Genetic and Functional Assessment of the Role of the rs13431652-A and rs573225-A Alleles in the G6PC2 Promoter That Are Strongly Associated With Elevated Fasting Glucose Levels

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OBJECTIVE—Genome-wide association studies have identified a single nucleotide polymorphism (SNP), rs560887, located in a G6PC2 intron that is highly correlated with variations in fasting plasma glucose (FPG). G6PC2 encodes an islet-specific glucose-6-phosphatase catalytic subunit. This study examines the contribution of two G6PC2 promoter SNPs, rs13431652 and rs573225, to the association signal.

RESEARCH DESIGN AND METHODS—We genotyped 9,532 normal FPG participants (FPG <6.1 mmol/L) for three G6PC2 SNPs, rs13431652 (proximal promoter), rs560887 (3rd intron). We used regression analyses adjusted for age, sex, and BMI to assess the association with FPG and haplotype analyses to assess comparative SNP contributions. Fusion gene and gel retardation analyses characterized the effect of rs13431652 and rs573225 on G6PC2 promoter activity and transcription factor binding.

RESULTS—Genetic analyses provide evidence for a strong contribution of the promoter SNPs to FPG variability at the G6PC2 locus (rs13431652; β = 0.075, P = 3.6 × 10⁻⁵⁸; rs573225 β = 0.073 P = 3.6 × 10⁻⁵⁸), in addition to rs560887 (β = 0.071, P = 1.2 × 10⁻⁵⁸). The rs13431652-A and rs573225-A alleles promote increased NF-Y and Foxa2 binding, respectively. The rs13431652-A allele is associated with increased FPG and elevated promoter activity, consistent with the function of G6PC2 in pancreatic islets. In contrast, the rs573225-A allele is associated with elevated FPG but reduced promoter activity.

CONCLUSIONS—Genetic and in situ functional data support a potential role for rs13431652, but not rs573225, as a causative SNP linking G6PC2 to variations in FPG, though a causative role for rs573225 in vivo cannot be ruled out. Diabetes 59:2662–2671, 2010

The glucose-6-phosphatase catalytic subunit gene family comprises three members, G6PC, G6PC2, and G6PC3 (1). G6PC2, also known as the IGRP gene (2–4), is principally expressed in the β-cells of pancreatic islets (5). Whether G6PC2 accounts for the low glucose-6-phosphatase enzyme activity detected in islets is unclear (2,3,6,7); however, a global knockout of G6pc2 results in a mild metabolic phenotype characterized by a ~15% decrease in fasting blood glucose (4). This observation suggests that G6PC2 may oppose the action of glucokinase and therefore modulate β-cell glycolytic flux and glucose-stimulated insulin secretion (8). This hypothesis is consistent with recent genetic studies in humans. Thus, using a genome-wide association approach to study the genetic basis for variations in fasting plasma glucose levels (FPG), we recently identified strong association signal in and around G6PC2 (9). FPG is an important metabolic trait that is correlated with cardiovascular-associated mortality (10,11). We showed that rs560887, a common variant located in the 3rd intron of G6PC2, may explain ~1% of the total variance in FPG. The rs560887-A allele is associated not only with elevated FPG, but also with long-term glucose regulation, as estimated by elevated glycated hemoglobin A1C levels, decreased basal insulin secretion, as assessed by Homa%B, and decreased risk of incidence of impaired fasting glucose over time using prospective data from the DESIR study (9). Data from an independent study reported similar findings (12), and another meta-analysis confirmed that the G6PC2 locus harbors the strongest genetic determinant of FPG in terms of effect size and significance (13). However, a functional link between the genetic variation in the G6PC2 locus and variations in FPG remains to be determined.

Two genetic variants located in the G6PC2 promoter
region, rs13431652 and rs573225, are the only common variants (MAF >0.05) that show high LD (r²>0.80) with rs560087, according to HapMap phase III data (r² = 0.88 and r² = 0.96, respectively), but their precise correlation with rs560087 and their contribution to the association with FPG in large and independent samples have not been investigated. In this report, we provide genetic support for the contribution of rs13431652 and rs573225 to the association signal between G6PC2 and FPG, but we do not exclude a role for rs560087. We also characterize the effect of these two G6PC2 promoter variants on transcription factor binding and G6PC2 fusion gene expression. We show that the rs13431652-A allele affects binding of the transcription factor NF-Y and that it is associated with both elevated FPG and elevated fusion gene expression, a correlation that is consistent with the function of G6PC2 (4) since elevated G6PC2 expression would oppose the action of glucokinase leading to elevated FPG. In contrast, we show that the rs573225-A allele affects binding of the transcription factor Foxa2 and that it is associated with elevated FPG but reduced fusion gene expression. These functional data support a potential role for rs13431652, but not rs573225, as a causative single nucleotide polymorphism (SNP) linking G6PC2 to variations in FPG.

RESEARCH DESIGN AND METHODS

Study participants. We analyzed 9,532 normal fasting glucose (NG) Europeans drawn from three general population studies (DESIR, N = 3,488; NFBC 1986, N = 4,372; and the Haguenau Study, N = 1,201) and one childhood obesity case population (N = 476).

DESIR. The data from the Epidemiological Study on the Insulin Resistance Syndrome (DESIR) cohort is a longitudinal French general population cohort that is fully described elsewhere (9,14). Because ethnicity could not be legally documented in the DESIR study, the proportion of subjects having non-European ancestry was estimated to be low (<0.30%) (15). We also excluded all individuals born outside metropolitan France before analysis. We analyzed 3,483 normoglycemic (NG) DESIR participants successfully genotyped for all SNPs with FPG available (supplementary Table 1) available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0389/DC1).

Haguenau. The Haguenau population is a community-based cohort of young obese children, NG obese French children with European ancestry were selected from obesity nuclear families recruited by the CNRS-UMR8090 unit (Lille, France) through an ongoing national media campaign (17). We analyzed 476 NG unrelated obese children (defined as BMI >97th percentile for age and sex according to a French population-based cohort) (18) who were successfully genotyped for all SNPs with FPG available (supplementary Table 1).

Haguenau. The Haguenau population is a community-based cohort of young adults that investigates the long-term consequences of being born small for gestational age and was fully described elsewhere (19). Briefly, subjects born between 1971 and 1985 were identified from a population-based registry of Haguenau, France. Non-European ancestry subjects are estimated to be <0.1% of the general population (19). At a mean age of 22 years, participants underwent a medical examination to assess anthropometric and clinical parameters. We analyzed 1,201 NG subjects successfully genotyped for all SNPs with FPG available (supplementary Table 1). The study protocol was approved by the Ethics Committee of Paris St. Louis University (Paris, France).

All study participants and children of parents signed an informed consent form. For each population, glycemic status was defined according to the 1997 American Diabetes Association criteria (20): normal glucose, defined as FPG <6.1 mmol/l without hypoglycemic treatment; impaired fasting glucose, defined as FPG between 6.1 and 6.99 mmol/l without hypoglycemic treatment; and type 2 diabetes, defined as FPG ≥7.0 mmol/l and/or treatment with antidiabetic agents.

Genotyping. Genotyping was performed using either TaqMan assays or SNP assay technology (Applied Biosystems) according to the manufacturer’s instructions. The genotyping success rate was at least 95% and genotype concordance between the two methods is more than 99% (internal data). No significant deviation (P > 0.05) from the Hardy-Weinberg equilibrium was observed. To allow optimal analytical conditions, all individuals in this study were successfully genotyped for the four SNPs studied here.

Genotype/expression correlation in human pancreatic cDNAs. Please see online appendix (available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0389/DC1).
G6PC2 promoter variants and fasting blood glucose

G6PC2-promoter region was used as a template with mutagenesis primers 5’-GGAAATGACTGTCAAAAAATTATTCAAGCATAATTCTACTTATGGA CATGATCCAGGTTCC3’ and 5’-GACAGGTGGATCTTGTTTGGAATTGGTTACAGTTACC3’ to convert rs573225 at position −259 from A to T allele. This study was performed with these templates and primers using a QuickChange II Kit (Stratagene) as described by Weigelt et al. (26).

G6PC2-luciferase reporter plasmids, containing promoter sequence from −8,563 to +11, were generated in the pGL3-Basic and pGL4.16 vectors (Promega) by a two-step process. In the initial cloning step, a SacI-HindIII fragment was isolated from the G6PC2-Pac294mpEplGEM7 plasmid, using a SacI site in G6PC2 at −4,305 and a HindIII site within the pGEM7 vector, and ligated into the SacI-HindIII digested pGL3-Basic and pGL4.16 vectors to generate gene (extracts that contain G6PC2 promoter sequence from −4,305 to +11. In the second cloning step a SacI fragment was isolated from the G6PC2-Pac294SacShuttleGEM7 plasmid and ligated into the SacI digested −4,305 pGL3-Basic and pGL4.16 fusion gene plasmids to extend the G6PC2 promoter sequences from −4,305 to −8,563.

A SacI fragment from the G6PC2-Pac294SacShuttleGEM7 plasmid containing the alternate rs1341652 allele and an XmaI fragment from the G6PC2-Pac294XmaShuttleGEM7 plasmid with the alternate rs573225 allele were then exchanged with the corresponding SacI and/or XmaI end fragments that contain the original Pac24 sequences to generate pGL3-Basic and pGL4.16 plasmids with the alternate SNP alleles within the context of the G6PC2 promoter region from −8,563 to +11.

Cell culture, transient transfection and luciferase assays. Hamster insulinoma cells INS-1, MIN6 and MIN8 were cultured and transfected using lipofectamine as previously described (23). Luciferase assays were performed as previously described (23).

G6PC2 promoter variants strongly associated with FPG. Only two common SNPs (minor allele frequency >0.05), namely rs1341652 and rs573225, are reported to be in high linkage disequilibrium (LD) with the previously identified intrinsic G6PC2 SNP rs560887 (r²=0.80, according to HapMap data [release XX]). rs1341652 is located in the distal G6PC2 promoter, at −4,405 relative to the transcription start site, whereas rs573225 is located in the proximal promoter at −259. We have assessed the effect of these two G6PC2 promoter variants on FPG, adjusted for age, sex and BMI in 9,532 NG Europeans from four independent populations. In the meta-analyses, we show strong associations of both rs1341652 (β = 0.075, P = 3.6 × 10⁻⁶⁰) and rs573225 (β = 0.073, P = 3.6 × 10⁻³⁴) with FPG, in similar magnitude to that for rs560887 (β = 0.071, P = 1.2 × 10⁻³¹) (Table 1). Conditioned regression model analyses to assess the independency of the three SNPs studied turn out to be noninformative (data not shown), as these analyses include a high variance inflation factor because of high LD that we observed between the SNPs in our populations (supplementary Table 2).

To compensate for this limited analytical situation, we analyzed the association of FPG with multiple haplotypes of the promoter variants and rs560887, specifically individual contributions of each variant and contributions when variants were analyzed in pairs and when we constrained the effect of rs560887 to be null (see supplementary Methods). Because of the LD discrepancies between populations (supplementary Table 2), these haplotype analyses were conducted in the four populations separately (supplementary Table 3). In DESIR, we found that rs560887 is significantly contributing to the haplotype association in combination with rs1341652 (P = 7.58 × 10⁻⁵) and rs573225 (P = 0.015). We also observed a significant contribution of rs1341652 (P = 4.09 × 10⁻⁷) and rs573225 (P = 1.21 × 10⁻⁶) to the haplotype associations, supporting independent effects of both promoter variants to the global association of the haplotype in combination with rs560887. In contrast, distinct results were obtained in the remaining cohorts in which we found that both promoter and intronic variants are not independently contributing to the haplotype associations, suggesting that promoter and intronic variants are dispensable to the two by two haplotype associations, with a modestly more significant contribution of rs560887 (P = 0.006 against rs1341652 and P = 0.07 against rs573225) in the NFBC 1986 cohort (supplementary Table 3). We speculate that these distinct results in some way reflect the differences in LD within the different cohorts. Despite these differences, haplotype analyses support a substantial role of promoter variants to the association signal with FPG, and do not discard the contribution of rs560887.

We have also assessed the effect of the intronic variant rs853789 located in the 19th intron of ABCB11, one of two SNPs, along with rs560887, that were reported by Chen et al. (12) to be associated with FPG. ABCB11 (ATP-binding...
TABLE 1

| Genetic Variation and Fasting Glucose in Four Cohorts and One Obese Population |
|-----------------------------------------------|
| EA (freq) | Meta-analysis (n = 9,532) | DESIR (n = 3,483) | NFBC86 (n = 4,372) | Haguenuau (n = 1,201) | Obese children (n = 476) |
| rs13431652 | A (0.68) | 0.075 (0.006) | 3.6 × 10^-35 | 0.083 (0.010) | 2.0 × 10^-15 | 0.069 (0.009) | 1.1 × 10^-14 | 0.071 (0.015) | 2.0 × 10^-6 |
| rs13431652 | A (0.66) | 0.073 (0.006) | 3.6 × 10^-34 | 0.080 (0.010) | 1.3 × 10^-14 | 0.066 (0.009) | 6.8 × 10^-14 | 0.073 (0.015) | 9.2 × 10^-7 |
| rs13431652 | G (0.69) | 0.071 (0.011) | 1.2 × 10^-31 | 0.069 (0.011) | 3.0 × 10^-11 | 0.072 (0.009) | 8.7 × 10^-16 | 0.076 (0.015) | 4.5 × 10^-7 |

Individual and meta-analyses data are displayed as regression coefficient (SE) adjusted for age, sex, BMI, and associated P values.
G6PC2 PROMOTER VARIANTS AND FASTING BLOOD GLUCOSE

FIG. 1. NF-Y binds the −4,425/−4,392 G6PC2 promoter region in vitro. A: The sense strand sequences of the oligonucleotides used in gel retardation assays are shown. SNP base pairs are shown in bold lower case letters. The consensus NF-Y binding motif, CCAAT, is taken from Quandt et al. (31). B: βTC-3 nuclear extract was incubated in the absence or presence of the indicated anti-serum for 10 min on ice. A labeled oligonucleotide representing the −4,425/−4,392 G6PC2 promoter region and containing the rs13431652-A allele (rs13431652-A; panel A) was then added and incubation continued for 20 min at room temperature. Protein binding was then analyzed using the gel retardation assay as described in RESEARCH DESIGN AND METHODS. In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess.

Gel retardation competition experiments, in which a varying molar excess of unlabeled DNA was included with the labeled rs13431652-A or rs13431652-G oligonucleotide, were used to compare the affinity of NF-Y binding to the rs13431652 variants of the G6PC2 NF-Y binding site. Fig. 2A shows that the rs13431652-A oligonucleotide competed more effectively than the rs13431652-G oligonucleotide for the formation of the NF-Y-DNA complex and quantitation of the results of several experiments (Fig. 2B) showed that NF-Y binds the rs13431652-A oligonucleotide with approximately fivefold higher affinity. Interestingly, in contrast to the competition experiment data (Fig. 2B), the direct analysis of NF-Y binding to the rs13431652-A and -G oligonucleotide probes, labeled with the identical specific activity, suggested a much more dramatic difference in NF-Y binding affinity to the rs13431652-A and -G oligonucleotides (Fig. 2C). We recently reported a similar discrepancy between the results of gel retardation competition and binding experiments in studies on FOXO1 binding (33). We hypothesize that such apparent discrepancies can arise when both the association and dissociation rate of factor binding to DNA are simultaneously increased since this would result in limited change in Kd (34), but a marked difference in the dissociation of the protein-DNA complex upon separation of bound and free probe during electrophoresis.

rs13431652 alters G6PC2 fusion gene expression. We next investigated the functional significance of altered NF-Y binding on human G6PC2 promoter activity. NF-Y can function as either an activator or repressor such that the effect of NF-Y on gene transcription is context dependent (35). Fusion genes containing each of the rs13431652 alleles, generated in the context of the −8,563 to +11 G6PC2 promoter region, were analyzed by transient transfection of βTC-3 cells. Fig. 3 shows that the rs13431652-G allele was associated with an −25% decrease in promoter activity in comparison with that observed with the rs13431652-A allele. Supplementary Fig. 2 shows that this difference was observed only when the G6PC2 promoter was analyzed in the context of the pGL4, but not the pGL3 vector. The former is an improved vector that lacks multiple transcription factor binding sites in the plasmid backbone and luciferase gene that are known to occasionally give rise to spurious data (23). These results indicate that NF-Y acts as an activator in the context of the G6PC2 promoter. More importantly, these data are consistent with the reduced FPG observed in G6pc2 knockout mice (4) in that the rs13431652-A allele is associated with both elevated FPG (Table 1) and G6PC2 promoter activity. As such, these functional data support a potential role for rs13431652 as a SNP linking G6pc2 to variations in FPG.

Fiox2 binds to the G6PC2 promoter in vitro. The G6PC2 promoter region that encompasses rs073225 is highly conserved in the mouse G6pc2 promoter (3). We
FIG. 3. The human G6PC2 rs13431652-G allele is associated with decreased G6PC2 promoter activity relative to the rs13431652-A allele. βTC-3 cells were transiently cotransfected, as described in Research Design and Methods, using a lipofectamine solution containing various G6PC2-luciferase fusion genes in the pGL4 MOD vector (2 μg) and an expression vector encoding Renilla luciferase (0.5 μg). The G6PC2-luciferase fusion genes represented the rs13431652-A or rs13431652-G alleles present in the context of the human G6PC2 promoter sequence located between −8,563 and +11. After transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested, and firefly and Renilla luciferase activity were assayed as described in Research Design and Methods. Results are presented as the ratio of firefly:Renilla luciferase activity, expressed as a percentage relative to the value obtained with the rs13431652-A allele, and represent the mean of 12 experiments ± SEM, each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *P < 0.05 vs. rs13431652-A allele.

recently characterized this conserved region in the context of the mouse G6pc2 promoter and showed that it contains a Foxa2 binding site (23). In addition, we demonstrated, using chromatin immunoprecipitation (ChIP) assays, that Foxa2 binds the endogenous G6pc2 promoter in mouse βTC-3 cells in situ (23). Fig. 4 shows that this region of the human G6PC2 promoter also binds Foxa2 in vitro. When labeled double-stranded oligonucleotides, designated rs573225-A and rs573225-G (Fig. 4A), representing the G6PC2 promoter sequence from −265 and −246 and the rs573225-A and -G alleles, respectively, were incubated with rat liver nuclear extract, a single major complex, designated A2, was detected with both alleles (Fig. 4B). When rat liver nuclear extract was preincubated with antisera specific for Foxa2, a clear supershift in the migration of complex A2 was observed (Fig. 4B), strongly suggesting that this complex represents Foxa2 binding. This experiment was performed using rat liver nuclear extract because, like pancreatic islets (36), it contains both Foxa1 and Foxa2 (37). In contrast, βTC-3 cells express only Foxa2 (23). The results indicate that rs573225 does not differentially affect binding of these related factors, instead both alleles strongly bind Foxa2.

Gel retardation competition experiments, in which a varying molar excess of unlabeled DNA was included with the labeled rs573225-A probe, were used to compare the affinity of Foxa2 binding to the rs573225 variants of the G6PC2 Foxa2 binding site. Fig. 5A shows that when using rat liver nuclear extract, both the rs573225-A and rs573225-G oligonucleotides competed equally effectively for the formation of the Foxa2-DNA complex. Quantitation of the results of several experiments confirmed that Foxa2 binds the rs573225-A and rs573225-G oligonucleotides with equal affinity (Fig. 5B). Identical results were obtained using mouse βTC-3 cell nuclear extract (Fig. 5A). We have previously shown that the two complexes detected using βTC-3 cell nuclear extract represent the binding of Foxa2 (A2) and an unknown factor that either represents a nonspecific (NS) interaction or binding to another region of the probe (23).

Interestingly, in contrast with the competition experiment data (Fig. 5A and B), the direct analysis of Foxa2 binding to the rs573225-A and rs573225-G oligonucleotide probes labeled with the identical specific activity showed that Foxa2 binds with slightly higher affinity to the rs573225-A allele (Figs. 4B and 5C). An identical observation was made using rat liver and rat H4IE hepatoma cell nuclear extract (Fig. 5C). As explained above in the analysis of NF-Y binding (Fig. 2C), this suggests that rs573225 alters the association and dissociation rates of Foxa2 binding without affecting binding affinity.

rs573225 alters G6PC2 promoter activity in an unexpected direction. We next investigated the functional significance of altered Foxa2 binding on human G6PC2 promoter activity. A block mutation in the G6PC2 Foxa2 binding site that abolishes Foxa2 binding (supplementary Fig. 3) also markedly reduces fusion gene expression (supplementary Fig. 4), indicating that Foxa2 is an activator in the context of the human G6PC2 promoter, as it is in the mouse G6pc2 promoter (23). Fusion genes containing each rs573225 variant generated in the context of the −324 to +3 (Fig. 6) and −8,563 to +11 (Fig. 7) G6PC2 promoter regions, were analyzed by transient transfection of βTC-3 cells. Since the loss of Foxa2 binding reduces G6PC2 promoter activity (supplementary Fig. 4), and because the rs573225-G allele decreases Foxa2 binding (Fig. 5C), we anticipated that the rs573225-G allele would also be associated with reduced G6PC2 promoter activity. Surprisingly, Figs. 6 and 7 show that the rs573225-G allele was associated with an increase in promoter activity in comparison with that observed with the rs573225-A allele. This result is not explained by the de novo creation of an activator binding site by the rs573225-G allele (Fig. 4B). Supplementary Figs. 5 and 6 show that a similar effect of
the rs573225-G allele was also seen in the HIT and Min6 cell lines and when the G6PC2 promoter was analyzed in the context of both the pGL4 and pGL3 vectors, respectively. Since the Foxa2 binding site incorporates the target sequence for the bacterial Dam methylase (GATC), we repeated this analysis using plasmids grown in SCS110 Dam and Dcm methylase-deficient bacteria. Suplementary Fig. 7 shows that Dam methylation of the Foxa2 binding site did not alter the stimulatory effect of rs573225 on fusion gene expression. Although the increased promoter activity observed with the rs573225-G allele was unexpected, given the reduction in Foxa2 binding, the key observation here is that the functional data obtained with rs573225 are not consistent with the reduced FPG observed in G6pc2 knockout mice (4) in that the rs573225-A allele is associated with elevated FPG (Table 1) but reduced G6PC2 promoter activity. As such, in contrast with the functional data obtained with rs13431652, these functional data do not support a potential role for rs573225 as a causative SNP linking G6PC2 to variations in FPG.

FIG. 5. Comparison of the affinity of Foxa binding to the rs573225-A and rs573225-G variants of the G6PC2 Foxa binding site in vitro. A: The labeled rs573225-A oligonucleotide (Fig. 4A) was incubated in the absence or presence of the indicated molar excess of the unlabeled human rs573225-A or rs573225-G oligonucleotide competitors (Fig. 4A) before the addition of βTC-3 nuclear extract. Protein binding was then analyzed using the gel retardation assay as described in Research Design and Methods. In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess. With βTC-3 nuclear extract, complex A2 represents Foxa2 binding, and complex NS represents nonspecific binding (23). With liver nuclear extract, complex A2 represents Foxa2 binding (37). B: Protein binding in experiments using liver nuclear extract was quantitated by using a Packard Instant Imager to count 32P associated with the retarded complex. The data represents the mean ± SEM of three experiments. C: The labeled rs573225-A and rs573225-G oligonucleotides (Fig. 4A) were incubated with either liver or H4IIE nuclear extract, and protein binding was analyzed using the gel retardation assay as described in Research Design and Methods. In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess. NS, nonspecific.

FIG. 6. The human G6PC2 rs573225-G allele is associated with increased G6PC2 promoter activity relative to the rs573225-A allele. βTC-3 cells were transiently cotransfected, as described in Research Design and Methods, using a lipofectamine solution containing various G6PC2-luciferase fusion genes in the pGL3 MOD vector (2 μg) and an expression vector encoding Renilla luciferase (0.5 μg). The G6PC2-luciferase fusion genes represented the rs573225-A or -G alleles present in the context of the human G6PC2 promoter sequence located between −324 and +3. After transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested, and firefly and Renilla luciferase activity was assayed as described in Research Design and Methods. Results are presented as the ratio of firefly:Renilla luciferase activity, expressed as a percentage relative to the value obtained with the rs573225-A allele, and represent the mean of three experiments ± SEM, each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *P < 0.05 vs. rs573225-A allele.

FIG. 7. Interaction between the G6PC2 rs13431652-G and rs573225-G alleles on G6PC2 promoter activity. βTC-3 cells were transiently cotransfected, as described in Research Design and Methods, using a lipofectamine solution containing various G6PC2-luciferase fusion genes in the pGL4 MOD vector (2 μg) and an expression vector encoding Renilla luciferase (0.5 μg). The G6PC2-luciferase fusion genes represented the rs573225-A or rs573225-G alleles and rs13431652-A or rs13431652-G alleles present in the context of the human G6PC2 promoter sequence located between −8,563 and +11. After transfection, cells were incubated for 18 to 20 h in serum-containing medium. The cells were then harvested, and firefly and Renilla luciferase activity was assayed as described in Research Design and Methods. Results are presented as the ratio of firefly:Renilla luciferase activity, expressed as a percentage relative to the value obtained with the rs573225-A and rs13431652-A alleles, and represent the mean of eight experiments ± SEM, each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *P < 0.05 vs. rs573225-A and rs13431652-A alleles.
Because endogenous G6PC2 gene expression will reflect the combined effects of multiple SNPs, we investigated the interaction between rs573225 and rs13431652 on G6PC2 fusion gene expression (Fig. 7). The results show a trend toward the rs13431652-G allele blunting the elevated expression conferred by the rs573225-G allele, although, because of the relatively small effects involved and the inherent variability in transient transfections, the decrease did not reach statistical significance.

**DISCUSSION**

Genome-wide association studies have recently provided important new insights about the genetics of common forms of type 2 diabetes and its related quantitative traits, especially for FPG (9,12,38). After this first discovery phase, attention is now beginning to focus on the study of the functional properties of the variants at the confirmed loci. In this study using a combination of genetic and functional data, we provide further genetic support for the primary contribution of G6PC2 to variations in FPG, a conclusion supported by other genetic studies (13,39 – 41). Furthermore, we specifically demonstrate that the functional properties of the variants at the confirmed loci are consistent with the reduced FPG observed in G6pc2 knockout mice (4). As such, unlike the functional data obtained with rs13431652, these functional data do not support a potential role for rs573225 as a causative SNP linking G6PC2 to variations in FPG. In contrast, Dos Santos et al. (43) recently concluded that rs573225 is the causative SNP that explains the association signal between FPG and G6PC2. As in our study, they observed that the rs573225-A allele is associated with elevated FPG but lower fusion gene expression, but they did not comment on the fact that these data are inconsistent with the function of G6PC2. Dos Santos et al. (43) also suggested that rs573225 is an epiSNP, because the rs573225-G allele is located at a Gpc dinucleotide within the Foxa2 binding site and methylation of the 'C' nucleotide affected Foxa2 binding. However, in contrast with the well-studied methylation of CpG dinucleotides (44), we can find no reports of the existence of methylated Gpc dinucleotides in mammals, although this modification does exist in fish (45). Finally, although our fusion gene data match that of Dos Santos et al. (43), our gel retardation results are the complete opposite. Dos Santos et al. (43) report that the rs573225-G allele binds Foxa2, whereas the rs573225-A allele completely abolishes Foxa2 binding. Their binding data therefore appear to correlate with their fusion gene data since the rs573225-G allele confers higher promoter activity. However, a detailed previous study by Overdier et al. (46) showed that both ‘G’ and ‘A’ nucleotides at this location within the Foxa2 binding site support Foxa2 binding. Our demonstration that Foxa2 binds with the same affinity to both alleles (Fig. 5A and B), though with different kinetics (Fig. 5C), is therefore consistent with the observations of Overdier et al. (46), but not with those of Dos Santos et al. (43). Moreover, given the importance of Foxa2 for G6PC2 fusion gene expression (supplementary Fig. 4), if the rs573225-A allele were to abolish Foxa2 binding, Dos Santos et al. (43) should have detected a major difference in the level of reporter gene expression conferred by the promoters containing the rs573225-A and rs573225-G alleles instead of the ~15% difference reported.

Using a combination of genetic and functional data, we show that rs573225-A allele is highly associated with elevated FPG (Table 1), enhanced Foxa2 binding (Figs. 4 and 5), and lower fusion gene expression (Fig. 6), a correlation that is inconsistent with the reduced FPG observed in G6pc2 knockout mice (4). As such, the functional data obtained with rs13431652, these functional data do not support a potential role for rs573225 as a causative SNP linking G6PC2 to variations in FPG. In summary, our study provides genetic and functional evidence supporting an important role for the promoter

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variant rs13431652 as a potentially causative SNP that contributes to the association signal between G6PC2 and FPG, but the data do not preclude a significant contribution of the intronic variant rs560887 or other additional unidentified variants.

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N.B.-N. supervised the design of the genetic and expression studies and wrote parts of the manuscript. A.B. contributed to the experiments and draft of the genetic expression studies. D.A.B. performed gel retardation and fusion gene analyses. M. Marchand performed genotyping and expression experiments. M.B. prepared pancreatic islet cDNA and DNA. P.M. prepared pancreatic islet cDNA and DNA and reviewed the manuscript. F.P. sorted β-cells, prepared β-cell cDNA, and reviewed the manuscript. R.L.P. cloned the human G6PC2 promoter. B.P.F., O.C.U., and N.L.C. performed gel retardation and fusion gene analyses. M.V. contributed to the design of the genetic experiments and reviewed the manuscript. O.L. and M. Marre collected data for the DESIR cohort. B.B. collected data for the DESIR cohort and reviewed the manuscript. C.L.-M. collected data for the Haguenau cohort. P.E. collected data for the NFBC 1986. M.-R.J. collected data for the NFBC 1986 and reviewed the manuscript. D.M. collected data for the obese children cohort and reviewed the manuscript. C.D. performed statistical analyses and reviewed the manuscript. J.K.O. performed fusion gene analyses. P.F. was the principle investigator for the genetic studies and reviewed the manuscript. R.M.O’B. was the principle investigator for the gel retardation and fusion gene studies and wrote parts of the manuscript.

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