The promoter for the gene encoding the catalytic subunit of rat glucose-6-phosphatase contains two distinct glucose-responsive regions

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Pedersen KB, Zhang P, Doumen C, Charbonnet M, Lu D, Newgard CB, Haycock JW, Lange AJ, Scott DK. The promoter for the gene encoding the catalytic subunit of rat glucose-6-phosphatase contains two distinct glucose-responsive regions. Am J Physiol Endocrinol Metab 292: E788–E801, 2007. First published November 14, 2006; doi:10.1152/ajpendo.00510.2006.—Glucose homeostasis requires the proper expression and regulation of the catalytic subunit of glucose-6-phosphatase (G-6-Pase), which hydrolyzes glucose 6-phosphate to glucose in glucose-producing tissues. Glucose induces the expression of G-6-Pase at the transcriptional and posttranscriptional levels by unknown mechanisms. To better understand this enzyme at the transcriptional and posttranscriptional levels, we analyzed the promoter region of the gene encoding the catalytic subunit of rat glucose-6-phosphatase (G-6-Pase). We first tested whether the promoter confers a glucose response to the gene in a reporter gene assay. A construct containing the rat G-6-Pase promoter was inserted into the pCMV-EGFP reporter construct. This construct was transfected into two glucogenic cell lines, HL1C rat hepatoma cells and the 832/13 INS-1 rat insulinoma cells, which are not glucogenic. Glucose strongly increased endogenous G-6-Pase mRNA levels in 832/13 cells and in rat pancreatic islets, although the induced levels from islets were still markedly lower than in untreated primary hepatocytes. A distal promoter region was glucose responsive in 832/13 cells and contained a carbohydrate response element with two E-boxes separated by five base pairs. A proximal promoter region was glucose responsive in both 832/13 and HL1C cells, with a hepatocyte nuclear factor 1 binding site and two cAMP response elements required for glucose responsiveness. Expression of dominant-negative versions of both cAMP response element-binding protein and CAAT/enhancer-binding protein blocked the glucose response of the proximal region in a dose-dependent manner. We conclude that multiple, distinct cis-regulatory promoter elements are involved in the glucose response of the rat G-6-Pase gene.

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were then tested in HL1C cells. We found that the rat G-6-Pase gene promoter possessed two glucose-responsive regions, a proximal glucose-responsive region (−230/−112 with respect to the transcription start site) that contains a hepatocyte nuclear factor 1 (HNF-1) binding site and two cAMP response elements (CREs) as key elements and a distal glucose-responsive region containing a typical ChRE sequence (−3702/−3686). The transcription factor ChREBP was recruited to the G-6-Pase gene promoter in a glucose-dependent manner to the distal glucose-responsive region, whereas transcription factors of the cAMP response element-binding protein (CREB) and CAAT/enhancer-binding protein (C/EBP) families were required for the glucose response from the proximal glucose-responsive region. In HL1C cells, the glucose response required the proximal, but not the distal region. We conclude that the rat G-6-Pase gene promoter contains several distinct cis-regulatory elements involved in its glucose responsiveness.

MATERIALS AND METHODS

Cell culture. HL1C rat hepatoma cells (10) were maintained in DMEM containing 33 μM biotin, 17 μM pantethione, 42 μM phenol red, 1 mM sodium pyruvate, 4 mM l-glutamine, and 15 mM HEPEP and further supplemented with 5% FBS, 5 mM glucose, 50,000 U/l penicillin, and 50,000 μg/l streptomycin. Rat insulinoma 832/13 cells (15) were maintained in RPMI 1640 medium with 10% FBS, 10 mM HEPEP, 2 mM l-glutamine, 1 mM sodium pyruvate, and 50 mM β-mercaptoethanol and further supplemented with 11 mM glucose. Pancreatic islets were harvested from male Wistar rats weighing ~250 g using the Liberase R1 enzyme (Roche Diagnostics, Indianapolis, IN) according to the guidelines of the manufacturer under a protocol approved by the Duke University Institutional Animal Care and Use Committee. Approximately 100 primary rat islets per condition were cultured for 24 h in 2 ml of RPMI 1640 medium containing 10% FCS plus 2 or 20 mM glucose. Primary hepatocytes were isolated using the collagenase perfusion method as previously described (9), under a protocol approved by the Louisiana State University Health Sciences Center, New Orleans Institutional Animal Care and Use Committee.

Plasmids. Expression plasmids for dominant-negative A-CREB and A-C/EBP proteins as well as the empty expression plasmid pRC/CMV500 (36) were kindly provided by Dr. Charles Vinson, National Cancer Institute (Bethesda, MD). cDNA was generated from RNA isolated from primary hepatocytes from male Wistar rats as previously described (9). The 58/1575 fragment of the glucokinase cDNA (numbering according to Ref. 4), including the entire coding region, was amplified by PCR using PfuUltra High-Fidelity DNA polymerase from Stratagene (La Jolla, CA). The sequences of the forward and reverse primers were 5′-gaaattcgctgagcaggacaa-3′ and 5′-aggaatccgagcatttcggtggctgaaagttc-3′, respectively. The amplified fragment was cloned into pcDNA3.1/Zeoc(−) from Invitrogen (Carlsbad, CA) between EcoRI and BamHI sites to generate the glucokinase expression plasmid pcDNA3.1/Zeo(−)GK. The cloned GK sequence is identical to the 58/1575 sequence published previously (4), except for a G instead of A at position 1466. The 286/685 sequence of rat G-6-Pase cDNA was likewise amplified from cDNA (numbering according to Ref. 22) prepared from primary hepatocytes. Forward and reverse primers had sequences 5′-tggctgctggacatttcggtggctgaaagaagttc-3′ and 5′-gaaattcgctgagcaggacaa-3′, respectively. Single oxyadenosines were added to the 3′ ends of the PCR product by treatment with 20 units of Tag DNA polymerase (Invitrogen) and 0.2 mM dATP at 70°C for 20 min. The treated PCR product was ligated into the pGEM-T Easy vector (Promega) to generate the plasmid 286/685 pGEM-T. The cloned 286/685 sequence differs from that reported previously (22) by a G instead of T at position 381. The plasmid −4712/+122 pBSSK was constructed by inserting the −4712/+122 promoter sequence of the rat G-6-Pase gene in PstI and SalI sites of the multicloning site of pBlueScript SK(+)1. Rat G-6-Pase gene promoter fragments −1642/+64, −729/+64, −600/+64, −400/+64, −350/+64, −300/+64, −250/+64, −230/+64, −226/+64, −200/+64, and −100/+64 were amplified by PCR from −4712/+122 pBSSK using primer sequences designed to flank the promoter sequences by an upstream Nhel site and a downstream XhoI site. The promoter fragments were inserted in the Nheli and XhoI restriction sites of pGL3-Basic vector from Promega (Madison, WI) to generate plasmids called X pGL3-Basic, where X denotes the inserted promoter fragment sequence. The plasmid −4078/+64 pGL3-Basic was made by a three-way ligation between the −4078/−1642 promoter fragment from −4712/+122 pBSSK generated by XhoI and HindIII digestion, the −1642/+64 promoter fragment from −1642/+64 pGL3-Basic generated by HindIII and XhoI digestion, and the vector backbone from −1642/+64 pGL3-Basic generated by XhoI and Nheli digestion. The human G-6-Pase gene promoter fragment −963/+67 was amplified from DNA isolated from Jurkat cells using Tri Reagent from Molecular Research Center (Cincinnati, OH) according to the guidelines of the manufacturer and cloned in the Nheli and XhoI sites of pGL3-Basic to generate the plasmid h-963/+67 pGL3Basic. Site-directed mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit from Stratagene according to instructions from the manufacturer. One and two copies of the rat G-6-Pase gene promoter sequence −230/−208, one and two copies of the rat G-6-Pase gene promoter sequence −3706/−3682, a scrambled nucleotide sequence of two copies of gene promoter sequence −3706/−3682, two copies of the rat ACCP1 sequence −126/−102 (numbering as in Ref. 35), two copies of the mouse G-6-Pase gene promoter sequence −2943/−2919, and two copies of the human G-6-Pase gene promoter sequence −2479/−2455 were cloned into vectors pGL3-Promoter (Promega) and pTA-Luc (Clontech Laboratories) between the SauI and Nheli sites using oligonucleotides of a defined sequence. Multiple copies of the rat G-6-Pase gene promoter sequences −167/−156 or −143/−132 were inserted in a similar fashion between the Nheli and XhoI restriction sites. The rat G-6-Pase gene promoter sequences −230/−187, −230/−162, −230/−137, and −230/−112 and the human G-6-Pase gene promoter sequence −230/−107 were inserted between the Nheli and XhoI sites of pTA-Luc. The fidelity of G-6-Pase gene promoter inserts and of site-directed mutations were confirmed by DNA sequencing.

Transfection. 832/13 cells and HL1C cells were transfected in 12-well tissue culture plates. 832/13 cells were seeded at 1 × 10⁴ cells/well in maintenance medium 1 day before transfection. Transfections were done with 1 μg Firefly Luciferase reporter plasmid, 0.25 μg pHRL-TK plasmid (Promega) as a control for transfection efficiency, and 5 μl Lipofectamine 2000 (Invitrogen) in 0.5 ml DMEM. After 2 h, an additional 0.5 ml of DME was added along with glucose to provide a final concentration of either 2 or 20 mM. HL1C cells were seeded in maintenance medium 1 day before transfection at a concentration to provide ~7 × 10⁴ cells/well at the time of transfection. Transfections were done with polystyrene-coated adenosine (3) purchased from Baylor College of Medicine (Houston, TX). The transfection mixture for one well contained 400 ng firefly luciferase reporter plasmid, with or without 10 ng pcDNA3.1/Zeo(−)GK or pcDNA3.1/Zeo(−) plasmid, 100 ng phRL-TK plasmid, 7 × 10⁴ virus particles, and 0.663 μg polylysine in HEPEP-buffered saline, pH 7.3. An aliquot of 51.7 μl of the transfection mixture was added to wells containing cells covered with 0.5 ml DMEM. After incubation for 2 h at 37°C, an additional 0.5 ml DMEM was added with glucose to obtain a final concentration of 2 or 20 mM. After the cells were cultured for an additional 20 h, they were lysed, and firefly and Renilla luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) in a TD-20/20 luminometer.
(Turner Designs, Sunnyvale, CA). Relative light units (RLU) were calculated as a ratio of firefly luciferase activity and Renilla luciferase activity. Transfection experiments were designed as generalized randomized complete block design with blocks being different transfection experiments, and with each treatment (a transfection mixture plus a certain glucose concentration) given to the experimental units, which were individual wells, with duplicate or triplicate treatments applied for each experiment. For each set of transfection experiments, at least two different sets of plasmid preparations were used. Transfection experiments were analyzed by ANOVA of a generalized randomized complete block design (24) assuming the blocks had random effects. Because the SDs of RLU measurements tended to be proportional with the means, the data were logarithm-transformed before ANOVA. Following the ANOVA, contrasts of reporter activity at 2 mM vs. at 20 mM glucose and contrasts comparing the fold expression (20 vs. 2 mM glucose) for different luciferase reporters were tested by the least-significant difference procedure. Means ± SE were calculated and retransformed back to linear scale for data presentation. Quantitative RT-PCR: Total RNA was isolated from 832/13 cells and primary hepatocytes using TRI Reagent and from pancreatic islets isolated and retransformed back to linear scale for presentation of the data. cDNA synthesis was done as previously described (38). The RNA concentration of which was determined relative to a total RNA standard from 832/13 cells, the RNA concentration of which was determined by optical density at 260 nm. The G-6-Pase transcript were done as previously described (38). The RNA concentration of the G-6-Pase RNA standard was determined by the optical density at 260 nm.

ChREBP antibodies. Polyclonal rabbit antibodies were raised against a synthetic peptide (residues 431–442 of rat ChREBP) that was conjugated to keyhole limpet hemocyanin (Sigma) via the sulfhydryl moiety of a Cys residue added at the end of the ChREBP sequence. Anti-ChREBP antibodies were affinity purified by column chromatography on a peptide-SulfoLink Plus column (Pierce) as previously described (13).

Chromatin immunoprecipitation assays. 832/13 cells, ~90% confluent, were pretreated overnight in medium containing 5 mM glucose and then were treated with medium containing either 2 or 20 mM glucose for 6 h before exposure to 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 0.125 M, and, after 5 min, the samples were washed two times and harvested in cold PBS with protease inhibitors (catalog no. 1183617001; Roche Diagnostics). Cells were collected by centrifugation for 4 min at 2,000 g and suspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). Chromatin immunoprecipitation (ChIP) assays were performed following the Upstate Biotechnology ChIP assay kit protocol (catalog no. 17–295), with slight modifications. Briefly, the cell lysate was sonicated with glass beads to yield 100–1,000 bp genomic DNA fragments. The lysate (2 ml) was precleared with 80 μl of a 50% slurry of protein G-agarose that contained 32 μg sonicated salmon sperm DNA, 80 μg BSA, 160 μg recombinant protein G-agarose suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.05% sodium azide for 30 min at 4°C with agitation. After centrifugation at 1,000 g for 2 min, aliquots of the supernatant were incubated with an antibody directed against ChREBP or with normal rabbit IgG (sc-2027; Santa Cruz Biotechnologies, Santa Cruz, CA) overnight with agitation at 4°C. Immunocomplexes were recovered by incubation with a 50% slurry of salmon sperm DNA/protein G-agarose, in the buffer described above, for 1 h at 4°C. The beads were washed 5 min each with low-salt (catalog no. 20–153), high-salt (catalog no. 20–154), LiCl buffer (catalog no. 20–155), and 10 mM Tris, pH 8.0, 0.5 mM EDTA buffer. The chromatin complexes were eluted by adding freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃) with rotation at room temperature for 30 min. The cross-linking was reversed by adding NaCl to a final concentration of 500 mM and heating at 65°C for 4 h. After incubation with 20 mg protease K for 1 h, the DNA was purified using a Qiagen PCR cleanup column, and target genes were quantified by real-time PCR with SyberGreen (iTaq SyberGreen Supermix with ROX, catalog no. 170–8853; Bio-Rad) using the purified DNA as template. Standard curves were constructed using twofold serial dilutions of the unbound DNA extracted from the 2 mM glucose IgG treatment (2.5 μl) as a reference input. The protein content of the different treatment groups was normalized using the Pierce BCA protein assay before immunoprecipitation, and the quantity of the amplicons were expressed as a percentage of the total reference input. The primer sequences used to amplify the distal glucose-responsive G-6-Pase gene promoter region by PCR were 5'-gcatagcctgttgtaa-3' and 5'-gagttgaggcaacaagagc-3'. The PCR primers for the proximal glucose-responsive G-6-Pase gene promoter region were 5'-aggccaggaagggctc-3' and 5'-gcctcgattgctgacta-3'. The ChIP experiments were analyzed as simple split-plot designs by ANOVA according to Ref. 24, with the two groups of formaldehyde-hyde-treated, sheared chromatin collected from cells grown at either 2 or 20 mM glucose representing whole plots and with the split plots being the two aliquots of the chromatin preparations that were precipitated with the antibody directed against ChREBP or with the control IgG. Separate ANOVAs were calculated for each target that was amplified by PCR. Because the SDs of target concentrations tended to be proportional to the means, the data were logarithm-transformed before the ANOVA. After ANOVA, group means were compared using the least-significant difference procedure. Inferences of the target concentration at 2 vs. 20 mM glucose for a particular antibody were made after calculation of the approximate degrees of freedom by Satterthwaite’s procedure. Means ± SE were calculated and retransformed back to linear scale for presentation of the data.

Computer analysis of promoter regions. Repetitive elements were determined using the web-based RepeatMasker program (A. F. A. Smit, R. Huebly, and P. Green, RepeatMasker Open-3.0. 1996–2004 http://www.repeatmasker.org). Sequence alignment of promoter regions from rat, mouse, and human were done with the web-based lalign program (37) from the GeneStream server (xylian.igh.cnrs.fr). Sequences of the G-6-Pase gene promoter regions from mouse and human were obtained from GenBank accession no. NT_039521 and accession no. NT_010755.15, respectively.

RESULTS

Characterization of the rat G-6-Pase promoter sequence from −4078 to +64. To map the cis-regulatory elements conferring glucose responsiveness to the rat G-6-Pase gene promoter, we cloned fragments of the promoter in the pGL3-Basic vector. The longest of the rat G-6-Pase gene promoter inserts was from −4078 to +64 relative to the transcription start site. The sequence from −1642 to +64 identical to one previously published (6), so the sequence from −4078 to
−1643 is shown in Fig. 1A. The rat and human G-6-Pase genes have several repetitive elements within 4 kb upstream of the transcription start site that do not share homology (Fig. 1B). However, most regions of nonrepetitive DNA in the human sequence have clear homologies in the rat sequence (Fig. 1B). The G-6-Pase gene promoter confers a glucose response in both hepatoma and insulinoma cells. We initially tested the glucose responsiveness of the G-6-Pase promoter in primary rat hepatocytes but found the glucose response to be too variable to allow fine mapping of the cis-regulatory elements involved in the response. We therefore decided to map the cis-regulatory elements using glucose-responsive cell lines.

HL1C cells lack expression of glucokinase, but, when glucokinase is introduced via an adenovirus, glucose increases endogenous rat G-6-Pase mRNA abundance in these cells (9). We found that the glucose response of a G-6-Pase gene promoter-reporter construct (−729/+64 pGL3-Basic) was dependent on cotransfection with the glucokinase expression vector pcDNA3.1/Zeo(−)GK (Fig. 2A). As shown in Fig. 2B, promoter fragments with 5′ ends ranging from −400 to −4078 supported greater luciferase activity, both in low (2 mM) and high (20 mM) glucose concentrations, than did shorter promoter fragments or the empty reporter construct. This shows that the −400/−200 promoter fragment contains elements required for basal G-6-Pase gene promoter activity in HL1C cells. Furthermore, there was a modest glucose response for the rat −400/+64 G-6-Pase gene promoter fragment and longer promoter fragments, and none for the shorter fragments, suggesting that elements required for the glucose response are located between −400 and −200.

Because the human G-6-Pase gene promoter provides a glucose response in INS-1 rat insulinoma cells (41), we tested whether the rat G-6-Pase gene promoter would be glucose responsive in INS-1-derived 832/13 cells. Note that, in INS-1 cells, endogenous glucokinase is constitutively expressed (30). As shown in Fig. 2C, the −400/+64 promoter fragment and longer promoter fragments conferred a robust glucose response, with the promoter being virtually inactive at 2 mM glucose. The −200/+64 and −100/+64 promoter fragments did not confer a significant glucose response, whereas the residual promoter activity of pGL3-Basic was slightly down-regulated by 20 mM glucose. These results show that the −400/−200 promoter fragment is required for a glucose response in 832/13 cells. Furthermore, glucose responsiveness increases when comparing the −1642/+64 and the −4078/+64 promoter fragments, suggesting that the −4078/−1642 promoter fragment contains elements that enhance glucose responsiveness in 832/13 cells. The glucose response in 832/13 cells was not an indirect response caused by insulin released after glucose stimulation, since additional human insulin supplemented in concentrations of up to 10 nM had no effect on expression from the −4078/+64 pGL3-Basic plasmid (data not shown). The fold expression was somewhat variable between experiments (see, e.g., the results for the −4078/+64 insert in Fig. 2C vs. 8/4) because of its calculation as a ratio between a marked promoter activity at 20 mM glucose and a very small promoter activity at 2 mM that was often below the background expression from the empty pGL3-Basic vector.

The human G-6-Pase gene promoter (h-963/+67), which is homologous to the rat −1642/+64 fragment, was not clearly glucose responsive in HL1C cells (Fig. 2B). However, the same human promoter fragment conferred a glucose response in 832/13 cells, although to a lesser degree than the rat G-6-Pase gene promoter (Fig. 2C). This observation confirms previous results from INS-1 cells (41).

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1 The nucleotide sequence of the −4078/−1643 of the rat G-6-Pase gene promoter has been deposited in the GenBank database under GenBank accession no. DQ100345.
The concentration of endogenous G-6-Pase mRNA is rapidly and potently induced (>100-fold after 24 h) by 20 mM glucose in 832/13 insulinoma cells (Fig. 3A). The upregulation occurred principally between 5 and 14 mM glucose, consistent with the $S_{0.5}$ of glucokinase (Ref. 34 and Fig. 3B). Induction of G-6-Pase mRNA by glucose also occurs in rat pancreatic islets, as shown in Fig. 3C, with the concentration of G-6-Pase mRNA being 12-fold higher in 20 mM glucose than in 2 mM glucose. It is important to note that the abundance of G-6-Pase mRNA from islets treated with 20 mM glucose was still ~80-fold lower than in primary hepatocytes freshly isolated from ad libitum-fed rats (data not shown).

Because the G-6-Pase gene promoter provides a more robust glucose response in 832/13 cells than in HL1C cells, our strategy was to identify elements that confer glucose responsiveness in 832/13 cells followed by testing whether these sequences play a similar role in HL1C cells.

An HNF-1 binding site is required, but not sufficient, for the complete glucose response of the G-6-Pase gene promoter. The -400/-200 rat G-6-Pase gene promoter fragment contains two E-boxes, -357/-352 (CAATTTG) and -316/-311 (CAGTGG), of which the former is preserved as an E-box in the human G-6-Pase gene promoter. The -400/-200 fragment also contains a sequence (-226/-212) homologous to mouse and human G-6-Pase gene promoter sequences that bind HNF-1α or HNF-1β (26, 45, 47). This HNF-1 binding site is important for the full promoter response to insulin, cAMP, and glucocorticoids, as well as for basal activity in various cell lines (7, 25, 45, 47, 52). We tested the involvement of these sites in the glucose response by deleting the E-boxes and mutating the HNF-1 binding site (-218/-216 ATT → CGG). In addition, we attempted to narrow down the location of important cis-regulatory elements by testing G-6-Pase gene promoter fragments -350/-64, -300/-64, and -250/-64 for glucose responsiveness.

Figure 4A shows that deletion of the E-boxes had no affect on the glucose response. Consistent with these results, the full glucose response was retained in the -250/64 fragment, which eliminated the E-boxes but retained the HNF-1 site. However, mutation of the HNF-1 binding site abolished the glucose response in 832/13 cells. In HL1C cells, mutation of the HNF-1 site also blocked glucose responsiveness, as well as basal activity, in the context of the -400/-64 G-6-Pase gene promoter construct (Fig. 4B). Thus the HNF-1 binding site is essential for the G-6-Pase glucose response.

To test whether the HNF-1 site is sufficient for a glucose response, we cloned the -230/-208 fragment in one or two copies into pGL3-Promoter and pTA-Luc. The HNF-1 sites did not cause glucose-dependent expression (see Fig. 7A, and data not shown). Likewise, insertion of the -400/-200 fragment in either pGL3-Promoter or pTA-Luc did not confer glucose responsiveness (data not shown). We conclude that the HNF-1 site is necessary, but not sufficient, for the glucose response of the proximal part of the G-6-Pase gene promoter.
A minimal −230/−112 G-6-Pase promoter fragment containing the HNF-1 and two CRE sites is glucose responsive. In 832/13 cells, the −226/+64 promoter fragment inserted in pGL3-Basic conferred a significant glucose response (data not shown), demonstrating that no sequence upstream of the HNF-1 binding site (−226/−212) is absolutely required for a response. Because the HNF-1 binding site was insufficient by itself to confer a glucose response, we searched for elements downstream of the HNF-1 site that promote responsiveness through interaction with the HNF-1 site. We tested the glucose responsiveness of a series of constructs wherein 25 bp were sequentially deleted downstream of the HNF-1 site in the context of the −230/+64 G-6-Pase promoter-reporter construct. Six of the 11 deletion constructs showed significantly reduced or no glucose responsiveness compared with −230/+64 pGL3-Basic (Fig. 5). Two of these, Δ−11/+14 and Δ−36/−12, lack the transcription start site and the TATA box, respectively. This suggests that interfering with the normal transcription initiation of the G-6-Pase gene promoter results with a reduced, albeit not abolished, glucose response. The Δ−211/−187 construct lost the 3′-flanking sequence of the HNF-1 site, which may have caused reduced responsiveness. The deletions of the remaining three constructs with reduced or absent glucose responsiveness (Δ−186/−162, Δ−161/−137,
CRE2, and the HNF-1 site, was fully glucose responsive. When the two CRE sites were mutated in the pase gene promoter in HL1C cells. sponsiveness of the G-6-P required for a glucose response, and CRE2 is required for the completely abolished it (Fig. 6). CRE1 alone, or in combination with the CRE2 mutation, significantly reduced the glucose response, whereas mutation of CTAGA) similar to that of Ref. 47. Mutation of CRE2 signif-

cation of the /H11002 reduced glucose induction compared with /H11001 64 pGL3-Basic. By contrast, insertion of the CGTAAA promoter construct, the glucose response was lost (Fig. 6 C).

We screened for the smallest promoter fragment that in-

del 230/+64 pGL3-Basic, −230/+64 pGL3-Basic, and a number of re-

porters made from −230/+64 pGL3-Basic by deleting a series of 25 bp sequences. Shown are results for 4 experiments with each treatment in duplicate. Statistically significant differences in expression between 2 and 20 mM glucose at the 5% (*), 1% (**), and 0.1% (***) significance level are shown. #, ##, and ### denote differences in fold expression compared with −230/+64 pGL3-Basic at the 5%, 1%, and 0.1% significance level.

and Δ−136/−112) remove sequences that, in the homologous regions of the mouse and human G-6-Pase gene promoters, are important cis-regulatory sequences such as insulin-responsive sequences, CREs, and glucocorticoid response elements (7, 25, 26, 40, 46, 52). Especially intriguing was that the −186/−162 deletion cuts through an upstream CRE at −165/−158 (termed CRE1 in Ref. 47), the −161/−137 deletion cuts through CRE1 and a downstream CRE at −141/−134 (termed CRE2 in Ref. 47), and the −136/−112 deletion cuts through CRE2, suggesting that CREs are important for the glucose response.

We subsequently tested the effects of CRE1 and CRE2 on glucose responsiveness in the context of −230/+64 pGL3-Basic. This was done by mutagenesis of CRE1 (−162/−157 CGTAAAA → AAGCTT) and CRE2 (−139/−135 GCATC → CTAGA) similar to that of Ref. 47. Mutation of CRE2 significantly reduced the glucose response, whereas mutation of CRE1 alone, or in combination with the CRE2 mutation, completely abolished it (Fig. 6A). CRE1 is thus absolutely required for a glucose response, and CRE2 is required for the full response, in the context of −230/+64 pGL3-Basic. We confirmed that these sites are also important for glucose responsiveness of the G-6-Pase gene promoter in HL1C cells. When the two CRE sites were mutated in the −230/+64 promoter construct, the glucose response was lost (Fig. 6B).

We screened for the smallest promoter fragment that in-

cludes the HNF-1 site that would confer glucose responsive-

ness to a heterologous promoter. When the −230/+64 G-6-Pase promoter fragment is inserted upstream of the minimal promoter of the pTA-Luc vector, the resulting luciferase activity increases after treatment with 20 mM glucose in 832/13 cells (Fig. 6C). No glucose-induced expression was seen with the pTA-Luc. By contrast, insertion of the −230/−112 fragment, which contains CRE1, CRE2, and the HNF-1 site, was fully glucose responsive.

Fig. 5. Elements downstream of the HNF-1 binding site are involved in the glucose responsiveness of the rat G-6-Pase gene promoter. 832/13 cells were transfected with pGL3-Basic, −230/+64 pGL3-Basic, and a number of reporters made from −230/+64 pGL3-Basic by deleting a series of 25 bp sequences. Shown are results for 4 experiments with each treatment in duplicate. Statistically significant differences in expression between 2 and 20 mM glucose at the 5%, 1%, and 0.1% significance level are shown. #, ##, and ### denote differences in fold expression compared with −230/+64 pGL3-Basic at the 5%, 1%, and 0.1% significance level.

Fig. 6. A minimal glucose-responsive G-6-Pase gene promoter fragment contains two cAMP response elements (CREs). A: 832/13 cells were transfected with pGL3-Basic, −230/+64 pGL3-Basic, or 230/+64 pGL3-Basic plasmids in which CRE1 or CRE2 were mutated. Shown are results for 4 experiments with each treatment in duplicate. B: HL1C cells were transfected with pGL3-Basic, −230/+64 pGL3-Basic, or 230/+64 pGL3-Basic wherein CRE1 and CRE2 were mutated. A glucokinase expression plasmid was cotransfected. Shown are results for 4 experiments with each treatment in triplicate. C: 832/13 cells were transfected with pTA-Luc and pTA-Luc containing fragments of the G-6-Pase promoter. Shown are results for 4 experiments with each treatment in duplicate. Statistically significant differences in expression between 2 and 20 mM glucose at the 5% (*), 1% (**), and 0.1% (###) significance level are shown. Differences in the fold expression compared with the plasmids containing the full −230/+64 rat G-6-Pase fragment at the 5% (#), 1% (##), and 0.1% (###) significance level are also shown.

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A combination of the HNF-1 binding site and the CREs is sufficient for a glucose response in a heterologous promoter context. We tested whether a combination of the HNF-1 binding site and the CREs from the rat G-6-Pase gene promoter would be sufficient for conferring a glucose response to the minimal promoter of pTA-Luc. Two copies of the HNF-1 binding site (−230/−208) were inserted between the SacI and NheI restriction sites. In addition, multiple copies of the CRE1 region (−167/−156) and/or CRE2 region (−143/−132) were inserted between the NheI and XhoI restriction sites. As shown in Fig. 7A, the combination of multiple HNF-1 binding sites and CREs promotes a glucose response in 832/13 cells. The construct with four CRE1 sequences promote a stronger glucose response and stronger basal expression than that with four CRE2 sequences. The strongest response was seen with the plasmid that contained two copies of the HNF-1 binding site and six copies of the CRE1 region. This plasmid also exhibited significant glucose-dependent Luciferase expression in HL1C cells (Fig. 7B). Finally, when four copies of the CRE 1 region were inserted in pTA-Luc, in the absence of an HNF-1 site, the resulting plasmid gave a glucose response in 832/13 cells (Fig. 7C). Thus multiple copies of a CRE sequence are sufficient for a glucose response in 832/13 cells.

The upstream G-6-Pase gene promoter region contains a ChoRE. We tested whether the −3702/−3686 rat G-6-Pase gene promoter sequence, CATATG-CTGAG-CATAG, consisting of two E-boxes separated by five base pairs, functions as a ChoRE in 832/13 cells. Deletion of the −3702/−3686 sequence from −4078/+64 pGL3-Basic resulted in a significant decrease in glucose responsiveness (Fig. 8A), showing its involvement in the total response. By comparison, deletion of the adjacent −3699/−3620 sequence, which is completely conserved between the rat and human promoters, had no significant effect on glucose responsiveness (data not shown). Mutation of the HNF-1 binding site (−218/−216 ATT → CGC) in −4078/+64 pGL3-Basic led to a drastic reduction in luciferase expression, but the residual activity was still significantly upregulated by glucose (Fig. 8A). This residual responsiveness was further decreased when the −3702/−3686 fragment was deleted in addition to the HNF-1 site mutation (Fig. 8A, bottom).

One or two copies of the −3706/−3682 G-6-Pase gene promoter fragment were inserted in pGL3-Promoter or pTA-Luc to determine if the fragment conferred a glucose response on the heterologous promoters of these vectors. As negative controls we used the vectors without inserts and vectors that contained a 50-bp scrambled sequence of the same nucleotide composition as two copies of the −3706/−3682 sequence. As a positive control, we used plasmids containing two copies of the rat ACCpI sequence −126/−102, known to contain the strongest ChoRE among three tested in hepatocytes (35). The results in Fig. 8, B and C, show that two copies of the G-6-Pase sequence conferred a highly significant glucose response, although not of the same magnitude as the ACCpI ChoRE. Even one copy of the G-6-Pase ChoRE-like sequence was sufficient to provide a significant glucose response in the context of pGL3-Promoter. We conclude that the −3702/−3686 rat G-6-Pase gene promoter sequence functions as a ChoRE in 832/13 cells.

We did not observe a glucose effect of the G-6-Pase ChoRE in HL1C cells in transient transfection experiments, nor did we observe a response from the potent ACCpI ChoRE (data not shown). This suggests that transcriptional enhancement from ChoREs is muted in HL1C cells under these experimental conditions.

Glucose responsiveness of the human G-6-Pase gene promoter. The proximal glucose-responsive region (−230/−112) and the distal ChoRE (−3702/−3686) of the rat G-6-Pase gene promoter occur in regions that have homology to the human G-6-Pase gene promoter. We investigated whether the homologous regions of the human promoter are involved in a glucose response.

We first tested whether the HNF-1 binding site and the two CREs are involved in the glucose response of the human G-6-Pase gene promoter. In the context of the human −963/
Fig. 8. The −3702/−3686 sequence functions as a ChoRE in 832/13 cells. A: 832/13 cells were transfected with Luciferase reporters containing the −4078/+64 fragment of the rat G-6-Pase gene promoter or −4078/+64 fragments wherein the −3702/−3686 sequence was deleted or the HNF-1 binding site was mutated. Both panels show results for 4 experiments with each treatment in duplicate. \( \text{A-CREB} \) and \( \text{A-C/EBP} \) were included in transfection experiments and compared with transfections with the empty expression plasmid \( \text{pcDNA3.1} \). **B**. 832/13 cells were transfected with pGL3-Promoter or pGL3-Promoter with one or two copies of the ChoRE-like sequence from the G-6-Pase gene promoter, two copies of the promoter PI of acetyl-coenzyme A carboxylase (ACCP1) ChoRE, or a scrambled sequence with the same nucleotide composition as two copies of the ChoRE-like sequence. Shown are results for 4 experiments with each treatment in duplicate. C: 832/13 cells were transfected with pTA-Luc or pTA-Luc with one or two copies of the ChoRE-like sequence from the G-6-Pase gene promoter, two copies of the ACCP1 ChoRE, or a scrambled sequence with the same nucleotide composition as two copies of the ChoRE-like sequence. Shown are results for 4 experiments with each treatment in duplicate. Statistically significant differences in fold expression between 2 and 20 mM glucose at the 5% (*), 1% (**), and 0.1% (###) significance level are shown. Statistically significant differences in fold expression at the 5% (#), 1% (##), and 0.1% (###) significance level are also shown.

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+67 G-6-Pase gene promoter fragment, we mutated CRE1 (−157/−152 CGTAAA → AAGCTT) and CRE2 (−134/−130 GCATC → CTAGA). In addition, two mutations of the HNF-1 site were introduced. Mutation m1 (−226/−221 AGT−TAAGCCGCGC) is similar to that of Ref. 26, and mutation m2 (−218/−216 ATT → CGC) is similar to the HNF-1 site mutation of the rat G-6-Pase gene promoter. The results in Fig. 9A show that, although the mutated fragments all conferred a modest glucose response, the degree of upregulation was significantly reduced when compared with the wild-type promoter sequence. This indicates that the HNF-1 binding site, CRE1, and CRE2 are all required for the full glucose response of the human G-6-Pase gene promoter. We further confirmed that the human −230/−107 promoter fragment, which is homologous to the rat −230/−112 promoter fragment, is capable of conferring glucose responsiveness to the minimal promoter of the pTA-Luc vector (Fig. 9B).

We compared the glucose response of the rat G-6-Pase ChoRE with the homologous sequences of the mouse and human G-6-Pase gene promoters listed in Fig. 9C. Although both mouse and human sequences contain the typical ChoRE pattern of two E-box-like sequences separated by 5 bp, only the 3′-most putative E-box of the mouse sequence is a consensus E-box (CANNTG), and only the 5′-most putative E-box is a consensus E-box in the human sequence. The results of Fig. 9D show that the mouse sequence can be classified as a ChoRE, although not as potent as the rat ChoRE, since it confers glucose responsiveness to the promoter of pTA-Luc. By contrast, the human sequence is not a ChoRE, or at best is a very weak ChoRE.

ChoREBP binds to the distal promoter region in a glucose-dependent manner: ChoREBP has recently been described as the transcription factor that is responsible for glucose-activated transcription from a ChoRE (16, 17, 29, 44, 53, 54). We tested whether ChoREBP binds to the rat G-6-Pase gene promoter in 832/13 cells in situ using a quantitative ChIP assay that amplified the −3793/−3580 or −274/−63 promoter fragments after immunoprecipitation of chromatin with an antibody directed against ChoREBP. Whereas the downstream G-6-Pase gene promoter region did not appear to bind ChoREBP, there was significant glucose-dependent binding of ChoREBP to the upstream G-6-Pase gene promoter containing the ChoRE (Fig. 10).
response of the −400/+64 fragment of the rat G-6-Pase promoter in a dose-dependent manner. A similar result was obtained for a reporter plasmid containing four copies of the G-6-Pase CRE1 region (−167/−156) upstream of the minimal promoter of pTA-Luc (data not shown). In contrast, Fig. 11, bottom, shows that the A-CREB and A-C/EBP expression plasmids had no effect on the glucose response on the pTA-Luc-derived plasmid containing two copies of the ChoRE from the rat G-6-Pase gene promoter or two copies of the homologous sequences from mouse or human. Shown are results for 4 experiments with each treatment in duplicate. Statistically significant differences in expression between 2 and 20 mM glucose at the 5% (*), 1% (**), and 0.1% (***) significance level are shown. Differences in the fold expression compared with the plasmid with the full −963/+67 human G-6-Pase gene promoter fragment at the 5% (#), 1% (##), and 0.1% (###) significance level are also shown.

DISCUSSION

In this work, we have demonstrated that the rat G-6-Pase gene promoter is glucose responsive. The glucose response in HL1C hepatoma cells is relatively modest and requires coexpression of glucokinase. The glucose response in 832/13 insulinoma cells is remarkably robust, as is the induction of endogenous G-6-Pase mRNA by glucose. We have shown that the rat G-6-Pase gene promoter contains two distinct regions that respond to glucose in 832/13 cells by different mechanisms.

A distal glucose-responsive region is a typical ChoRE: the −3793/−3580 sequence consists of two E-boxes (CANNTG) separated by five base pairs, and its deletion from −4078/+64 pGL3-Basic reduced the glucose induction approximately threefold and conferred glucose responsiveness to heterologous promoters. The E-box half-site CACG has previously been
described as being important for the function of a ChoRE (35). The rat G-6-Pase ChoRE does not contain a CACG motif. However, it is a near-perfect palindrome that contains the sequence 5'-CATGTG-3', which is also part of the ChoREs of the rat ACCpI and the rat FAS promoter (35, 39). The sequences in the mouse and the human G-6-Pase gene promoters that are homologous to the rat G-6-Pase ChoRE each have only one consensus E-Box. The mouse sequence, which retains the CATGTG motif, also functioned as a ChoRE. The human sequence, which does not, at most functioned as a very weak ChoRE. This further suggests that the CATGTG motif is important for the function of the G-6-Pase ChoRE.

The mechanism by which glucose-responsive transcriptional activation occurs from a ChoRE has lately been described (16, 17, 29, 44, 54). Increased glucose flux leads to dephosphorylation of the cytoplasmic ChREBP transcription factor, which allows nuclear localization and increased binding of ChREBP together with its dimerization partner Mix to the ChoRE with ensuing activation of target genes. The G-6-Pase ChoRE might work by such a mechanism, since we have observed binding of ChREBP in 832/13 cells in situ to the distal glucose-responsive region of the promoter in a glucose-dependent manner. It has been reported that the mRNA and the transcription rate of ChREBP are upregulated by glucose in INS-1 cells (53). However, preliminary experiments indicated that the binding of ChREBP to the G-6-Pase gene promoter occurs rapidly, within 15 min, after exposure to 20 mM glucose (P. Zhang, and D. K. Scott, unpublished observations). Thus increased binding of ChREBP to the G-6-Pase ChoRE is not likely to be due simply to an increased concentration of ChREBP protein in these cells.

A proximal glucose-responsive region (−230/−112) provides a glucose response to a heterologous minimal promoter in 832/13 cells. There are no obvious ChoREs, nor any E-boxes in this region. Within this region, an HNF-1 binding site is necessary, but not sufficient, for glucose responsiveness. Therefore, it can be described as an accessory factor for the glucose response. The HNF-1 binding site plays a similar role for the response of the mouse or human G-6-Pase gene promoter to insulin, cAMP, and glucocorticoids (25, 45, 47).

Two CREs are surprisingly also required for the full glucose response. These CREs are completely conserved between mouse, rat, and human G-6-Pase gene promoters. In the context of the −200/+64 pGL3/Basic construct, the CREs are not sufficient for a response. The glucose response thus seems to involve a glucose-dependent interplay of transcription factors binding to the HNF-1 and CRE sites. Indeed, a combination of multiple CREs and HNF-1 binding sites from the rat G-6-Pase gene promoter is sufficient for conferring glucose responsiveness to the promoter of the pTA-Luc vector in both 832/13 and HL1C cells. Furthermore, multiple copies of the CRE1 region are sufficient for conferring a glucose response to the promoter of the pTA-Luc vector in 832/13 cells. We therefore suggest that the transcription factors activating the proximal G-6-Pase gene promoter in a glucose-dependent manner interact with the CREs of the promoter. The CRE1 site is a bona fide CRE, since it can confer responsiveness to cAMP and protein kinase A to a heterologous promoter and allow in vitro binding of the CREB transcription factor (40, 47). It may also be involved in glucocorticoid responsiveness and basal activity of the promoter (40), and it overlaps an insulin-responsive sequence and a glucocorticoid response element (7, 52). The CRE2 site overlaps binding motifs for HNF-3 and C/EBP, and gel shift assays and in vitro footprinting have revealed binding of CREB, C/EBP-α, C/EBP-β, HNF-3β, HNF-3γ, and the forkhead transcription factor FKHR to the region of CRE2 (26, 52). Recently, C/EBP-α and C/EBP-β have been shown to be constitutively bound to the rat G-6-Pase gene promoter in rat FAO hepatoma cells in situ, whereas CREB binds after induction with forskolin (11). A potential candidate for mediating a glucose response through the CREs in insulinoma cells is CREB which is activated by glucose in INS-1 cells (51). Indeed, we found that CREB or a closely related family member, as well as a member of the C/EBP family of transcription factors, are required for the full glucose responsiveness.
In HL1C cells, the G-6-Pase gene promoter is glucose responsive through the proximal glucose-responsive region. The CREs and the HNF-1 binding site are also required elements for the response in this cell line. However, there was no clear glucose response from the G-6-Pase ChoRE sequence, and the potent ChoRE of ACC1 also failed to confer glucose responsiveness in transient transfection experiments in this cell line. It is thus apparent that HL1C cells do not elicit a strong glucose-mediated response from traditional ChoREs, at least not under the employed experimental conditions. A recent report indicates that Mix is required for the full glucose response of the G-6-Pase gene in primary rat hepatocytes (28). This observation provides evidence to suggest that the ChREBP-Mix dimer acts through a ChoRE, with the distal ChoRE identified here as a likely cis-regulatory element.

To our knowledge, this is the first report of glucose-responsive regions in the rat G-6-Pase gene promoter, although the human G-6-Pase gene promoter has previously been described as glucose responsive. In the rat insulinoma cell line INS-1, luciferase reporter constructs with −1227/+57 and −161/+4 sequences of the human G-6-Pase gene promoter were reported to exhibit glucose responsiveness (41). Consistent with these results, we observed glucose activation from the −963/+67 fragment of the human G-6-Pase gene promoter in 832/13 cells and found that the HNF-1 binding site, the CRE1, and the CRE2 are required for full glucose responsiveness. The human G-6-Pase gene promoter was also found to be glucose responsive in the human enterocyte cell line Caco-2/TC7 and the human hepatoma cell line HepG2 (8), but different promoter regions seemed involved. In HepG2 cells, the −299/+57 region was sufficient for a glucose response, but in Caco-2/TC7 cells, elements between −299 and −1227 were required. A binding site for the transcription factor aryl receptor nuclear translocator (ARNT) was required for the full response, and a decrease in the concentration of ARNT protein by RNA interference diminished the glucose response (8). The ARNT binding site, as well as three other regions for putative transcription factor-binding sites between −299 and −1227 of the human promoter, are located in Alu elements with no clear homology in the −4078/+64 rat G-6-Pase gene promoter. Thus there appears to be both cell-line and species-specific differences with regard to the glucose-responsive regions of the G-6-Pase gene promoter.

Upregulation of G-6-Pase by glucose in hepatocytes counteracts the effects of insulin in the fed state. Metabolic control analysis of gluconeogenesis in rat hepatocytes indicated that G-6-Pase has a very low flux control coefficient (12). The induction of G-6-Pase by glucose may thus have a limited effect on the gluconeogenesis rate. On the other hand, an increase in the G-6-Pase activity causes a noticeable decrease in the concentration of glucose 6-phosphate and in the rates of glycolysis and glycogen synthesis in rat hepatocytes (2). The authors of the latter study hypothesized that the main regulatory function of G-6-Pase is to buffer the glucose 6-phosphate concentration and that the induction by glucose is a compensatory mechanism. This could limit the glycogen storage in the postprandial period, as has been previously suggested (5). Consistent with this view, among the metabolic changes of rats with a 1.6- to 3-fold increase in hepatic G-6-Pase activity, there was a significant decrease in hepatic glycogen content (50).

We observed that glucose induces G-6-Pase mRNA in rat islets. This glucose induction may explain the increased amounts of G-6-Pase expression in islets from diabetic and/or obese rodents (19, 23, 48). Glucose-mediated release of insulin from pancreatic β-cells occurs through glucose metabolism and an increase in the ATP-to-ADP ratio (reviewed in Ref. 14). If G-6-Pase were sufficiently overexpressed, increased cycling between glucose and glucose 6-phosphate with concomitant hydrolysis of ATP as well as a decrease in glycolytic flux would be expected to result in decreased insulin secretion. This has been demonstrated in INS-1 cells overexpressing the G-6-Pase via recombinant adenovirus (49). It has also been reported that pancreatic islets from hyperglycemic ob/ob mice show increased glucose cycling (18). However, even after treatment with 20 mM glucose, the concentration of G-6-Pase mRNA in rat islets remains ~80-fold lower than the concentration that we have observed in freshly isolated hepatocytes from ad libitum-fed male Wistar rats (data not shown). It is thus questionable whether the induction of G-6-Pase by glucose is sufficient to acutely affect insulin secretion or other aspects of β-cell physiology.

It is remarkable that G-6-Pase mRNA is induced by glucose in several cell types of human and rodent origin and by several different mechanisms, suggesting an important physiological role for this glucose induction. From the present study, we conclude that the rat G-6-Pase gene promoter responds to glucose through multiple cis-regulatory elements.

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