cells (expressed as fold induction). In panels B and C, CAT activity, corrected for β-galactosidase activity in the cell lysate, is expressed as a percentage of that obtained with the -231 G6Pase-CAT fusion gene in the presence of the PKA expression vector (B) or the same vector with the PKA open reading frame deleted (C). Results in panel B and C represent the mean ± S.E. of 5–18 experiments assayed in duplicate, while results in panel C represent the mean ± S.D. of 5–18 experiments assayed in duplicate.

FIG. 3. Multiple regions of the G6Pase promoter are required for the full induction of G6Pase-CAT fusion gene transcription by PKA in HepG2 cells. HepG2 cells were transiently co-transfected, as described under “Experimental Procedures,” with various G6Pase-CAT fusion genes (15 μg), containing either distinct lengths of wild-type (WT) promoter sequence, assayable as described previously (32, 33). In panel A, results are presented as the ratio of CAT activity, corrected for β-galactosidase activity in the cell lysate, in PKA transfected versus empty vector transfected cells (expressed as fold induction). In panels B and C, CAT activity, corrected for β-galactosidase activity in the cell lysate, is expressed as a percentage of that obtained with the -231 G6Pase-CAT fusion gene in the presence of the PKA expression vector (B) or the same vector with the PKA open reading frame deleted (C). Results in panels B and C represent the mean ± S.E. of 5–18 experiments assayed in duplicate, while results in panel C represent the mean ± S.D. of 5–18 experiments assayed in duplicate.

with nuclear extract prepared from LLC-PK cells, a single major protein-DNA complex was observed (Fig. 5A, see arrow, upper panel). In competition experiments, a 25-fold molar excess of the unlabeled HNF-1 oligonucleotide competed effectively for the binding of this complex (Fig. 5A, upper panel), indicating that this represents a specific protein-DNA interaction. By contrast, a 25-fold molar excess of an unlabeled, double-stranded oligonucleotide, in which the HNF-1 site has been mutated (HNF-1MUT; Table I), was unable to effectively compete with the labeled probe for protein binding (Fig. 5A, upper panel). This oligonucleotide contains the same HNF-1 binding site mutation that almost abolished the stimulatory effect of PKA on G6Pase-CAT fusion gene expression in LLC-PK cells (Fig. 4B). Thus, the binding of this complex correlates with the PKA response. Next, LLC-PK cell nuclear extract was pre-incubated with 0.1 or 1.0 μl of antisera specific to either HNF-1α or HNF-1β (Fig. 5A, lower panel). Both concentrations of HNF-1β antiserum resulted in the slower migration of the major protein-DNA complex. However, neither concentration of HNF-1α antiserum had any effect on the migration of the major protein-DNA complex (Fig. 5A, lower panel). A positive control demonstrating that the HNF-1α antiserum functions in super-shift assays is described below. These results indicate that, in LLC-PK cells, only HNF-1β binds to the HNF-1 site in the G6Pase promoter.

We have previously shown that HNF-1 present in HepG2 nuclear extracts binds the HNF-1 motif in the mouse G6Pase promoter (32). However, the antiserum we used, a generous gift from Dr. Moshe Yaniv, recognizes both HNF-1α and HNF-1β, although it binds HNF-1α with a much greater affinity (62). Therefore, to conclusively determine whether the HNF-1 binding activity detected in HepG2 nuclear extract represents HNF-1α or HNF-1β, we made use of commercially available antisera raised specifically to each isoform of HNF-1. As previously reported (32), when a labeled oligonucleotide representing the G6Pase HNF-1 motif was incubated with nuclear extract prepared from HepG2 cells multiple protein-DNA

with nuclear extract prepared from LLC-PK cells, a single major protein-DNA complex was observed (Fig. 5A, see arrow, upper panel). In competition experiments, a 25-fold molar excess of the unlabeled HNF-1 oligonucleotide competed effectively for the binding of this complex (Fig. 5A, upper panel), indicating that this represents a specific protein-DNA interaction. By contrast, a 25-fold molar excess of an unlabeled, double-stranded oligonucleotide, in which the HNF-1 site has been mutated (HNF-1MUT; Table I), was unable to effectively compete with the labeled probe for protein binding (Fig. 5A, upper panel). This oligonucleotide contains the same HNF-1 binding site mutation that almost abolished the stimulatory effect of PKA on G6Pase-CAT fusion gene expression in LLC-PK cells (Fig. 4B). Thus, the binding of this complex correlates with the PKA response. Next, LLC-PK cell nuclear extract was pre-incubated with 0.1 or 1.0 μl of antisera specific to either HNF-1α or HNF-1β (Fig. 5A, lower panel). Both concentrations of HNF-1β antiserum resulted in the slower migration of the major protein-DNA complex. However, neither concentration of HNF-1α antiserum had any effect on the migration of the major protein-DNA complex (Fig. 5A, lower panel). A positive control demonstrating that the HNF-1α antiserum functions in super-shift assays is described below. These results indicate that, in LLC-PK cells, only HNF-1β binds to the HNF-1 site in the G6Pase promoter.

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complexes were observed (Fig. 5B, upper panel). In competition experiments, a 25-fold molar excess of the unlabeled HNF-1 oligonucleotide competed effectively for two protein-DNA complexes (Fig. 5B, see arrows, upper panel) indicating that the other complexes represent nonspecific interactions. By contrast, a 25-fold molar excess of the unlabeled, double-stranded HNF-1MUT oligonucleotide (Table I) was unable to effectively compete for protein binding with the labeled probe (Fig. 5B, upper panel). Pre-incubation of HepG2 nuclear extract with either 0.1 or 1.0 μl of the antisera specific for HNF-1α resulted in the slower migration of the specific protein-DNA complexes, however, neither 0.1 nor 1.0 μl of the antisera specific for HNF-1β had any effect on the migration of these complexes (Fig. 5B, lower panel). These data indicate that, using the gel retardation assay, only HNF-1β binding to the HNF-1 site in the G6Pase promoter is detected using LLC-PK cell nuclear extracts, whereas, using HepG2 nuclear extract, only HNF-1α binding is detected.

Both HNF-1α and HNF-1β, as Well as HNF-3 and HNF-4, Are Capable of Enhancing the Stimulatory Effect of PKA on G6Pase-CAT Fusion Gene Expression in LLC-PK Cells—Although only HNF-1β binding was detected in LLC-PK cell nuclear extracts, we wanted to determine whether HNF-1α was also capable of enhancing the stimulatory effect of PKA on G6Pase-CAT fusion gene expression in LLC-PK cells. To address this question, a construct, designated −231 HNF-1→GAL4, was generated in which the HNF-1 binding site in the G6Pase promoter was replaced with the binding site for the yeast transcription factor GAL4 (Fig. 6A). Expression vectors encoding chimeric proteins consisting of the GAL4-DBD fused to either the coding sequence of HNF-1α or HNF-1β were also generated. As predicted, substitution of the HNF-1 binding site in the G6Pase promoter for the GAL4 binding site resulted in a severely diminished stimulation of G6Pase-CAT gene expression by PKA (Fig. 6B), equivalent to that seen when the HNF-1 binding site was mutated or deleted (Fig. 4B). However, co-transfection of the −231 HNF-1→GAL4 fusion gene construct into LLC-PK cells with either the HNF-1α or HNF-1β GAL4-DBD chimeras fully restored the stimulatory effect of PKA on G6Pase-CAT fusion gene expression (Fig. 6B). Furthermore,
co-transfection with an expression vector encoding a chimeric protein representing the transactivation domain of HNF-1α (63) fused to the GAL4-DBD was sufficient to fully restore the induction of −231 HNF-1→GAL4 expression by PKA to a level that was greater than seen with the wild-type −231 G6Pase-CAT fusion gene construct (data not shown). These data indicate that HNF-1α and β are both capable of enhancing the stimulatory effect of PKA on G6Pase-CAT fusion gene transcription. Surprisingly, co-transfection with an expression vector encoding the GAL4-DBD alone also resulted in a modest stimulation of G6Pase-CAT gene expression (Fig. 6B). This observation raised the possibility that any factor bound to this region of the G6Pase promoter would enhance the stimulatory effect of PKA on fusion gene expression. Indeed, co-transfection of the −231 HNF-1→GAL4 fusion gene with expression vectors encoding chimeric GAL4-DBD-HNF-3 and GAL4-DBD-HNF-4 proteins fully restored the stimulatory effect of PKA (Fig. 6B).

A Cyclic AMP Response Element Is Located between −162 and −155 in the Mouse G6Pase Promoter—Previously, two regions of the human G6Pase promoter were reported to be involved in cAMP responsiveness (38, 39). Burchell and colleagues (39) demonstrated that, in H4IE hepatoma cells, the sequence from −161 to −152 of the human G6Pase promoter contains a CRE based on the ability of this sequence to confer a direct stimulatory effect of cAMP on the expression of a heterologous fusion gene. By contrast, Chou and colleagues (38) showed that in HepG2 cells mutation of the human G6Pase promoter sequence between −136 and −134 completely blocked the stimulatory effect of cAMP. For clarity, the G6Pase promoter sequence between −136 and −134 has been termed CRE1, while the region encompassed by the mutation between −136 and −134 has been termed CRE2. Table I compares the sequence of the human CRE1 and CRE2 motifs with the equivalent sequences in the mouse G6Pase promoter and demonstrates that both cAMP responsive regions are well conserved. In order to determine whether these regions participate in the stimulatory effect of PKA on mouse G6Pase gene transcription in LLC-PK cells, we separately mutated CRE1 and CRE2 in the context of the −231 to +66 G6Pase-CAT fusion gene. Compared with the wild-type −231 G6Pase-CAT fusion gene, site-
directed mutagenesis of either the mouse CRE1 or CRE2 sequence resulted in approximately a 95% and an 85% decrease in the ability of PKA to stimulate G6Pase-CAT expression, respectively (Fig. 7A). These data suggest that both sites are required for the stimulation of G6Pase-CAT fusion gene expression by PKA in LLC-PK cells. However, this experiment does not reveal whether these sites are acting directly as bona fide CREs or indirectly as accessory factor binding sites to enhance the effect of cAMP mediated through another element.

In order to determine whether CRE1 and CRE2 act as bona fide CREs, multiple copies of double-stranded oligonucleotides representing either the mouse G6Pase sequence from −175 to −142 (CRE1) or from −155 to −119 (CRE2) (see Table I) were ligated into the heterologous XMB-CAT expression vector (48) and transiently transfected into LLC-PK cells (Fig. 7B). The G6Pase promoter sequence from −175 to −142 (CRE1) mediated a direct stimulatory effect of PKA on reporter gene expression (Fig. 7B), whereas the sequence from −155 to −119 was unable to mediate a PKA response (Fig. 7B). These data indicate that the CRE1 region, but not the CRE2 region, contains a bona fide CRE. The CRE1 region contains the sequence TTACGTAAA, located between −162 and −155 in the mouse G6Pase promoter (Table I), which is similar to the consensus CRE sequence of TGACGTCA. A double-stranded oligonucleotide containing a mutation of this CRE-like motif within the CRE1 sequence between −175 and −142 (CRE1MUT; see Table I) was inserted in multiple copies into the heterologous XMB-CAT expression vector in order to determine whether this CRE-like motif was responsible for the stimulatory effect of PKA on CRE1 XMB-CAT expression. CAT expression directed by the resulting construct (CRE1MUT XMB) was not stimulated by PKA following transient transfection into LLC-PK cells (Fig. 7B). Taken together, these data indicate that in LLC-PK cells the mouse G6Pase promoter sequence between −162 and −155 (CRE1) is a bona fide CRE while the CRE2 region contains an accessory factor binding site that likely enhances the effect of cAMP mediated through CRE1.

**DISCUSSION**

In the postabsorptive state, the liver is generally believed to be responsible for approximately 95% of whole body glucose production, with the kidneys contributing the remaining 5% (64). However, some recent reports have suggested that the renal contribution may be as large as 20–25% (65, 66). Conditions that challenge normal glucose homeostasis, such as exercise, prolonged fasting, insulin-induced hypoglycemia, and diabetes, require the kidney to play a more prominent role in the production of glucose (67). Despite the increased importance of the kidney in times of glucose demand, little is known about the molecular mechanisms regulating renal gluconeogenesis. In the present paper, we have examined the regulation of G6Pase gene transcription by the cAMP pathway in both the kidney-derived LLC-PK and the liver-derived HepG2 cell lines. The data presented demonstrate that multiple cis-acting elements are required for the full stimulatory effect of cAMP on G6Pase-CAT gene expression (Figs. 3, 4, and 7). This type of multi-element promoter structure is termed a hormone response unit (CRU). Although a CRU is required for the full stimulatory effect of cAMP on G6Pase gene transcription in both the HepG2 and LLC-PK cell lines, it is apparent that the quantitative importance of the various cis-acting elements that comprise this CRU varies in the two cell lines. In particular, deletion or mutation of the CRE1 site in the G6Pase promoter reduces PKA-stimulated G6Pase-CAT gene expression by −95% in LLC-PK cells (Fig. 4), whereas this same deletion is less deleterious in HepG2 cells (Fig. 3). These results on G6Pase gene expression are somewhat similar to those of Hanson and colleagues (69), who were the first to use the PKA co-transfection technique to define a CRU in a promoter, namely that of another gluconeogenic enzyme-encoding gene, phosphoenolpyruvate carboxykinase (PEPCK). They showed that four cis-acting elements (designated the CRE, P3[I], P3[II], and P4) were required for PKA-stimulated PEPCK fusion gene transcription in the HepG2 cell line, results that were subsequently confirmed in transgenic animals (70). HNF-1 binds to the P2 site in the PEPCK promoter but is not required for PKA-induced PEPCK gene expression in HepG2 cells (70). However, as with G6Pase, HNF-1 does play a key role in the maximal induction of PEPCK gene expression by cAMP in the kidney (60).

Previous studies by Nagamine and colleagues (71, 72) on the urokine-type plasminogen activator promoter suggest that HNF-1 acts as an accessory factor to enhance the stimulatory effect of the cAMP signal transduction pathway on renal gene expression rather than acting as a direct target of PKA itself. Thus, these investigators demonstrated that an HNF-1 site in the urokine-type plasminogen activator promoter was required for the full cAMP response (71, 72). Moreover, they showed that HNF-1β was capable of interacting with CREB and ATF-1 in both a mammalian two-hybrid assay and a co-precipitation assay, providing a potential mechanism to explain the accessory factor action of HNF-1 (72). In addition, Nagamine and co-workers (72) demonstrated that HNF-1β was not phosphorylated by PKA in vitro or in LLC-PK cells treated with cAMP, arguing against HNF-1 directly mediating a PKA response. It remains to be determined whether HNF-1α is phosphorylated by PKA, but there is no obvious consensus PKA phosphorylation site located within the HNF-1α protein. Moreover, since both HNF-3 and HNF-4 can substitute for HNF-1 over, since both HNF-3 and HNF-4 can substitute for HNF-1 role in the G6Pase promoter, acting by altering DNA conformation or accessibility, rather than directly affecting some component of the cAMP signal transduction pathway. Finally, HNF-1 can also act as an accessory factor to enhance the action of both insulin (32) and glucocorticoids (73) on gene transcription.

GSD type 1 results from an inborn error of metabolism in which glucose-6-phosphatase activity is either absent or severely diminished. Patients present to the clinic with a variety of ailments including hypoglycemia, hyperlipidemia, hyperuricemia, lactic acidemia, hepatomegaly, kidney enlargement, and growth retardation (9, 11–13). Poor metabolic control can result in severe systemic complications including pulmonary hypertension and even renal failure (9, 11–13). Some GSD type 1 patients with poor metabolic control are afflicted with a renal Fanconi-like syndrome characterized by proximal renal tubular defects including β2-microglobulinuria, generalized amino aciduria, phosphaturia, and renal tubular acidosis (11). Interestingly, Pontoglio and co-workers (74) reported that HNF-1α-deficient mice are also characterized by a renal Fanconi-like syndrome. This observation, in combination with our results demonstrating the requirement of HNF-1 to induce G6Pase gene expression in response to the cAMP pathway, make it enticing to speculate that dysregulation of G6Pase gene expression in HNF-1α-deficient animals may contribute to the renal
Fanconi-like syndrome associated with these animals. One caveat here is that the proximal tubule of the kidney, the site of G6Pase gene expression, expresses both HNF-1α and HNF-1β, whereas the LLC-PK cell line used in our studies only expresses HNF-1β (Fig. 5), even though this cell line is derived from the proximal tubule (58). Nevertheless, it is apparent that both HNF-1α and HNF-1β can enhance the action of the cAMP signal transduction pathway on G6Pase-CAT gene expression in the LLC-PK cell line (Fig. 6).

Two regions of the human G6Pase promoter, which we have designated CRE1 and CRE2 for clarity (Table I), have previously been shown to be important for the stimulatory effect of cAMP on G6Pase gene transcription (38, 39). Both CRE1 and CRE2 are involved in the induction of G6Pase gene transcription by the cAMP pathway in LLC-PK cells (Fig. 7A). However, in LLC-PK cells only CRE1, and not CRE2, represents a bona fide CRE, as it is the only one of these elements that is shown by Chou and colleagues (38) to have consensus CRE homology and can enhance the action of the cAMP signal transduction pathway on G6Pase-CAT gene expression (53). The sequence of the CRE2 region (136TTGACATCA-129, see Table I), only has weak homology to a consensus CRE (TGACGTCA), but Chou and colleagues (38) have shown that it can bind CREB. Interestingly, the CRE2 region of the G6Pase promoter overlaps with a putative HNF-3 binding site (38). The mutation shown in Table I that was introduced into the CRE2 motif would be predicted to disrupt HNF-3 binding, so it is possible that HNF-3, which is expressed in the kidney (75), is the factor that is mediating the accessory element function of CRE2. Certainly, as with HNF-1, HNF-3 has been shown previously to act as an accessory factor to enhance the effect of glucocorticoids on gene transcription (53).

From the data presented in this report, it is apparent that regulation of G6Pase gene transcription by the cAMP signal transduction pathway is accomplished through a complex, tissue-specific mechanism in which multiple promoter elements are required for the full stimulatory effect. In contrast to hepatoma cells, we demonstrate that an HNF-1 binding site in the G6Pase promoter is critical for the stimulation of G6Pase gene transcription by the cAMP pathway in the LLC-PK kidney cell line. Finally, we demonstrate that two regions of the G6Pase promoter previously identified as CREs in hepatoma cell experiments are also utilized by the cAMP signal transduction pathway in LLC-PK cells as well, although only one of these elements is a bona fide CRE in LLC-PK cells.

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Differential Role of Hepatocyte Nuclear Factor-1 in the Regulation of Glucose-6-phosphatase Catalytic Subunit Gene Transcription by cAMP in Liver- and Kidney-derived Cell Lines

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