Selective stimulation of G-6-Pase catalytic subunit but not G-6-P transporter gene expression by glucagon in vivo and cAMP in situ

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Submitted 9 October 2003; accepted in final form 6 January 2004

Hornbuckle, Lauri A., Carrie A. Everett, Cyrus C. Martin, Stephanie S. Gustavson, Christina A. Svitek, James K. Oeser, Doss W. Neal, Alan D. Cherrington, and Richard M. O’Brien. Selective stimulation of G-6-Pase catalytic subunit but not G-6-P transporter gene expression by glucagon in vivo and cAMP in situ. Am J Physiol Endocrinol Metab 286: E795—E808, 2004. First published January 13, 2004; 10.1152/ajpendo.00455.2003.—We recently compared the regulation of glucose-6-phosphatase (G-6-Pase) catalytic subunit and glucose 6-phosphate (G-6-P) transporter gene expression by insulin in conscious dogs in vivo (Hornbuckle LA, Edgerton DS, Ayala JE, Svitek CA, Neal DW, Cardin S, Cherrington AD, and O’Brien RM. Am J Physiol Endocrinol Metab 281: E713—E725, 2001). In pancreatic-clamped, euglycemic conscious dogs, a 5-h period of hypoinsulinemia led to a marked increase in hepatic G-6-Pase catalytic subunit mRNA; however, G-6-P transporter mRNA was unchanged. Here, we demonstrate, again using pancreatic-clamped, conscious dogs, that glucagon is a candidate for the factor responsible for this selective induction. Thus glucagon stimulated G-6-Pase catalytic subunit but not G-6-P transporter gene expression in vivo. Furthermore, cAMP stimulated endogenous G-6-Pase catalytic subunit gene expression in HepG2 cells but had no effect on G-6-P transporter gene expression. The cAMP response element (CRE) that mediates this induction was identified through transient transfection of HepG2 cells with G-6-Pase catalytic subunit chloramphenicol acetyltransferase fusion genes. Gel retardation assays demonstrate that this CRE binds several transcription factors including CRE-binding protein and CCAAT enhancer-binding protein.

insulin; fatty acids; gene transcription; cyclic adenosine monophosphate; glucose-6-phosphatase; glucose 6-phosphate

DURING FASTING CONDITIONS, the liver supplies the body with glucose through the breakdown of glycogen stores and through de novo synthesis from gluconeogenic precursors. Glucose-6-phosphatase (G-6-Pase) catalyzes the final step in both of these pathways, hydrolyzing glucose 6-phosphate (G-6-P) to glucose and inorganic phosphate. In 1975, Arion et al. (6) proposed that G-6-Pase exists as an enzyme system of multiple components, each embedded in the endoplasmic reticulum (ER) membrane. According to this so-called substrate-transport model, G-6-P translocates across the membrane via a specific transporter. Once in the lumen, G-6-P is then hydrolyzed by the system’s catalytic subunit, a relatively nonspecific phosphatase. Glucose and inorganic phosphate transporters then shuttle the reaction products, glucose and inorganic phosphate, to the cytoplasm. Although no model of the G-6-Pase system appears to account fully for all the reported kinetic data (22, 55, 76, 77), the validity of the substrate-transport model has been supported by the identification of a G-6-P transporter located in the ER membrane (26, 42).

Pathophysiological conditions arising from aberrant activity of either the G-6-Pase catalytic subunit or the G-6-P transporter have demonstrated the importance of the G-6-Pase system. Inactivating mutations in the G-6-Pase catalytic subunit or G-6-P transporter result in glycogen storage disease types 1a and 1b, respectively; patients with such mutations suffer from severe hypoglycemia in the postabsorptive state, among other defects (12). Conversely, increased expression of both the G-6-Pase catalytic subunit and G-6-P transporter may contribute to the elevated hepatic glucose production (HGP) characteristic of both poorly controlled type 1 and type 2 diabetes. In the liver of diabetic animal models, mRNA levels of both genes are markedly elevated (4, 29, 40, 44, 50, 56). Furthermore, adenoaviral overexpression of either gene in hepatocytes results in enhanced rates of G-6-P hydrolysis and altered glycogen metabolism (1, 70).

We have been interested in investigating whether the expression of the genes encoding the G-6-Pase catalytic subunit and the G-6-P transporter are regulated in parallel in the liver or whether hormones and nutrients differentially regulate their expression. We first addressed this question by examining the effects of insulin and glucose on the expression of these genes in vivo in the liver of conscious dogs. In hyperinsulinemic dogs, the expression of both the G-6-Pase catalytic subunit and G-6-P transporter genes was suppressed (35). This suppression of the expression of both genes by insulin was also apparent in the liver-derived H4IIE cell line (35). In contrast, in hypoinsulinemic dogs, G-6-Pase catalytic subunit mRNA levels were fourfold greater than those of control dogs whereas G-6-P transporter gene expression was unchanged (35). This result indicates that insulin tonically suppresses hepatic G-6-Pase catalytic subunit but not G-6-P transporter gene expression in vivo (35).

Unfortunately, tissue culture studies cannot directly address the question of what factor, or factors, mediate the selective elevation of G-6-Pase catalytic subunit gene expression in the absence of insulin in vivo. Nevertheless, the observation that cAMP selectively stimulated G-6-Pase catalytic subunit gene expression in primary hepatocytes and in H4IIE hepatoma cells suggested that glucagon may contribute to the differential regulation observed in vivo (35). However, fatty acids and glyceral were also candidates to explain this differential regulation because insulin inhibits lipolysis, and therefore the
hypoinsulinemia induced in these studies also resulted in an increase in the levels of both nonesterified fatty acids (NEFAs) and glycerol (18). Although the effects of fatty acids and glycerol on G-6-P transporter gene expression have not been reported, both these agents regulate G-6-Pase catalytic subunit gene expression. Thus infusion of conscious rats with triglycerides (51) or treatment of primary hepatocytes with long-chain fatty acids (11) elevates G-6-Pase catalytic subunit mRNA. Additionally, at low concentrations, the gluconeogenic substrate glycerol increases G-6-Pase catalytic subunit mRNA levels in primary hepatocytes (48).

The studies presented here were designed to address the question of what factor, or factors, are candidates to mediate the selective elevation of G-6-Pase catalytic subunit gene expression in the absence of insulin in vivo. This was achieved by using the metabolically clamped, conscious dog model to assess the effects of glucagon, fatty acids, and glycerol on hepatic G-6-Pase catalytic subunit and G-6-P transporter gene expression.

METHODS

Materials. [α-32P]dATP (>3,000 Ci/mmol), [γ-32P]ATP (>5,000 Ci/mmol), and [α-32P]UTP (>3,000 Ci/mmol) were obtained from Amer sham. d-[3-3H]glucose (10–20 Ci/mm) was obtained from Perkin Elmer. Somatostatin was purchased from Bachem. For dog and tissue culture studies, glucagon was obtained from Bedford Laboratories. For dog studies, porcine insulin was purchased from Eli Lilly, whereas for tissue culture studies recombinant human insulin was obtained from Collaborative Bioproducts. Dexamethasone 21-phosphate and 8-(4-chlorophenylthio)adenosine-3’5’-cyclic monophosphate (pCPT-cAMP) were purchased from Sigma Chemical and Boehringer Mannheim, respectively. Human genomic DNA was purchased from Promega. Antiseria raised against various CCAAT enhancer-binding protein (C/EBP) isoforms were generously provided by Dr. Steven L. McKeith (8). Antiserum raised against CRE-binding protein (CREB) was purchased from NEB-Cell Signaling Technology (cat. no. 9192).

Animal care. Experiments were conducted on fourteen 18-h-fasted conscious mongrel dogs (19–30 kg) of either sex that had been fed a standard diet of meat (Pedigree canned beef, Vernon, CA) and chow (Laboratory Canine Diet no. 5006; PMI Nutrition International, Brentwood, MO) once daily. The diet totaled 33% protein, 12% fat, 49.5% carbohydrates, and 5% fiber, based on dry weight. Only dogs that had a good appetite, a leukocyte count <18,000/mm³, a hematocrit >35%, and normal stools were used for studies. The animal housing and surgical facilities met American Association for the Accreditation of Laboratory Animal Care standards. Protocols were approved by the Vanderbilt University Medical Center Animal Care Committee.

Animal surgical procedures. Each dog underwent a laparotomy performed under general anesthesia (15 mg/kg pentothal sodium presurgery and 1% isoflurane during surgery) 14–16 days before the experiment to implant catheters into the appropriate vessels. Silastic catheters (0.03 in. ID; Dow-Corning, Midland, MI) were placed into jejunal and splenic veins for the intraportal infusion of pancreatic hormones. Catheters (0.04 in. ID) for blood sampling were placed into the left hepatic vein, the hepatic portal vein, and left femoral artery, as previously described (16). All catheters were filled with heparinized saline (200 U/ml; Abbott Laboratories, North Chicago, IL), and their free ends were knotted before closure of the skin. The catheters were placed in a subcutaneous pocket before closure of the abdominal skin. On the day of the experiment, the catheters were externalized under local anesthesia (2% lidocaine; Abbott Laboratories). The contents of each catheter were aspirated and the catheters flushed with saline. The intraproval catheters (splenic and jejunal) were used for the infusion of glucagon and insulin. Angiocaths were inserted percutaneously into peripheral veins for the infusion of [3H]glucose plus indocyanine green (Becton Dickinson, Cokeysville, MD), as well as the infusion of somatostatin and Intralipid plus heparin infusions. Each animal was allowed to rest quietly in a Pavlov harness for 30 min before the start of the experiment.

Animal experimental procedures. Each experiment consisted of a tracer and dye equilibration period (~140 to ~40 min), a basal period (~40 to 0 min), and an experimental period (0 to 195 min). At ~140 min, a priming dose of [3H]glucose (33.3 μCi) was given and a constant infusion of [3H]glucose (0.35 μCi/min) was begun to allow the assessment of HGP. Constant infusions of indocyanine green (0.077 mg/min), to assess hepatic blood flow, and somatostatin (0.8 μg·kg⁻¹·min⁻¹), to inhibit endogenous insulin and glucagon secretion, were also started at ~140 min. [3H]Glucocycloneogenic, and somatostatin were given via a leg vein. A constant intraportal infusion of glucagon (0.55 ng·kg⁻¹·min⁻¹) was given (t = ~140 min) to replace basal endogenous glucagon secretion. Endogenous insulin was replaced at a variable intraportal infusion rate from ~140 to ~40 min. The plasma glucose level was monitored every 5 min, and euglycemia was maintained by adjusting the rate of insulin infusion. Once the plasma glucose level had been stabilized at euglycemic levels for 30 min, basal sampling began and the infusion rate of insulin remained unchanged.

The study included four protocols. In protocol 1, [hyperglycemic (HG); n = 3], a hyperglycemic clamp was performed. At 15 min, 20% dextrose was infused via a leg vein to clamp the arterial glucose levels at the levels seen in the groups receiving three times basal glucagon. In protocol 2 [HG and elevated glucagon (HG + GGN); n = 4], at 15 min, the intraportal glucagon infusion was increased from 0.55 ng·kg⁻¹·min⁻¹ to three times the basal rate (1.65 ng·kg⁻¹·min⁻¹). In protocol 3 [HG and elevated fatty acids and glycerol (HG + NEFA); n = 3], a constant Intralipid (0.02 ml·kg⁻¹·min⁻¹) 20% fat emulsion (Baxter Healthcare, Deerfield, IL) plus heparin (0.5 U·kg⁻¹·min⁻¹) infusion was started at 0 min in the presence of a hyperglycemic clamp. At 15 min, 20% dextrose was infused via a leg vein to clamp the arterial glucose levels at the increasing levels seen in the groups receiving three times basal glucagon.

In protocol 4 (HG + GGN + NEFA; n = 4), a constant Intralipid (0.02 ml·kg⁻¹·min⁻¹) plus heparin (0.5 U·kg⁻¹·min⁻¹) infusion was started at 0 min via a leg vein. After a NEFA-glycerol equilibration period (15 min), the intraportal glucagon infusion was increased from 0.55 to 1.65 ng·kg⁻¹·min⁻¹, as seen in protocol 2.

The glucagon infusion rate was increased by 7% every hour to correct for glucagon degradation in the groups receiving three times basal glucagon. Arterial blood samples were taken every 10 min during the control period and every 15 min during the experimental period. In the HG and HG + NEFA groups, arterial blood samples were also taken every 5 min to monitor glucose levels. The total blood volume withdrawn did not exceed 20% of the dog’s total blood volume. No significant decrease in hematocrit was observed with this procedure.

Immediately following the final blood sample, each animal was anesthetized with pentobarbitol sodium. The animal was then removed from the harness while the hormones, Intralipid-heparin, and/or glucose continued to be infused. A midline laparotomy incision was made, and clamps cooled in liquid nitrogen were used to freeze the liver. The liver was processed in a similar manner as described in the Sections and Results. A 1-ml aliquot of whole blood was lysed with 3 ml of 4% perchloric acid. The
solution was centrifuged and the supernatant stored for the future analysis of whole blood glycerol. A second 1-ml aliquot of whole blood was treated with 20 μl of an antioxidant (0.2 M glutathione) and centrifuged. This supernatant was then stored for the future determination of catecholamines, epinephrine and norepinephrine. A 1-ml aliquot of plasma received 50 μl of 100,000 kallikrein inhibitor units/ml (Trasylol; FBA Pharmaceuticals, New York, NY) and was stored for analysis of immunoreactive glucagon. The remainder of the plasma was used for analysis of insulin and NEFA. All samples were kept in an ice bath during the experiment and were then stored at −70°C until the assays were performed. Whole blood glycerol concentrations were determined according to the enzymatic methods of Lloyd et al. (45) for the Technicon Autoanalyzer (Tarrytown, NY) and were modified for the Monarch 2000 centrifugal analyzer (Lexington, MA). The levels of epinephrine and norepinephrine were assessed using high-performance liquid chromatography as previously described (57). Immunoreactive insulin was measured using a double-antibody radioimmunoassay (59). Immunoreactive glucagon was measured using a modification of the double-antibody insulin method (59). Plasma NEFA were determined spectrophotometrically using the Monarch 2000 centrifugal analyzer and a kit obtained from Wako Chemicals (Richmond, VA).

Because the total blood flow entering the liver comes from two sources, 20% from the hepatic artery and 80% from the portal vein, the hepatic sinusoidal concentration of a substance can be calculated as the arterial concentration multiplied by 0.2 plus the portal concentration multiplied by 0.8 ([A]·[P]·0.8).

**Cell culture.** Human HepG2 hepatoma cells were grown in Dulbecco’s modified Eagle’s medium containing 2.5% (vol/vol) newborn calf serum, 2.5% (vol/vol) fetal calf serum, and 5% (vol/vol) Nu serum IV (Collaborative Research), as previously described (61, 74). RNA isolation. After a 16-h period of serum starvation, HepG2 cells were incubated in fresh serum-free Dulbecco’s modified Eagle’s medium supplemented with various combinations of pCPT-cAMP (100 μM), dexamethasone (500 nM), and insulin (100 nM), as indicated in the figure legends. Total RNA was then isolated at the time indicated in the figure legends by cesium chloride centrifugation (see Fig. 5), as previously described (17). TRI Reagent (Molecular Research Center) was used to isolate total RNA from dog liver samples, according to the manufacturer’s instructions.

**Isolation of genomic DNA fragments for the generation of antisense ribonuclease protection assay probes.** With human genomic DNA as the template, a fragment of exon 3 of the human G-6-P transporter gene (26) was generated using PCR in conjunction with the following primers: 5'-GGAATTCCGGAGTCCAACATCAG-3' and 5'-SacGTTGGCAATCTCAGTTGGGA-3' (SacI and HindIII cloning sites underlined). The isolated PCR fragment was then digested with EcoRI and HindIII and ligated into the EcoRI- and HindIII-digested pGEM7 vector (Promega) and then sequenced using the USB sequenase kit. The sequence of the human G-6-P transporter fragment was identical to that previously reported (26). The plasmid was linearized with HindIII such that in vitro transcription using T7 polymerase generated a 254-nucleotide antisense RNA probe.

The generation of plasmids containing genomic DNA fragments of the dog genes encoding the G-6-P transporter, the G-6-P catalytic subunit, and cyclophilin A has been previously described (35). These plasmids were linearized with HindIII or SacI, as previously described (35), such that in vitro transcription using T7 polymerase generated 254-, 335-, or 121-nucleotide antisense RNA probes, respectively. A linearized plasmid containing a fragment of the human cyclophilin A gene was purchased from Ambion and was used to generate a 165-nucleotide antisense probe.

**Ribonuclease protection assay.** [α-32P]UTP-labeled antisense dog G-6-P transporter, G-6-P catalytic subunit, and cyclophilin A probes, as well as human G-6-P transporter and cyclophilin A probes, were generated using the linearized plasmids described above and the MAXScript T7 kit (Ambion) according to the manufacturer’s instructions. Ribonuclease protection assays (RPAs) were performed using 10 μg of total dog liver or HepG2 RNA and the RPA III kit (Ambion), again according to the manufacturer’s instructions except that the combined RNA and probe precipitate were dissolved in 1 μl of water before the addition of 10 μl of hybridization buffer. After RNase A/T1 digestion, RNA products were resolved on 5% polyacrylamide-urea-TBE gels and sizes estimated by comparison with coelectrophoresed DNA sequencing reactions. The sizes of the human G-6-P transporter and human cyclophilin A products were close to the calculated sizes of 201 and 103 nucleotides, respectively. The human G-6-P transporter product appeared as a doublet (see Fig. 5), probably because the EcoRI site used in the cloning of the PCR product is partially homologous to the gene sequence. The sizes of the dog G-6-Pase catalytic subunit, G-6-P transporter, and cyclophilin A probes were close to the calculated sizes of 256, 201, and 68 nucleotides, respectively. Data were quantitated through the use of a Packard Instant Imager.

**Primer extension analysis.** A 100-nucleotide primer (5'-AACACGTCTCTGGGAGTCCTTGGTAATTAC-3'), complementary to exon 1 of the human G-6-Pase catalytic subunit gene (39), was synthesized for the analysis of G-6-Pase catalytic subunit gene expression in HepG2 cells (Fig. 5). A 30-nucleotide primer (5'-ATGTGAAAGAACAACGTTGGGTTGACCATG-3'), complementary to exon 1 of the mouse, rat, and human cyclophilin A genes (15, 30, 32), was synthesized for use as a nonhybrinized responsive internal control. After gel purification, these primers were 5' end-labeled with [γ-32P]ATP to a specific activity of ~2 Ci/μmol (66). The labeled primers (~3 × 107 cpm) were then annealed to 50 μg of total HepG2 RNA for 1 h at 60°C, and then primer extension was performed as previously described (21). Extension products were visualized by electrophoresis on polyacrylamide-urea-TBE gels (21). The sizes of the extension products were calculated by comparison with a DNA sequencing ladder. The human G-6-Pase catalytic subunit primer gave the predicted extension product of 168 nucleotides (39). With HepG2 RNA, the cyclophilin A primer gave a cluster of extension products between 73 and 75 nucleotides (Fig. 5); the published transcription start site predicts a product of 73 nucleotides with this primer (30). Data were quantitated through the use of a Packard Instant Imager.

**Fusion gene plasmid construction.** The generation of mouse G-6-Pase catalytic subunit-chloramphenicol acetyltransferase (CAT) and G-6-Pase catalytic subunit-luciferase fusion genes, both containing promoter sequence spanning nucleotides −231 to +66 relative to the transcription start site and the site-directed mutants (SDM) of the two putative G-6-Pase CRE motifs (see Fig. 6) in the context of the −231 G-6-Pase catalytic subunit-CAT fusion gene (designated −231 CRE1 SDM and −231 CRE2 SDM) have all been previously described (47, 72, 75).

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 (10), as have the CRE1 WT XMB, CRE1 MUT XMB, and CRE2 WT XMB plasmids, which contain multiple (3–4) copies of double-stranded, complementary oligonucleotides representing the wild-type or mutated G-6-Pase catalytic subunit CRE1 and CRE2 motifs ligated into the XMB vector (75).

Expression vectors encoding the α- and β-forms of the catalytic subunit of protein kinase A (PKA) were a generous gift from Dr. Richard A. Maurer (53). An empty-vector control was generated by digesting the PKA-β plasmid with Xhol and HindIII to remove the open reading frame, filling in the noncompatible ends with the Klenow fragment of Escherichia coli DNA polymerase I and then religating. An expression vector encoding the glucagon receptor was a generous gift from Dr. Thomas P. Sakmar (9). All plasmid constructs were purified by centrifugation through cesium chloride gradients (66).
Transient transfections, CAT, β-galactosidase, and luciferase assays. HepG2 cells were transiently transfected in suspension with the plasmids indicated in the figure legends by use of the calcium phosphate-DNA coprecipitation method as previously described (61, 74). Where indicated in the figure legends, the CAT or firefly luciferase reporter gene construct (15 μg) was cotransfected with expression vectors encoding either β-galactosidase (2.5 μg), Renilla luciferase (0.15 μg), the glucagon receptor (5 μg), the catalytic subunit of PKA-α (5.0 μg), or the same vector with the PKA open reading frame deleted (5.0 μg). CAT, β-galactosidase, and luciferase assays were performed exactly as previously described (47, 61, 74). In contrast to previous experiments (73, 75), we observed that PKA slightly stimulated Rous sarcoma virus-β-galactosidase expression in the HepG2 cells used in these experiments. Therefore, CAT activity in control and PKA-cotransfected cells was corrected for β-galactosidase activity or for the protein concentration in the cell lysate, respectively. In experiments using glucagon (see Fig. 4), firefly luciferase activity directed by the −231 G-6-Pase catalytic subunit-luciferase fusion gene construct was corrected for the Renilla luciferase activity in the same samples. Each construct was analyzed in duplicate in multiple transfections, as specified in the figure legends.

**Gel retardation assay.** HepG2 nuclear extracts were prepared by a modification of the method of Andrews and Fallier (2) using NP-40 to isolate nuclei (69). Briefly, cells were lysed in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40, 0.5 mM DTT, and 0.5 mM PMSF, and 1X protease inhibitor cocktail (Roche), and the isolated nuclei were extracted using 20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 25% glycerol, 2 mM DTT, and 1 mM PMSF. The protein concentration of the nuclear extract was determined by the Bio-Rad assay and was typically ~5 μg/μl.

Oligonucleotides representing the sense and antisense wild-type mouse G-6-Pase catalytic subunit CRE1 promoter sequence (sense: AG(−175)CTGTTTTGGCTATTTCAGTAATATCAGCCCTGACA (−142); HindIII compatible end underlined; CRE core in italics) and a consensus CRE (sense: GATCGAGTTGCGCTAGTGCAAGAGCT; BamHI compatible end underlined; CRE core in italics) were synthesized and subsequently gel purified, annealed, and labeled with [α-32P]dATP by using the Klenow fragment of E. coli DNA Polymerase I to a specific activity of ~2.5 Ci/pmol (66). The labeled double-stranded oligonucleotide probes (~7 fmol, ~50,000 cpm) were incubated with HepG2 nuclear extract (8 μg) in a final reaction volume of 20 μl containing 20 mM HEPES (pH 7.9), 100 mM NaCl, 0.06 mM spermidine, 0.01 mM spermine, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 12.5% glycerol (vol/vol), 0.5 μg of poly(dI-dC)·poly(dI-dC), and 0.5 μg of poly(dA-dT)·poly(dA-dT). After incubation for 20 min at room temperature, the reactants were loaded onto a 6% polyacrylamide gel and electrophoresed for 90 min at 150 V at room temperature in a buffer containing 25 mM Tris (pH 7.8), 190 mM glycine, and 1 mM EDTA. After electrophoresis, the gels were dried and exposed to Kodak XAR5 film, and binding was analyzed by autoradiography.

For competition experiments, a 100-fold molar excess of various unlabeled double-stranded oligonucleotides (see Fig. 8) were incubated with the labeled oligomer before the addition of HepG2 nuclear extract. Gel supershift assays were carried out by incubating HepG2 nuclear extract with the indicated antisera (1 μl) for 10 min on ice before the addition of the labeled oligonucleotide probe and binding buffer and incubation for an additional 20 min at room temperature. Binding was then analyzed by polyacrylamide gel electrophoresis as described above.

**Statistical analysis.** The animal study data were analyzed for differences between basal period and experimental period values by use of a paired Student’s t-test and for differences from the control group values using an unpaired Student’s t-test. The level of significance was P < 0.05 (two-sided test).

**RESULTS**

**Metabolic parameters of in vivo conscious dog studies.** Four protocols were utilized in which glucagon, free fatty acids, and glycerol were selectively manipulated in conscious dogs in vivo (Fig. 1). This was achieved, following an 18-h fast, by the continuous administration of somatostatin throughout the study so as to block endogenous pancreatic hormone secretion. In all four protocols, hyperglycemia was achieved during the experimental period either directly through glucose infusion or as a consequence of elevated glucagon levels (Fig. 1). We have previously shown that the level of hyperglycemia achieved (~200 mg/dl) has no effect on G-6-Pase catalytic subunit or G-6-P transporter gene expression in conscious dogs in vivo (35). Insulin was infused at a rate sufficient to achieve euglycemia during the basal period; this insulin infusion rate was maintained during the experimental period. This strategy was designed to offset variations in insulin sensitivity between animals and so ensure a similar degree of insulin signaling in each animal. Thus the actual basal insulin concentration required to achieve euglycemia varied modestly between animals. Either glucagon was supplied at basal rates during the basal and experimental periods with glucose and then clamped at a hyperglycemic level during the experimental period, or it was supplied at approximately threefold the basal rate during the experimental period, in which case glucose was permitted to respond to the change in glucagon concentration (Fig. 1).

The study was designed so that the level of hyperglycemia achieved was similar in each case. Finally, infusions of the triglyceride emulsion Intralipid allowed manipulation of both fatty acid and glycerol concentrations to a level approximately threefold above basal. The actual changes in sinusoidal plasma glucose, insulin, glucagon, NEFA, and glycerol levels in the four experimental groups during the transition between the basal and experimental periods are described in the legend to Fig. 1. The mean levels ± SE during the final 2 h of the experimental period are shown in Fig. 2. The data in Fig. 2 show that these experimental manipulations achieved the desired goals. Thus glucose (Fig. 2A) and insulin (Fig. 2B) levels were statistically no different among the four groups of animals, whereas glucagon levels were selectively increased in protocols 2 and 4 (Fig. 2C). Similarly, glycerol (Fig. 2D) and NEFA levels (Fig. 2E) were selectively increased in protocols 3 and 4.

**Selective regulation of G-6-Pase catalytic subunit and G-6-P transporter gene expression in vivo.** Freeze-clamped liver samples were removed from anesthetized dogs immediately after the experimental period. Total RNA was then isolated and RPAs were performed to quantify G-6-Pase catalytic subunit, G-6-P transporter, and cyclophilin A mRNA levels. RPA probes representing fragments of the dog G-6-Pase catalytic subunit, G-6-P transporter, and cyclophilin A genes were all generated using PCR in conjunction with primers representing conserved sequences in the mouse, rat, and human genes. Expression of the latter is not responsive to hormones or metabolites so that it serves as an internal control. Therefore, the effects of glucagon, fatty acids, and glycerol on G-6-Pase
catalytic subunit and G-6-P transporter mRNA levels are quantitated relative to the level of cyclophilin A mRNA in the same samples.

As shown in Fig. 3, when glucagon levels were elevated above basal, and glucose levels were allowed to rise in response (protocol 2), G-6-Pase catalytic subunit mRNA levels increased ~2.5-fold over those assayed in hyperglycemic animals (protocol J (P < 0.05). In contrast, G-6-P transporter gene expression was not significantly changed (Fig. 3). This stimulation of G-6-Pase catalytic subunit gene expression cannot be due to the secondary rise in glucose levels induced by the elevation in glucagon concentration, since the glucose level achieved was identical to that in hyperglycemic animals (Fig. 2). In contrast to the effect of glucagon, infusion of Intralipid failed to stimulate an increase in either G-6-Pase catalytic subunit or G-6-P transporter gene expression (Fig. 3). Moreover, the magnitude of the stimulatory effect of glucagon on G-6-Pase catalytic subunit gene expression in combination with Intralipid was no different from that seen in the presence of glucagon alone (Fig. 3). G-6-Pase catalytic subunit and G-6-P transporter mRNA levels do not vary with location within the liver. The canine liver can be functionally divided into seven lobes according to the relative contributions of the hepatic artery and portal vein to the blood supply (19). However, the data shown in Fig. 3 were obtained using RNA isolated from only hepatic lobe 7 of each dog. Therefore, we were concerned about the theoretical possibility that the basal and/or hormone-regulated expression of the G-6-Pase catalytic subunit and G-6-P transporter genes might vary between different liver lobes. Indeed, the expression levels of some genes, such as c-fos, do vary between lobes (33), although others, such as collagen, do not (25). We therefore compared the relative expression level of these genes in liver lobes 2, 3, and 7 in eight individual metabolically clamped dogs. Four of these dogs had been subjected to hyperglycemia (28), and four had been subjected to hyperglycemia plus elevated glucagon (protocol 2; Fig. 1). There were no significant differences in the expression level of either gene among the three lobes examined, regardless of the experimen-

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**Table 1.** Schematic representation of the 4 in vivo conscious dog protocols.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Glucose Level</th>
<th>Hormone Levels</th>
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<tbody>
<tr>
<td>Protocol 1 (n=3)</td>
<td>Peripheral glucose (~200 mg/dL)</td>
<td>Glucagon (3X basal; 1.65 ng/kg/min)</td>
</tr>
<tr>
<td>Protocol 2 (n=4)</td>
<td>Glucagon (3X basal; 1.65 ng/kg/min)</td>
<td>Pe20% Intralipid (0.02 mL/kg/min)</td>
</tr>
<tr>
<td>Protocol 3 (n=3)</td>
<td>Peripheral glucose (~200 mg/dL)</td>
<td>Pe20% Intralipid (0.02 mL/kg/min)</td>
</tr>
<tr>
<td>Protocol 4 (n=4)</td>
<td>Glucagon (3X basal; 1.65 ng/kg/min)</td>
<td>Pe20% Intralipid (0.02 mL/kg/min)</td>
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Fig. 1. Schematic representation of the 4 in vivo conscious dog protocols. Protocol 1 (hyperglycemia, HG): beginning at 15 min, the glucose infusion rate was increased to raise arterial plasma glucose levels to ~200 mg/dL. No changes were made in hormone infusion rates after the equilibration period. Sinusoidal plasma glucose levels rose from 104 ± 4 mg/dL during the basal period to 178 ± 22 mg/dL (P = 0.058) during the final 2 h of the study. Sinusoidal insulin, glucagon, nonesterified fatty acids (NEFA), and glycerol concentrations remained at basal levels and relatively unchanged throughout the experimental period. Protocol 2 (HG + elevated glucagon; HG + GGN): beginning at 15 min, the intraportal glucagon infusion rate was increased to 1.65 ng/kg·min⁻¹. Glucose and insulin infusion rates were not altered after the equilibration period. Sinusoidal glucagon levels rose from 69 ± 3 pg/ml during the basal period to 168 ± 21 pg/ml (P < 0.05) and sinusoidal glucose levels from 116 ± 8 to 196 ± 10 mg/dl (P < 0.05) during the final 2 h of the study. Sinusoidal insulin remained at basal levels and relatively unchanged throughout the experimental period. However, NEFA levels fell slightly but significantly from 690 ± 113 µmol/l during the basal period to 454 ± 69 µmol/l (P < 0.05) during the final 2 h of the study, and glycerol levels declined from 104 ± 21 to 70 ± 14 µmol/l (P < 0.05). Protocol 3 (HG + elevated fatty acids and glycerol; HG + NEFA): at 0 min, peripheral infusion of Intralipid (20%, 0.2 ml/kg·min⁻¹) was begun, accompanied by simultaneous heparin infusion (0.5 U/kg·min⁻¹) to stimulate lipolysis. Beginning at 15 min, the arterial plasma glucose level was raised to ~200 mg/dl by an increase in the glucose infusion rate. Hormone infusion rates were not changed after the equilibration period. Sinusoidal glucose levels rose from 94 ± 4 mg/dl during the basal period to 192 ± 10 mg/dl (P < 0.05) during the final 2 h of the study. Sinusoidal NEFA and glycerol levels increased from 496 ± 33 to 1,570 ± 158 µmol/l (P < 0.05) and from 61 ± 8 to 179 ± 9 µmol/l (P < 0.05), respectively, during the final 2 h of the study. Sinusoidal insulin and glucagon concentrations remained at basal levels and relatively unchanged throughout the experimental period. Protocol 4 (HG + GGN + NEFA); beginning at 0 min, infusions of Intralipid and heparin were begun as in protocol 3, and beginning at 15 min the glucagon infusion rate was increased to 1.65 ng/kg·min⁻¹ as in protocol 2. Sinusoidal glucagon levels increased from 56 ± 4 pg/ml during the basal period to 164 ± 27 pg/ml (P < 0.05) during the final 2 h of the study. Sinusoidal NEFA levels increased from 544 ± 83 to 1,473 ± 150 µmol/l (P < 0.05) and glycerol levels from 64 ± 7 to 189 ± 12 µmol/l (P < 0.05) during the final 2 h of the study. Sinusoidal glucose levels rose from 108 ± 7 to 219 ± 23 mg/dl (P < 0.05) during the final 2 h of the study. Insulin infusion rates were not changed after the equilibration period; however, sinusoidal insulin levels rose slightly from 16 ± 1 to 22 ± 2 µU/ml (P < 0.05) during the final 2 h of the study.
tal conditions (data not shown). This suggests that the results shown in Fig. 3 are representative of G-6-Pase catalytic subunit and G-6-P transporter mRNA levels throughout the liver.

Glucagon stimulates G-6-Pase catalytic subunit fusion gene expression in HepG2 hepatoma cells. The results from the in vivo dog liver studies described above suggest that elevation of glucagon levels can selectively induce G-6-Pase catalytic subunit gene expression. This stimulation of G-6-Pase catalytic subunit gene expression by glucagon in vivo cannot be due to the secondary rise in glucose levels induced by the elevation in glucagon concentration, since the glucose level achieved was identical to that in the control hyperglycemic animals (Fig. 2). However, this experiment cannot formally rule out the possibility that glucagon alters the abundance of another metabolite/factor that then mediates the induction of G-6-Pase catalytic subunit gene expression.

To address this caveat, a G-6-Pase catalytic subunit-luciferase fusion gene, containing G-6-Pase catalytic subunit promoter sequence from −231 to +66, relative to the transcription start site at +1 (47, 72), was transiently transfected into HepG2 cells, and the effect of glucagon on fusion gene expression was assessed (Fig. 4). Although it has been reported that HepG2 cells respond to glucagon treatment (71), most studies suggest that glucagon receptors are markedly downregulated in hepatoma cells (20, 54). Not surprisingly, therefore, glucagon failed to induce G-6-Pase catalytic subunit-luciferase fusion gene expression in HepG2 cells (Fig. 4A). However, glucagon did stimulate reporter gene expression when the G-6-Pase catalytic subunit-luciferase fusion gene was cotransfected with an expression vector encoding the glucagon receptor (Fig. 4A). In the absence of glucagon, cotransfection with the glucagon receptor alone had little effect on G-6-Pase catalytic subunit-luciferase gene expression, suggesting a low level of signaling through the basal receptor (Fig. 4B). The maximal effect of glucagon on G-6-Pase catalytic subunit-luciferase gene expression was seen at ~100 nM (Fig. 4C). This result suggests that glucagon signaling can directly stimulate G-6-Pase catalytic subunit gene expression.

cAMP stimulates endogenous G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression in HepG2 hepatoma cells. Because HepG2 cells are transfected with a low efficiency, it was not possible to look at the effect of glucagon on fusion gene expression. However, it has been reported that HepG2 cells spontaneously express G-6-Pase catalytic subunit (71). This expression is not due to the transfected plasmids, since transfection with the empty vector did not alter the expression of the endogenous G-6-Pase catalytic subunit (Fig. 4D). Because the HepG2 cells used in this study express significant levels of endogenous G-6-Pase catalytic subunit, the increase in luciferase activity observed in response to glucagon treatment may not fully reflect the increase in G-6-Pase catalytic subunit gene expression that occurs in vivo. However, it is clear that cAMP stimulates the expression of both the endogenous and transfected G-6-Pase catalytic subunit genes in HepG2 cells (Fig. 4E).
on endogenous G-6-Pase catalytic subunit gene expression. Therefore, we treated cells with the cAMP analog pCPT-cAMP for 3 h after overnight serum starvation and then harvested total RNA. Primer extension analyses and RPAs were used to quantify G-6-Pase catalytic subunit and G-6-P transporter mRNA levels, respectively, and expression of the cyclophilin A gene was again used as an internal control. As shown in Fig. 5, pCPT-cAMP treatment stimulated endogenous G-6-Pase catalytic subunit mRNA expression approximately threefold but did not affect G-6-P transporter mRNA levels. In addition, insulin repressed the endogenous basal expression of both genes. Both of these observations are consistent with the regulation of the equivalent hepatic dog genes by glucagon (Fig. 3) and insulin (35). Figure 5 also shows that insulin repressed the endogenous basal expression of the equivalent hepatic dog G-6-Pase catalytic subunit gene in vivo. This observation is also consistent with the regulation of the hepatic dog G-6-Pase catalytic subunit gene in vivo. Thus, because the induction of G-6-Pase catalytic subunit gene expression during hypoinsulinemia (35) may be explained by the unopposed action of glucagon (Fig. 3), this suggests that basal insulin normally suppresses the stimulatory effect of basal glucagon. Although the hormonal regulation of endogenous G-6-Pase catalytic subunit gene expression in HepG2 cells...
closely matches that seen in dog liver, this does not extend to the effect of glucocorticoids. Thus glucocorticoids induce expression of the endogenous hepatic G-6-Pase catalytic subunit gene (24), but the synthetic glucocorticoid analog dexamethasone did not significantly stimulate expression of this gene, or the G-6-P transporter gene, in HepG2 cells (Fig. 5). We have previously shown that dexamethasone stimulates the expression of both genes in H4IIE rat hepatoma cells (35), suggesting that these cells are a better model, at least with respect to glucocorticoid signaling.

**Analysis of CREs in mouse G-6-Pase catalytic subunit promoter.** Two regions of the human G-6-Pase catalytic subunit promoter have been reported to be involved in cAMP responsiveness (43, 68). Schmoll et al. (68) demonstrated that, in H4IIE hepatoma cells, deletion of the sequence from −161 to −151 of the human G-6-Pase catalytic subunit promoter markedly reduced the combined stimulatory effect of cAMP and dexamethasone on fusion gene expression. In contrast, Lin et al. (43) showed that, in HepG2 cells, deletion of the human G-6-Pase catalytic subunit promoter sequence between −146 and −125 completely blocked the stimulatory effect of cAMP. Both of these promoter regions contain sequences that are similar to the consensus CRE sequence TGACGTCAT, and both elements bind CREB (43, 68). For clarity, the G-6-Pase catalytic subunit promoter sequence between −161 and −152 has been termed CRE1, and the region between −146 and −125 has been termed CRE2. The reason for this discrepancy between the results of Schmoll et al. and those of Lin et al. is unclear, but these elements are both well conserved in the mouse G-6-Pase catalytic subunit promoter (75).

We (75) have previously shown that, in HepG2 cells, co-transfection with a plasmid encoding the catalytic subunit of PKA more robustly stimulates G-6-Pase catalytic subunit-CAT fusion gene expression than treatment with a cAMP analog (~15- vs. 2- to 3-fold). Using this approach, we demonstrated that multiple promoter elements are required for the stimulatory effect of PKA on G-6-Pase catalytic subunit-CAT fusion gene expression (75), rather than the single elements described by other investigators (43, 68). One of these elements, located between −114 and −99, was subsequently identified as a hepatocyte nuclear factor-6 (HNF-6)-binding site (73); however, the results also showed that deletion of the regions encompassing either CRE1 or CRE2 resulted in a reduction in the maximal effect of PKA on fusion gene expression (75).

To explore the role of CRE1 and CRE2 in the stimulatory effect of PKA on mouse G-6-Pase catalytic subunit gene transcription in HepG2 cells, we separately mutated CRE1 and CRE2 in the context of the −231 to +66 G-6-Pase catalytic subunit-CAT fusion gene. Figure 6A shows that, when the results are expressed in terms of maximal induction of expression by PKA, mutation of both CRE1 and CRE2 results in a decrease in the ability of PKA to stimulate G-6-Pase catalytic subunit-CAT fusion gene expression compared with the wild-type −231 G-6-Pase catalytic subunit-CAT fusion gene. However, the interpretation of this experiment is complex, because mutation of CRE1 and CRE2 also results in a reduction in basal G-6-Pase catalytic subunit-CAT fusion gene expression (Fig. 6B). Nevertheless, Fig. 6C shows that, even when the results are expressed in terms of fold induction of expression by PKA, mutation of both CRE1 and CRE2 decreases the ability of PKA to stimulate G-6-Pase catalytic subunit-CAT fusion gene expression. These data suggest that both sites are required for the stimulation of G-6-Pase catalytic subunit-CAT fusion gene expression by PKA in HepG2 cells. However, this experiment does not reveal whether CRE1 and CRE2 are acting directly as bona fide CREs or indirectly as accessory factor binding sites to enhance the effect of cAMP mediated through another element.

**CRE1, but not CRE2, acts as a bona fide CRE in HepG2 cells.** Multiple copies of double-stranded oligonucleotides representing the mouse G-6-Pase catalytic subunit sequence either from −175 to −142 (CRE1) or from −155 to −119 (CRE2)
were ligated into the heterologous XMB-CAT expression vector and transiently transfected into HepG2 cells (Fig. 7). The G-6-Pase catalytic subunit promoter sequence from −175 to −142 (CRE1) mediated a direct stimulatory effect of PKA on reporter gene expression (Fig. 7), whereas the sequence from −155 to −119 was unable to mediate a PKA response (Fig. 7). As a control, a double-stranded oligonucleotide containing a mutation of the CRE-like motif within the CRE1 sequence, the same mutation as present in the −231 CRE1 SDM fusion gene (Fig. 6), was inserted in multiple copies into the heterologous XMB-CAT expression vector. CAT expression directed by the resulting construct (CRE1 MUT XMB) was not stimulated by PKA following transient transfection into HepG2 cells (Fig. 7). These data indicate that the CRE1 region, but not the CRE2 region, contains a bona fide CRE and that the CRE-like motif within CRE1 is responsible for the stimulatory effect of PKA on G-6-Pase catalytic subunit gene expression. In addition, this result suggests that the CRE2 region contains an accessory factor binding site that likely enhances both basal G-6-Pase catalytic subunit gene expression (Fig. 6C) and the effect of cAMP mediated through CRE1.

**CREB and C/EBP bind the G-6-Pase catalytic subunit CRE.** When a labeled oligonucleotide representing the wild-type G-6-Pase catalytic subunit promoter sequence from −175 to −142 that encompasses CRE1 was incubated with nuclear extract prepared from HepG2 cells, several protein-DNA complexes were detected in gel retardation assays (Fig. 8A). Competition experiments, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, were used to correlate protein binding with cAMP-regulated G-6-Pase catalytic subunit gene expression. The wild-type CRE1 oligonucleotide competed effectively for the formation of four protein-DNA complexes (Fig. 8A; see arrows). In contrast, an oligonucleotide designated CRE1 MUT (75), which contains a
the CREB antisera. To identify the factors present in the other three complexes, gel retardation assays were performed in which HepG2 cell nuclear extract was preincubated with antisera specific for different members of the C/EBP transcription factor family (64). It has been previously shown that members of the C/EBP transcription factor family bind the CRE motif in the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter (60, 62). Figure 8B shows that addition of antibodies recognizing C/EBPα resulted in a selective disruption in the formation of only complexes 2 and 3, with no effect on the formation of complexes 1 and 4, whereas addition of antibodies recognizing C/EBPβ resulted in a selective disruption in the formation of only complex 4, with no effect on the formation of complexes 1–3. Concomitant with the disruption of complexes 2–4, a clear supershift was apparent upon addition of the C/EBPα and C/EBPβ antisera (Fig. 8B). These results suggest that complex 1 contains CREB, complexes 2 and 3 contain C/EBPα, and complex 4 contains C/EBPβ. Previous studies suggest that C/EBPα is susceptible to proteolysis such that complexes 2 and 3 may represent the binding of full-length and truncated C/EBPα, respectively (60).

It is unclear why Schmoll et al. (68) detected binding only of CREB to CRE1, whereas we detect the binding of CREB, C/EBPα, and C/EBPβ. However, when a labeled oligonucleotide representing a consensus wild-type CRE sequence was incubated with nuclear extract prepared from HepG2 cells, only a single protein-DNA complex was detected (Fig. 8A). Competition experiments, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, showed that the wild-type consensus CRE oligonucleotide competed effectively for the formation of this protein-DNA complex, whereas an unrelated, random (RN) oligonucleotide failed to compete with the labeled probe for formation of this complex (Fig. 8A). This indicates that this complex represents a specific protein-DNA interaction. Addition of antibodies recognizing CREB resulted in a selective disruption in the formation of this complex concomitant with the formation of a clear supershift (Fig. 8A). Antisera to C/EBPα, C/EBPβ, and C/EBPδ had no effect on complex formation (data not shown). The core sequence of the G-6-Pase catalytic subunit CRE (TTACGTAA) differs from the consensus CRE (TGACGTCA), which may explain this difference in transcription factor binding.

**DISCUSSION**

We have previously shown that, in pancreatic-clamped, euglycemic conscious dogs, a 5-h period of hypoinsulinemia leads to a marked increase in hepatic G-6-Pase catalytic subunit mRNA, whereas G-6-P transporter mRNA remains unchanged (35). Although the dog studies described here were initially designed for a different purpose, namely to determine the effect of elevated circulating NEFA levels on glucagon action in vivo, we were able to use the samples already generated to address the question of what factor is likely to be responsible for the selective induction of G-6-Pase catalytic subunit gene expression in hypoinsulinemic dogs. We show that glucagon is a prime candidate for this factor. Thus glucagon selectively stimulated G-6-Pase catalytic subunit but not G-6-P transporter gene expression in vivo (Fig. 3). This stimulation of G-6-Pase catalytic subunit gene expression cannot be
due to the secondary rise in glucose levels induced by the elevation in glucagon concentration, since the resulting glucose level was identical to that in the control, hyperglycemic animals (Fig. 2). Moreover, it is unlikely that glucagon alters the abundance of another metabolite/factor that then mediates the induction of G-6-Pase catalytic subunit gene expression, since glucagon can directly stimulate G-6-Pase catalytic subunit fusion gene expression in HepG2 hepatoma cells (Fig. 4).

Furthermore, cAMP also stimulated endogenous G-6-Pase catalytic subunit gene expression in HepG2 cells but had no effect on G-6-P transporter gene expression (Fig. 5). This result is consistent with previous results showing that cAMP selectively stimulates G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression in rat H4IE cells and rat primary hepatocytes (35). Moreover, although Li et al. (40) initially reported that, in HepG2 cells, cAMP stimulated G-6-P transporter gene expression, more recently Li and van de Werve (41) reported that cAMP had no effect.

In the substrate-transport model for G-6-Pase, the translocation of G-6-P across the ER membrane was identified as a major control point in the G-6-Pase reaction on the basis of the phenomenon of latency, which is defined as the difference in the rate of G-6-P hydrolysis by intact vs. disrupted microsomes (5). Disruption of microsomes with detergents increases the rate of G-6-P hydrolysis, suggesting that G-6-P transport is limiting (5). However, Newgard and colleagues have shown, using adenoviral technology, that overexpression of either the G-6-Pase catalytic subunit [Seoane et al. (70)] or G-6-P transporter [An et al. (1)] is sufficient to cause an increased rate of HGP in primary hepatocytes. These experiments suggest that both the G-6-Pase catalytic subunit and G-6-P transporter contribute significantly to the overall rate of the G-6-Pase reaction. The data presented here and in our previous study (35) suggest that hormones differentially regulate G-6-Pase catalytic subunit and G-6-P transporter gene expression. This, then, may explain the observations that the degree of latency varies in microsomes prepared from fed or fasted rats (5) and with aging (27). One caveat with this hypothesis is that it is based on the assumption that G-6-Pase catalytic subunit and G-6-P transporter mRNA and protein/activity levels correlate. Studies on gene expression changes during liver regeneration show that this is not necessarily the case. G-6-Pase catalytic subunit mRNA is induced 30-fold during liver regeneration, but there is little (29) or no change (81) in G-6-Pase catalytic subunit protein or G-6-Pase enzyme activity. Furthermore, it cannot be assumed that the changes in G-6-Pase catalytic subunit and G-6-P transporter mRNA are due to transcriptional changes, since many hepatic genes are regulated at the level of mRNA stability (37), including the G-6-Pase catalytic subunit (48).

Multiple promoter elements are required to mediate the stimulatory effect of cAMP on G-6-Pase catalytic subunit gene expression in HepG2 cells (73, 75). However, we show here that the main target for cAMP signaling is the element CRE1, located between −162 and −155 (Fig. 7). We speculate that other elements in the G-6-Pase catalytic subunit promoter, such as CRE2, bind accessory factors that act to enhance cAMP signaling through CRE1. Such an arrangement is referred to as a cAMP response unit (46, 65). One exception is an HNF-6 motif located between −110 and −101 that can directly mediate cAMP signaling by a mechanism that involves direct phosphorylation of HNF-6 by PKA (73). However, cAMP signaling through CRE1 appears to be quantitatively more important than cAMP signaling through the HNF-6 motif (73).

A key question that remains to be addressed is which CRE1-bound factor mediates cAMP signaling. Gel retardation assays show that CRE1 can bind CREB, C/EBPα, and C/EBPβ in vitro (Fig. 8). Moreover, studies on the PEPCK promoter, using chimeric GAL4 fusion proteins, show that each of these factors can directly mediate cAMP signaling (79). The mechanism of cAMP signaling through CREB is well established and involves the direct phosphorylation of CREB by PKA, which then leads to binding of the coactivator CREB-binding protein (14, 58). In contrast, when bound to the PEPCK promoter, cAMP activates C/EBPα and C/EBPβ through an unknown mechanism that does not involve the direct phosphorylation of these factors by PKA (79). It is therefore possible that cAMP can activate G-6-Pase catalytic subunit gene expression through CRE1 regardless of which specific factor is bound. However, it is more likely that the selective binding of each of these factors will be important for different aspects of G-6-Pase catalytic subunit gene expression. For example, binding of C/EBPβ, but not C/EBPα or CREB, to the CRE in the PEPCK promoter is required for the maximal glucocorticoid-stimulated expression of that gene (80). Studies of mice expressing dominant negative CREB (34) and studies in which the genes encoding C/EBPα (78) and C/EBPβ (7) have been selectively deleted, support the involvement of all of these factors in the regulation of G-6-Pase catalytic subunit gene expression. However, the changes in G-6-Pase catalytic subunit gene expression observed in these animals could be indirect. In addition, C/EBPα and C/EBPβ may bind to elements in the G-6-Pase catalytic subunit promoter other than CRE1 (4, 43).

Commerford et al. (13) have recently shown that high-fat diets do not affect G-6-P transporter gene expression in rats. However, the lack of a stimulatory effect of fatty acids and glycerol on G-6-Pase catalytic subunit gene expression in conscious dogs in vivo (Fig. 3) was surprising, because feeding rats high-fat diets (13), infusing conscious rats with a triglyceride emulsion (51), or treating primary rat hepatocytes with either short-chain (49) or long-chain fatty acids (11) all elevate rat G-6-Pase catalytic subunit gene expression. Similarly, glycerol increases G-6-Pase catalytic subunit mRNA levels in rat primary hepatocytes (48). The reason for this lack of stimulation of G-6-Pase catalytic subunit gene expression by NEFA in conscious dogs is unclear. The concentration of NEFA achieved in our study (~1,500 μmol/l; Fig. 2E) is similar to that reported by Massillon et al. (51) in studies involving the infusion of conscious rats with a triglyceride emulsion. However, Massillon et al. used Liposyn (Abbott) rather than Intralipid (Baxter) as the source of fatty acids; the fatty acid composition of these products is not identical. This may be significant, since the regulation of G-6-Pase catalytic subunit gene expression by fatty acids is complex. Thus, although short- and long-chain fatty acids stimulate G-6-Pase catalytic subunit gene expression, polyunsaturated fatty acids actually suppress expression of the gene (63). Perhaps more significant, in the study by Massillon et al., the animals were mildly hyperinsulinemic, and glucagon was not replaced following somatostatin administration. It is therefore possible that basal G-6-Pase catalytic subunit gene expression was reduced rela-
tive to the level seen in our dogs, in which insulin and glucagon were both at basal levels. Such a reduction in basal G-6-Pase catalytic subunit gene expression might make a stimulatory effect of fatty acids more apparent.

Another possible explanation for the lack of fatty acid-stimulated G-6-Pase catalytic subunit gene expression in conscious dogs in vivo is that this gene is regulated differently in dogs and rats. Interestingly, we (35) have previously reported that, in conscious dogs in vivo, glucose also fails to stimulate G-6-Pase catalytic subunit gene expression. In contrast, in conscious rats (50, 52), primary rat hepatocytes (3, 11, 48), and rat FAO hepatoma cells (3, 38), glucose stimulates rat G-6-Pase catalytic subunit gene expression. We propose to explore this potential differential regulation of rat and dog G-6-Pase catalytic subunit gene expression in future experiments that will involve the cloning of the dog G-6-Pase catalytic subunit gene promoter. The comparative analysis of the rat and dog G-6-Pase catalytic subunit gene expression. Such primary hepatocyte studies will also be critical for confirming the results of our analysis of PKA-stimulated G-6-Pase catalytic subunit fusion gene transcription in HepG2 cells. This is an important issue, since HepG2 cells (36) and hepatoma cells in general (31) differ from normal liver cells in several respects, including reduced gluconeogenic capacity, and therefore may be lacking other key transcription factors or proteins important for understanding the regulation of this gene.

In summary, this study demonstrates that glucagon and its second messenger cAMP differentially regulate the hepatic expression of the genes encoding the G-6-Pase catalytic subunit and the G-6-P transporter in vivo and in situ. In contrast, insulin suppresses the expression of both genes, although the magnitude of the effect of insulin is much greater on G-6-Pase catalytic subunit gene expression (35). Interestingly, there have been two recent reports that described a differential regulation of G-6-Pase catalytic subunit and the G-6-P transporter gene expression in the liver of mice bearing Ehrlich ascites tumor cells (23) and in the clear cell type of human renal cell carcinoma (67), although the mechanisms responsible for this differential regulation were not elucidated.

ACKNOWLEDGMENTS

We thank Drs. Richard A. Maurer and Thomas P. Sakmar for providing the PKA and glucagon receptor expression vectors, respectively. We also thank Dr. Steven L. McKnight for providing antisera raised against various C/EBP isoforms.

GRANTS

This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grants DK-56374 to R. O’Brien and DK-18243 to A. D. Cherrington and by the Vanderbilt Diabetes Core Laboratory (P60 DK-20593). L. A. Hornbuckle and S. S. Gustavson were supported by the Vanderbilt Molecular Endocrinology Training Program (5 T32 DK-07563–12). C. A. Everett was supported by NIDDK Grant R37 DK-18243–27S1 (to A. D. Cherrington).

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