

A Single-Nucleotide Polymorphism in a Methylatable Foxa2 Binding Site of the *G6PC2* Promoter Is Associated With Insulin Secretion In Vivo and Increased Promoter Activity In Vitro

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OBJECTIVE—The *G6PC2* gene encoding islet-specific glucose-6-phosphatase related protein (IGRP) has a common promoter variant, rs573225 (–231G/A), located within a Foxa binding site. We tested the *cis*-regulatory effects of rs573225 on promoter activity and its association with insulin response to oral glucose.

RESEARCH DESIGN AND METHODS—Functional effects of rs573225 were explored in transfected INS-1 and HIT-T β -cell lines. A total of 734 young obese subjects of European ancestry were genotyped for rs573225. Insulin and glucose levels were measured in response to oral glucose, and the insulinogenic index (IGI) of insulin secretion was calculated.

RESULTS—In vitro, the G allele showed a higher affinity for binding Foxa2 transcription factor and increased *G6PC2* promoter activity. Foxa2 binding is modified if the C adjacent to the G allele is methylated. IGI was associated with rs573225 by linear regression analysis and was 30% greater in AA or AG than in GG obese children. rs573225 was also associated with fasting glucose.

CONCLUSIONS—rs573225 is a functional *cis*-regulatory (epi)-single-nucleotide polymorphism (SNP) of *G6PC2* associated with glucose-insulin homeostasis in obese children, likely to explain the results of recent genome-wide association studies in nondiabetic adults. *Diabetes* 58:489–492, 2009

The flux through glycolysis is the sensed process that makes β -cells glucose responsive. Thus, altered kinetics of glucose-induced insulin secretion could result from a changed intrinsic activity, cellular level, or allosteric regulation of several enzymes including glucokinase, phospho-fructokinase, *G6PC2* (glucose-6-phosphatase, catalytic, 2), or others involved in the regulation of cellular glucose-6-phosphate (G6P) content. Whereas glucokinase, a high K_m enzyme, phosphorylates glucose into G6P in β -cells, *G6PC2*, a low K_m enzyme, catalyzes G6P dephosphorylation (1), which could antagonize insulin secretion (2). In mice, *G6PC2* has only ~5% of the activity of liver *G6Pase* (1), sufficient

however for the knockout of *G6PC2* to result in a small decrease in glycemia (3). The human *G6PC2* gene is located in 2q24 in a region that we found linked to glycemia (4). The *G6PC2* promoter is inactive in HepG2 cells but has 150-fold more activity in HIT-T15 β -cells, due to the region located between –306 and +3 (5). This proximal promoter region regulates *G6PC2* expression in transiently transfected β TC-3, HIT-T15, and Min6 cells through the binding of Foxa2 and MafA transcription factors (6). We found three single-nucleotide polymorphisms (SNPs) in the *G6PC2* promoter in public banks, among which we selected rs573225, a G/A variant located at position –231. Our choice was based on the fact that this variant is common among European populations and belongs to a binding site for Foxa transcription factors (5,7) that carries a potentially methylatable CG motif created by the G allele. The Foxa proteins, previously designated HNF-3, are known to modulate the expression of pancreatic genes involved in glucose homeostasis (8–11).

We performed a functional analysis of rs573225 in β -cell lines and found that this promoter SNP binds Foxa2 with variable affinity and can act as a transcriptional regulator and then explored whether rs573225 genotypes were associated with insulin responses to oral glucose in 734 obese children of European ancestry. Recently, two independent genome-wide association studies revealed that SNPs in linkage disequilibrium with rs573225, but with no known function, were associated with fasting glucose levels in nondiabetic adults (12,13). We thus suspect rs573225 to be the causative variant for the observed associations.

RESEARCH DESIGN AND METHODS

Methylation-specific PCR. Human pancreatic DNAs were obtained from human islets provided by T. Berney; 1 μ g was fragmented with *Xba*I and denaturated by incubation in 3 mol/l NaOH for 15 min at 37°C. Bisulfite conversion was carried out in a solution of sodium bisulfite and hydroquinone at 55°C overnight. After desalting, DNAs were desulfonated by incubation in NaOH at 37°C for 15 min, the solution was neutralized with Na-acetate, and DNAs were ethanol precipitated. Pellets were dissolved in distilled H₂O, and DNA aliquots were used for PCR amplifications by *Taq*DNA polymerase (Invitrogen) under the following conditions: 94°C, 5 min; 45 amplification cycles (94°C, 30 s; 50°C, 30 s; 72°C, 30 s); and finally at 72°C, 10 min. The PCR products were purified using QIAquick kit (Qiagen) and used for automated sequencing (DNA Sequencer, Roche).

Electrophoretic mobility shift assay. Nuclear extracts were prepared from HepG2 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Binding reactions used binding buffer with 1 μ l dIdC (Pierce). A total of 4 μ g of the protein extracts was used per gel mobility shift sample. Double-stranded oligonucleotides used to detect protein binding on the *G6PC2*-231 element were end-labeled with biotin (Operon). The double-strand oligonucleotides were “G” probe, 5'-biotin-AATTTC AAGCAAACATGATC

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CAACTGTT-3'; "A" probe, 5'-biotin-AATTTCAAACAACATGATCCAAGTT-3'; and "GC^m" probe, 5'-biotin-AATTTCAAAGC^mAAACATGATCCA ACTGTT-3'. Samples were subjected to electrophoretic separation on a 6% nondenaturing polyacrylamide gel. The gel was transferred to a Nylon membrane (Pierce), which was cross-linked using a UV-Light cross linker instrument (Bio-Rad) and incubated with Nucleic Acid Detection buffer (Pierce). Signals were visualized using Chemiluminescent Substrate.

For supershift assays, binding reactions were incubated with 1 μ l of antibodies (Foxa 1, 2, or 3; Santa Cruz) before addition of biotin probes. DNA protein complexes were electrophoresed through 6% nondenaturing polyacrylamide gel and revealed with the Nucleic Acid Detection Buffer.

Cell cultures. INS-1 cells derived from a rat insulinoma cell line developed and propagated at the Division of Biochimie Clinique (C.B. Wollheim) were cultured in RPMI-1640 media (GIBCO) with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical), 50 μ mol/l 2-mercaptoethanol, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. HIT-T15 cells were cultured in the same media.

Transfections. We amplified the proximal promoter (-306 + 14 bp) segment of the human *G6PC2* carrying either -231G or -231A allele. We inserted each form into the pGl3 basic vector plasmid (Promega). Sequencing confirmed that constructs differed uniquely at the -231 position. A total of 100,000 INS-1 or HIT-T15 cells were plated on 24-well plates in RPMI-1640 (GIBCO) and cotransfected with vector plasmids: 200 ng pGl3-231G or -231A per well and 70 ng pRL-TK plasmid (Promega) per well using Fugene HD (Roche). Luciferase activities were determined 24 h after transfection using dual-luciferase reporter assays (Promega) using a Centro LB 960 luminometer (Berthold).

Cohort. The 734 studied obese children were recruited in the pediatric endocrinology department of Saint Vincent de Paul Hospital as reported (4). All were of Western European ancestry assessed by family history and grandparents' birthplace. Inclusion criteria were a BMI reaching the 95th percentile at time of study, exceeding the 85th percentile before 6 years of age, and a monotonic weight curve from birth to study time. After 12 h of overnight fasting after 3 days of a standardized isocaloric diet, the oral glucose tolerance test (OGTT) consisted of 1.75 g/kg glucose in 200 ml lemon-flavored water at 10°C. Plasma insulin concentrations were measured in duplicate (14) at 0, 30, 60, 90, and 120 min. The insulinogenic index (IGI) was calculated as $[\text{Ins}_{30} - \text{Ins}_0 \text{ (pmol/l)}] / [\text{Gly}_{30} - \text{Gly}_0 \text{ (mmol/l)}]$, and homeostasis model assessment (HOMA)- β was calculated as $[20 \times \text{Ins}_0 \text{ (pmol/l)}] / [\text{Gly}_0 \text{ (mmol/l)}]$ (14).

Genotyping. The rs573225 polymorphism was detected by fluorescent hybridization probe melting curves using real-time PCR. Samples were run on a Roche LightCycler380; data were analyzed using LightCycler Software 3.5 (10 min denaturation; 40 cycles at 95°C, 1 s; 50°C, 5 s; 72°C, 15 s). Primer sequences are available on request. Three internal controls made of samples that were previously genotyped and sequenced were systematically introduced into each 96-well plate.

Statistical analysis. Values are reported as means \pm SD. For all tests, a *P* value <0.05 was considered significant. General linear regression models were used to assess the relation between rs573225 and glucose or insulin values. Regression model coefficients were determined to be significant with the use of the standard *t* test. For glycemia and IGI analyses, the covariates included age and BMI (15). Normality of residuals was systematically verified, and robustness analyses were performed by dropping outliers from analyses. For in vitro analyses, a *t* test was used to compare luciferase activity in the genotypic groups for each cell type. Data analyses used the *R* statistical software.

RESULTS

In vitro. We found that rs573225 alleles modified the binding of Foxa to *G6PC2* promoter by comparing the binding of nuclear proteins from HepG2 cells to probes carrying the "G" or the "A" allele or to a "GC^m" probe (Figs. 1 and 2). Electrophoretic mobility shift assay experiments revealed that the G probe showed a specific binding to HepG2 cell nuclear protein complex, whereas the A probe could not bind this complex and the GC^m probe bound this complex with a lower affinity (Fig. 2A). The presence of Foxa2 proteins in the DNA-protein complex was confirmed by gel supershift assay experiments (Fig. 2B). These observations indicate that rs573225 alleles create a functional variation in a Foxa2 binding motif, with an added effect of the methylation of the cytosine residue adjacent to the G allele of rs573225. When the proximal

Sequence: AATTTCAA GCAAACATGATCCAA

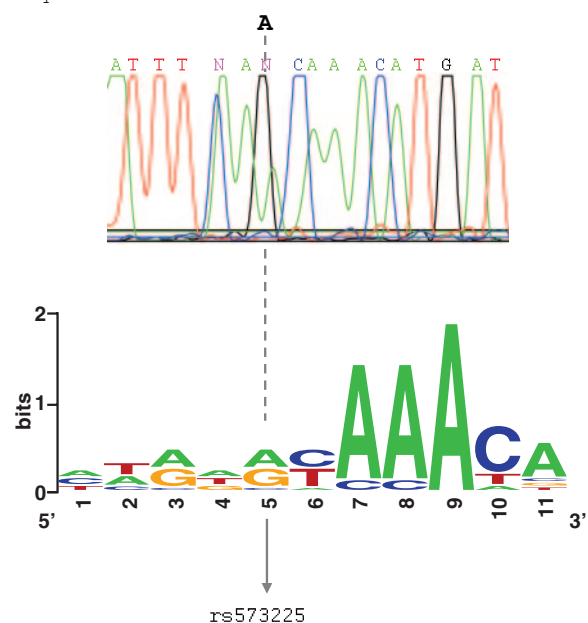


FIG. 1. Representative standard sequencing diagram for bisulfite direct sequencing of *G6PC2* gene promoter. Human pancreatic DNA provided from a rs573225 heterozygote is shown. Signals of false conversion were not observed. All cytosines in this sequence were not converted to uracil. The *G6PC2* gene promoter region was methylated. On the bottom, the Foxa consensus site in humans is shown. (Please see <http://dx.doi.org/10.2337/db08-0587> for a high-quality digital representation of this image.)

segment of *G6PC2* gene promoter bearing rs573225 and placed upstream of the luciferase gene in the pGl3 basic vector was transfected into INS-1 or HIT-T15 cells, the G allele was consistently associated with a higher promoter activity than the A allele (Fig. 2C).

In vivo. Our hypothesis that the candidate rs573225 variant was associated with parameters of insulin-glucose homeostasis was supported by several findings (Table 1). Linear regression analysis found that rs573225 was associated with IGI ($P = 0.0191$ under a recessive model and $P = 0.0102$ under an additive model). Indeed, IGI was $\sim 30\%$ greater in these obese children with AA (36.8 ± 38.1 mmol/l) or AG (33.5 ± 24.2 mmol/l) genotypes compared with GG homozygotes (21 ± 16.9 mmol/l). Insulin levels showed no significant differences *sensu stricto*, but showed a trend for higher insulin levels in obese children of AA or AG genotype ($P = 0.058$ for Ins0 and $P = 0.059$ for Ins30). In addition, linear regression analysis found that rs573225 was associated with fasting glucose ($P = 0.032$ under a recessive model and $P = 0.037$ under an additive model) with higher glucose levels in obese children with AA or AG alleles.

DISCUSSION

When studied during their dynamic period of fat accumulation, obese children have glucose-insulin homeostasis yet are unaltered by dietary restriction or drugs, thus allowing bona fide genotype-phenotype studies. This population has the additional advantage of having normal fasting glucose levels, which avoids the interference of glucotoxicity with the studied β -cell parameters. Obese children oversecrete insulin in response to mounting insulin resistance; thus, we could explore genotypic effects over a wide range of insulin levels. Being aware that insulin and glucose levels collected during OGTTs are not

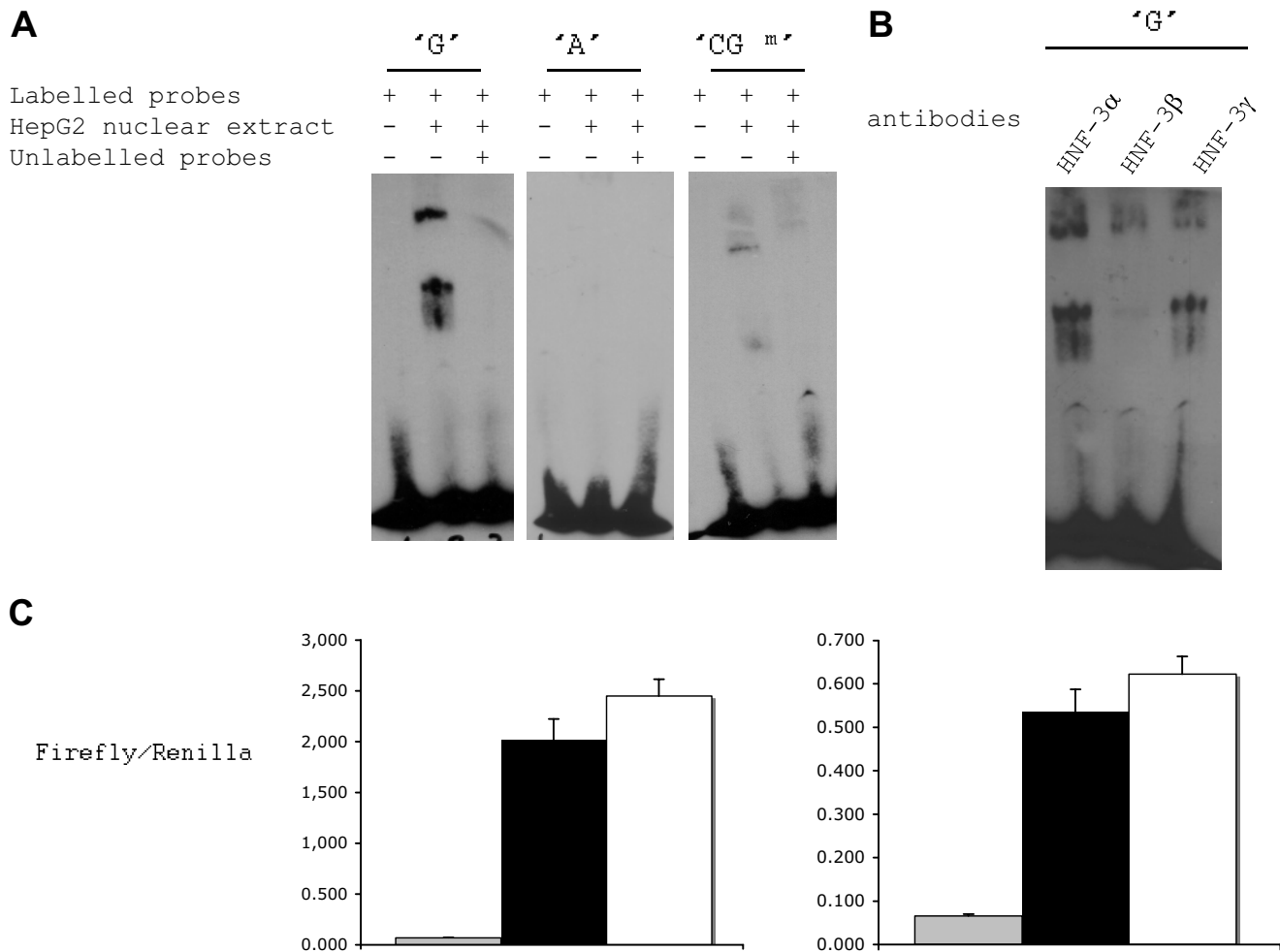


FIG. 2. The G allele of the rs573225 ($-231G/A$) binds Foxa2 and is associated with increased transcriptional activity of the *G6PC2* gene. **A:** Electrophoretic mobility shift assay (EMSA) carried out with HepG2 nuclear extracts and unmethylated G probe or A probe and methylated CG^m probe. The oligonucleotide sequence includes the *G6PC2* promoter region -239 to -267 . **B:** Specific antibodies was added in the gel supershift assay and carried out with the G probe only. **C:** Mutation of the rs573225 *G6PC2* promoter variant reduces *G6PC2* transcriptional activity in HIT-T15 cells (*left*) and in INS-1 cells (*right*). For each cell type, the following is shown: in gray, luciferase activity in pG13 basic vector without *G6PC2* promoter ($n = 24$); in black, luciferase activity in pG13 basic vector with *G6PC2* promoter bearing the $-231A$ allele ($n = 24$); and in white, luciferase activity in pG13 basic vector with *G6PC2* promoter bearing the $-231G$ allele ($n = 24$). Data are means \pm SD. $P = 2.55 \times 10^{-7}$ $-231G$ allele vs. $-231A$ allele, after Bonferroni correction.

TABLE 1

Clinical and biological characteristics of insulin secretion in our young obese Caucasian cohort, according to rs573225 variant

	G/G	G/A	A/A
<i>n</i>	60	293	381
Age (years)	12.4 \pm 2.8	11.8 \pm 3	11.7 \pm 2.8
BMI (kg/m ²)	30.7 \pm 5.6	29.7 \pm 5.3	29.6 \pm 5.5
Fasting glucose (mmol/l)	4.41 \pm 0.51*	4.53 \pm 0.53	4.56 \pm 0.57
Glycemia at 30 min (mmol/l)	7.47 \pm 1.22	7.67 \pm 1.23	7.53 \pm 1.3
Fasting insulin (μ U/ml)	15 \pm 7.7	15.7 \pm 8.7	15.9 \pm 9.9
Insulin at 30 min (μ U/ml)	87 \pm 52	109 \pm 77	106 \pm 85
IGI	21 \pm 16.9 \dagger	33.5 \pm 24.2	36.8 \pm 38.1
HOMA- β	68.5 \pm 34.1	69.6 \pm 38.8	69.9 \pm 41.4

Data are means \pm SD. *P* values under a recessive model: **P* = 0.032 and *P* = 0.037 under an additive model, $\dagger P$ = 0.0191 under a recessive model, and *P* = 0.0102 under an additive model.

entirely replicable in the same obese child (16), we minimized stress and diet variations in controlled in-hospital conditions. These strict criteria do not allow the study of the thousands of subjects necessary to well-powered association studies. We therefore consider our results as tentative and needing further replication. Already, two genome-wide association studies have reported the association of *G6PC2* variants and fasting glycemia. In 9,353 normoglycemic subjects, Bouatia-Naji et al. (13) found an association between fasting glycemia and rs560887. The magnitude of the genotypic effect on glycemia was only 0.09 mmol/l, explaining why thousands of subjects were necessary to reach significance. Chen et al. (12) found a significant association between rs563694 and fasting glucose in 5,088 nondiabetic individuals from Finland and Sardinia and a *P* value of 5.7×10^{-7} for rs573225. Because our rs573225 is in almost complete linkage disequilibrium with rs560887 ($r^2 = 0.96$) and with rs563694 ($r^2 = 0.95$) (according to the HapMap CEU reference panel), it is likely that these three SNPs represent the same association signal. Bouatia-Naji et al. (13) found an association with the HOMA- β index of insulin secretion derived from

fasting insulin and glucose values, whereas our study found only an association of rs573225 with IGI, an index derived from OGTT responses, not with HOMA- β . IGI is an index of insulin secretion that is based on the first-phase response to oral glucose and possibly more sensitive to acute changes in β -cell G6P level modulated by *G6PC2* activity than is HOMA- β . We suspect that these apparent differences between results in large genome-wide association studies and our candidate variant study may be due to differences in sample size and nature of proxies used to estimate insulin secretion.

Our functional analysis of the rs573225 variant in β -cell lines found that the G allele was able to bind Foxa2 factor more efficiently than the A allele and was associated with a higher *G6PC2* promoter activity. Previous studies have found that several mutated transcription factor sites located in *G6PC2* promoter could modify its expression (6,17). Studies reviewed in Martin et al. (6) support a positive role for Foxa2 in *G6PC2* expression and a negative impact on insulin secretion in vitro (18–20). *G6PC2* appears to negatively affect stimulus-secretion coupling (3). Based on these studies and our own observations, we postulate that the higher affinity of Foxa factor for the G allelic form of the binding promoter motif could result in 1) a higher expression of *G6PC2*, 2) a diminution of β -cell G6P content, and 3) a diminution of insulin secretion in GG carriers. The cytosine adjacent to the G allele creates a CG/CGm epi-allele that showed variable Foxa2 binding capacity, which could possibly add allele-specific epigenetic effects to the Foxa2-dependent transcriptional activity of the promoter. Rs573225 can thus be called an epiSNP.

In summary, the present study reports that the *G6PC2* promoter carries an rs573225 variant that modulates Foxa2 binding and promoter activity and could influence insulin secretion consistently with the known role of *G6PC2* in β -cell physiology.

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C.D.S. and P.B. report no other dualities of interest.

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