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Promoter Polymorphism in *PCK1* (Phosphoenolpyruvate Carboxykinase Gene) Associated with Type 2 Diabetes Mellitus

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We sequenced the promoter and coding regions of *PCK1* encoding cytosolic phosphoenolpyruvate carboxykinase from genomic DNA of subjects with type 2 diabetes mellitus (DM). We found nine single nucleotide polymorphisms (SNPs) that were present with varying allele frequencies and pairwise linkage disequilibrium relationships in different ethnic groups. The −232C→G promoter SNP was within a *cis*-acting element required for basal and cAMP-mediated *PCK1* gene transcription. The expression of a luciferase reporter construct containing −232G in three different cell lines showed significantly increased basal expression with no down-regulation by insulin compared with a construct containing −232C. The odds ratios for type 2 DM among subjects with one or two copies of −232G compared with −232C/C homozygotes were 1.9 (95% confidence interval, 1.2–3.0) in a Canadian aboriginal sample and 2.8 (95% confidence interval, 1.7–4.7) in a Caucasian sample. Thus, we report a promoter SNP in *PCK1* that was resistant to down-regulation by insulin *in vitro* and was associated with type 2 DM in two independent study samples. (*J Clin Endocrinol Metab* 89: 898–903, 2004)

CYTOSOLIC PHOSPHOENOLPYRUVATE carboxykinase (EC 4.1.1.32) catalyzes the first committed step in hepatic gluconeogenesis (1, 2). Altered regulation of *PCK1* (phosphoenolpyruvate carboxykinase gene) expression might arise from genomic DNA variations that elevate basal promoter activity and/or result in loss of negative regulation by insulin, contributing to hyperglycemia in the context of underlying insulin resistance (3). Previously (4) the *PCK1* promoter region was screened using single strand conformation polymorphism (SSCP) analysis of genomic DNA from 117 subjects with type 2 diabetes mellitus (DM). Because no mutations or single nucleotide polymorphisms (SNPs) were found, genetic investigators have avoided *PCK1* as a candidate gene for type 2 DM. We previously discovered several diabetes-causing mutations by directly sequencing candidate genes from genomic DNA (5–8). Based on this, we reevaluated *PCK1* as a candidate gene for type 2 DM using direct sequencing of genomic DNA.

Subjects and Methods

Subjects

The study received approval from the University of Western Ontario ethics review panel. *PCK1* genomic sequence was studied in 12 unrelated Caucasians with type 2 DM diagnosed between age 30–54 yr (median, 44 yr), and mean body mass index (BMI) of 30.8 kg/m^2 (range, 27.0–40.3 kg/m^2). This sample size provided 100% power to detect alleles with a frequency greater than 0.15, and 70% power to detect alleles with a frequency greater than 0.05. Disease association was further evaluated in 115 clinic-based Caucasian type 2 DM subjects (42% female) with mean ± SE age and BMI of 51.6 ± 5.6 yr and 31.2 ± 3.5 kg/m^2, respectively. All type 2 DM subjects were treated with oral hypoglycemic agents and/or insulin. We also studied 260 clinic-based normoglycemic Caucasian controls (52% female) with age and BMI of 50.6 ± 6.5 yr and 25.8 ± 3.0 kg/m^2, respectively. Control subjects had normal fasting glucose according to 1997 American Diabetes Association diagnostic criteria. *PCK1* SNP allele frequencies were evaluated in population-based normal African, European, Chinese, East Indian, Oji-Cree, and Inuit subjects (n = 50 from each ethnic group). Disease association of *PCK1* −232C→G SNP genotype was evaluated in a well characterized, community-based sample of 170 Oji-Cree aged 18 yr or older either with type 2 DM or glucose intolerance and 392 normoglycemic controls (5, 9, 10). All Oji-Cree subjects were diagnosed based on a standardized glucose challenge (5, 9, 10).

DNA analysis

Primers to amplify the *PCK1* promoter and exons 1–9, including at least 50 bp of intron sequence at each boundary (Table 1) were designed using reference sequences from the National Center for Biotechnology Information (GenBank accession no. L_12760 and NM_002591). Amplification conditions were: denaturation at 94 C for 5 min; followed by 30 cycles comprised of 30 sec each at 94, 59, and 72 C; followed by a final extension step at 72 C for 10 min. Amplification products were run on 1.5% agarose and purified using QIAEX II (Qiagen, Mississauga, Canada). DNA was bidirectionally sequenced using amplification primers, sequences were detected on a 377 PRISM Automated DNA Sequencer (PE Applied Biosystems, Mississauga, Canada), and results were analyzed with Sequence Navigator software (PE Applied Biosystems). Each *PCK1* mutation was seen in both directions and was confirmed using a second, independent amplification and resequencing on another day.

Genotyping of −1237C→T and −1107C→T SNPs was performed

Abbreviations: BMI, Body mass index; C/EBP, CCAAT/enhancer-binding protein; CI, confidence interval; db, National Center for Biotechnology Information database; DM, diabetes mellitus; IRS, insulin response sequence; LD, linkage disequilibrium; nt, nucleotide; OR, odds ratio; *PCK1*, phosphoenolpyruvate carboxykinase gene; PGC-1, PPARγ coactivator-1; RLU, reporter luciferase units; SNP, single nucleotide polymorphism; SSCP, single strand conformation polymorphism.

TABLE 1. Primer sequences and product sizes

Region	Primer sequence	Product size (bp)
Promoter	F: 5′-TCT AAG TGA GTT TGG TCG GAG G-3′ R: 5′-CTG CAG AGT GCT GCT AAG GG-3′	1640
Exon 1	F: 5′-CTG CAG AAA TGC CTC CTC AG-3′ R: 5′-GTC TGA AAC TAC CCG AAG GC-3′	341
Exon 2	F: 5′-CTG AAG GAG ATG GTT CGC TG-3′ R: 5′-CTG AAG GAG ATG GTT CGC TG-3′	351
Exons 3 and 4	F: 5′-GGT CAT TTC TCA CCA GTG CC-3′ R: 5′-CAT CTG CAC ATC AGG GCT T-3′	590
Exon 5	F: 5′-GGT CAT TTC TCA CCA GTG CC-3′ R: 5′-ATT CGC TTG AGT CCA ATC GT-3′	269
Exons 6 and 7	F: 5′-GCC TGG CAC TCA CTA CTG GT-3′ R: 5′-GAT TTC ACA GAT GCC TTC GG-3′	554
Exon 8	F: 5′-GTG TCT TTC CGT GTT GTG AA-3′ R: 5′-CCC ACT TCT CTG TGG TTT CAG-3′	227
Exon 9	F: 5′-TGT CAA CAA TCA ATG GCG TC-3′ R: 3′-GCG TCA ATT TGG GAA CAC TT-3′	622

F, Forward; R, reverse.

using direct genomic DNA sequencing. Exon 3 574G→A and 610A→G SNPs were genotyped using DNA amplification, followed by purification using serum alkaline phosphatase and exonuclease I (*Exo*I; PE Applied Biosystems). The treatment reaction mixture included 2.0 μl serum alkaline phosphatase (1.0 U/μl), 0.2 μl *Exo*I, 6.0 μl deionized water, and 4.0 μl amplification product. This was incubated at 37 C for 1 h and then at 72 C for 15 min to inactivate the enzyme. Additional primers were designed to terminate one nucleotide before the SNP. For the 574G→A and 610A→G SNPs, these primers were 5′-ATG GGG CCG CTG GGC TC-3′ and 5′-ATC GAG CTG ACG GAT TC-3′, respectively. The PCR template and primer were treated with a SNaPshot ddNTP Primer Extension Kit and analyzed on a PRISM 377 DNA Sequencer (PE Applied Biosystems). Altered restriction endonuclease recognition sites permitted genotyping of the remaining SNPs using 2% agarose gels. Digestion of the 1640-bp promoter product with *Mae*III yielded fragments of 494, 483, 393, 180, 38, 34, and 18 bp for the −232C allele and fragments of 674, 483, 393, 38, 34, and 18 bp for the −232G allele. Digestion of the 341-bp exon 1 product with *Cac*I yielded fragments of 195, 71, and 71 bp for the 190G allele and 270 and 71 bp for the 190A allele. Digestion of the 590-bp product that contained exons 3 and 4 with *Bsr*I yielded fragments of 199, 160, 120, 85, and 17 bp for the L184 allele and 289, 199, 85, and 17 bp for the V184 allele. Digestion of the same product with *Taq*I yielded fragments of 484, 93, and 13 bp for the 583G allele and 484 and 106 bp for the 583A allele. Digestion of the 554-bp exon 6 product with *Bsa*HI yielded fragments of 334 and 220 bp for the 1261C allele and 554 bp for the 1261T allele.

Expression assays

The *PCK1* promoter was mutagenized at position −232 by the two-primer pair method with primers: 5′-GTT GTG TCA AAA GTC ACT ATG GTT GGT-3′ and 5′-ACC AAC CAT AGT GA**C** TTT TGA CAC AAC-3′ (mutated nucleotide in underlined bold). A DNA fragment with 1.6 kb of *PCK1* promoter was amplified using primers: 5′-TCT AAG TGA GTT TGG TCG GAG G-3′ and 5′-CTG CAG AGT GCT GCT AAG GG-3′. The amplified promoter fragments were ligated into a TA cloning vector (Invitrogen, Carlsbad, CA). The promoter fragment was subcloned into the luciferase reporter PGL3 basic vector (Promega, Madison, WI) after *Sac*I and *Xho*I digestion. The fidelity of amplification, replication and the introduction of −232C→G were confirmed by DNA sequencing. Both wild-type and mutant constructs contained −1237C and −1107C as the genomic backbone.

The human hepatoma HepG2 and the mouse preadipocyte NIH-3T3 cell lines were obtained from American Type Culture Collection (Manassas, VA). Mouse dermal fibroblasts were also studied (11). Cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% (vol/vol) heated-inactivated fetal calf serum (Invitrogen). Cell cultures grew to 70% confluence in six-well culture plates and then were transfected using calcium phosphate (www.stanford.edu/group/nolan/index.html). For each transfection, 4 μg DNA and 6.7 mg $CaCl_2$ in HEPES-buffered saline were added. For each transfection with the *PCK1*-containing vector, 2 μg pSV-β-galactosidase vector (ProFection Mammalian Transfection System, Promega, Madison, WI) was cotransfected. The transfection solution was removed after 20 h at 37 C and replaced by fresh medium for further culture. Insulin was added to achieve final concentrations of 0, 10, 25, 50, and 100 nmol/liter. Cells were harvested 48 h posttransfection. Luciferase activity was assayed (Promega Corp., Madison, WI), and luminescence was determined using a Lumat LB9507 luminometer (Berthold Systems, Pittsburgh, PA). Luciferase activities were normalized per unit of activity of β-galactosidase [reporter luciferase units (RLU)]. The β-galactosidase assay (Promega Corp.) used 150 μl cell lysate mixed with 150 μl 2× assay buffer and was incubated at 37 C for 3 h. The reaction was stopped by 500 μl 1 M sodium carbonate. Spectrophotometric absorbance at 420 nm was read. A blank was prepared using the lysate from nontransfected cells. Each data point was the mean of three triplicate assays from two experiments performed on different days.

Statistical analysis

SAS version 8 (SAS Institute, Cary, NC) was used for statistical analyses. Deviation of genotype frequencies from those predicted by Hardy-Weinberg equilibrium for each locus in each group were evaluated using the conditional χ^2 goodness of fit test using 1 df (12), with adjustment for small numbers as described (12). Pairwise linkage disequilibrium (LD) between SNP genotypes was estimated as described using the maximal likelihood estimate of Hill and Robertson (12, 13). Significance and confidence intervals for type 2 DM odds ratios (ORs) were calculated based on case-control χ^2 analysis (with 1 df). For comparisons of data from luciferase expression experiments, paired *t* tests were used to compare individual insulin concentrations, and repeated measures ANOVA in SAS was used to compare expression curves over the dose range of insulin. A nominal value of $P < 0.05$ was used for all statistical comparisons.

Results

SNP identification

Genomic DNA sequencing in 12 Caucasian subjects with type 2 DM uncovered nine *PCK1* polymorphisms (Table 2). The observed genotype frequencies in all subsequent experiments and sample subgroups followed Hardy-Weinberg expectations (data not shown). Our primers captured the full *PCK1* promoter (14), and we found three promoter SNPs, namely, −1237C→T, −1107C→T, and −232C→G; one nonsynonymous coding SNP, namely, 671C→G (trivial name L184V); and five synonymous coding SNPs, namely, 190A→G, 574G→A, 583A→G, 610A→G, and 1261C→T. Although the promoter SNPs were not present in the National Center for Biotechnology Information database (db), the 190A→G, 574G→A, 583A→G, 610A→G, 671C→G, and 1261C→T SNPs were already assigned dbSNP reference numbers 1042521, 1062600, 1042523, 1062601, 707555, and 2070756, respectively. All SNPs were present with varying frequencies in different ethnic groups (Table 2). Pairwise linkage disequilibrium relationships differed according to ethnicity (Tables 3–5).

Association of −232C→G SNP genotype with diabetes

Genotype and allele frequencies of the *PCK1* −232C→G genotype in Oji-Cree and Caucasian type 2 DM case-control samples are shown in Table 6. For both samples, the distribution of genotypes was significantly different between cases and controls, with ahigher number of −232G/G homozygotes in each diabetic subgroup. For both samples, the −232G allele frequency was significantly increased in subjects with type 2 DM. Compared with −232C/C homozygotes, Oji-Cree and Caucasian subjects with at least one −232G allele (dominant model, −232G/G plus −232G/C subjects) had type 2 DM OR of 1.9 [95% confidence interval (CI), 1.2 − 3.0; $P = 0.009$] and 2.8 (95% CI, 1.7–4.7; $P = 0.00004$), respectively. Compared with −232C/C homozygotes, Oji-Cree and Caucasian subjects with two −232G alleles (recessive model) had type 2 DM OR of 2.3 (95% CI, 1.4–4.0; $P = 0.002$) and 3.1 (95% CI, 1.6–6.2; $P = 0.001$), respectively. In Oji-Cree, we previously showed that the *HNF1A* G319S genotype was associated with increased risk of type 2 DM (5). In Oji-Cree more than 40 yr of age, subjects with one or more *HNF1A* S319 allele had type 2 DM OR of 3.0 (95% CI, 1.2–7.5; $P = 0.009$), but subjects with one or more *HNF1A* S319 allele and one or more *PCK1* −232G allele had type 2 DM OR of 3.2 (95% CI, 1.5–6.7; $P = 0.002$) compared with a reference group homozygous for the wild-type allele for each polymorphism.

Expression analysis of −232C→G SNP

DNA sequencing confirmed that all constructs contained both −1237C and −1107C, and that the only sequence variation was either C or G at nucleotide (nt) −232. The negative control construct contained the wild-type sequence in the reverse orientation, with no significant difference in activity compared with the blank (data not shown). Normalized luciferase activities (RLU) from three triplicate experiments performed on 2 separate d are shown in Fig. 1. Basal RLU (insulin concentration, 0 nmol/liter) was between 1.5- and 15-fold higher for the −232G-containing construct in HepG2, 3T3L1 preadipocytes and mouse dermal fibroblasts. An elevated basal RLU of the −232G-containing construct was confirmed in three additional transfection experiments using an independent fusion gene preparation, with an overall mean 1.8-fold increase compared with the −232C-containing construct (data not shown; $P < 0.0001$).

Repeated measures analysis of RLU across the range of insulin concentrations showed significant differences between the −232G- and −232C-containing constructs transfected in all three cell lines (Fig. 1). Paired *t* tests showed that at 50 nmol/liter insulin, reporter activity was reduced from baseline for the −232C-containing construct in HepG2 and 3T3L1 cells, but not in fibroblasts. These differences were not significant for the −232G-containing construct in any cell line. These results indicate that the −232G-containing construct had both increased basal activity and failure of suppression of activity with insulin compared with the −232C-containing construct.

Discussion

These observations support earlier speculations regarding putative common diabetes-related *PCK1* promoter SNPs associated with increased basal *PCK1* expression and/or failure to down-regulate in the presence of insulin (14). Transcription of *PCK1* is down-regulated by insulin (15) and responds to several hormonal and nutritional stimuli (16). Although regulation of *PCK1* transcription involves several response elements (14, 17, 18), the region between −490 and +73 nt contains the minimal information for major transcriptional properties (19). Insulin suppresses human *PCK1* ex-

TABLE 2. *PCK1* SNPs

Region	SNP name[a]	Genotyping method	Allele frequencies in control samples					
			African	European	South Asian	Chinese	Inult	Oji-Cree
Promoter	−1237C→T	Direct sequencing	−1237T: 0.41	0.45	0.47	0.37	0.50	0.52
Promoter	−1107C→T	Direct sequencing	−1107T: 0.41	0.45	0.47	0.37	0.50	0.52
Promoter	−232C→G	*Mae*III digestion	−232G: 0.20	0.31	0.40	0.30	0.42	0.56
Exon 1	190A→G	*Cac*I digestion	190G: 0.30	0.46	0.40	0.40	0.55	0.57
Exon 3	574G→A	Allele specific method	574A: 0.35	0.14	0.48	0.09	0.16	0.36
Exon 3	583A→G	*Taq*I digestion	583G: 0.33	0.45	0.43	0.08	0.17	0.31
Exon 3	610A→G	Allele specific method	610G: 0.33	0.45	0.36	0.09	0.15	0.33
Exon 3	671C→G (L184V)	*Bsr*I digestion	671G: 0.03	0.13	0.25	0.31	0.21	0.33
Exon 6	1261C→T	*Bsa*HI digestion	1261T: 0.05	0.29	0.17	0.25	0.27	0.32

[a] Nucleotide numbering refers to numbers used in the cDNA sequence (GenBank accession no. NM_002591).

TABLE 3. *PCK1* SNP pairwise linkage disequilibrium estimates in African (upper half) and Caucasian (lower half) samples

	−1237C→T	−1107C→T	−232C→G	190A→G	574G→A	583A→G	610A→G	671C→G	1261C→T
−1237C→T		1.00[a]	0.150	0.017	0.010	0.037	0.014	0.268	0.264
−1107C→T	1.00[a]		0.150	0.017	0.010	0.037	0.014	0.268	0.264
−232C→G	0.235	0.235		0.732[a]	0.080	0.601[a]	0.056	0.105	0.103
190A→G	0.218	0.218	0.904[a]		0.172	0.983[a]	0.109	0.045	0.258
574G→A	0.075	0.075	0.001	0.108		0.124	0.944[a]	0.223	0.162
583A→G	0.244	0.244	0.938[a]	0.829[a]	0.016			0.139	0.179
610A→G	0.036	0.036	0.035	0.010	0.828[a]	0.010		0.340	0.179
671C→G	0.036	0.036	0.179	0.007	0.022	0.169	0.006		0.910[a]
1261C→T	0.060	0.060	0.191	0.103	0.135	0.103	0.496[a]	0.101	

[a] $P < 0.05$.

TABLE 4. *PCK1* SNP pairwise linkage disequilibrium estimates in East Indian (upper half) and Chinese (lower half) samples

	−1237C→T	−1107C→T	−232C→G	190A→G	574G→A	583A→G	610A→G	671C→G	1261C→T
−1237C→T		1.00[a]	0.886[a]	0.794[a]	0.187	0.864[a]	0.287	0.087	0.031
−1107C→T	1.00[a]		0.886[a]	0.794[a]	0.187	0.864[a]	0.287	0.087	0.031
−232C→G	0.861[a]	0.861[a]		0.847[a]	0.158	0.927[a]	0.150	0.041	0.376
190A→G	0.010	0.010	0.062		0.244	0.923[a]	0.227	0.066	0.046
574G→A	0.299	0.299	0.141	0.241		0.517[a]	0.884[a]	0.010	0.210
583A→G	0.394[a]	0.394[a]	0.137[a]	0.264	0.964[a]		0.140	0.075	0.371
610A→G	0.299[a]	0.299[a]	0.141	0.426[a]	1.00[a]	0.916[a]		0.018	0.121
671C→G	0.066	0.066	0.001	0.066	0.010	0.075	0.018		0.634[a]
1261C→T	0.507[a]	0.507[a]	0.042	0.338	0.012	0.023	0.012	0.296	

[a] $P < 0.05$.

TABLE 5. *PCK1* SNP pairwise linkage disequilibrium estimates in Oji-Cree (upper half) and Inult (lower half) samples

	−1237C→T	−1107C→T	−232C→G	190A→G	574G→A	583A→G	610A→G	671C→G	1261C→T
−1237C→T		1.00[a]	0.700[a]	0.815[a]	0.052	0.334[a]	0.006	0.208	0.653[a]
−1107C→T	1.00[a]		0.700[a]	0.815[a]	0.052	0.334[a]	0.006	0.208	0.653[a]
−232C→G	0.405[a]	0.405[a]		0.629[a]	0.216	0.574[a]	0.045	0.490[a]	0.299[a]
190A→G	0.208	0.208	0.139		0.168	0.166	0.254	0.606[a]	0.690[a]
574G→A	0.104	0.104	0.418[a]	0.229		0.146	0.952[a]	0.288	0.119
583A→G	0.456[a]	0.456[a]	0.456[a]	0.450	0.955[a]		0.042	0.004	0.670[a]
610A→G	0.282	0.282	0.592[a]	0.487[a]	0.918[a]	1.00[a]		0.463[a]	0.151
671C→G	0.177	0.177	0.425[a]	0.606[a]	0.288[a]	0.004	0.463[a]		0.332[a]
1261C→T	0.104	0.104	0.562[a]	0.091	0.016	0.076	0.162	0.759[a]	

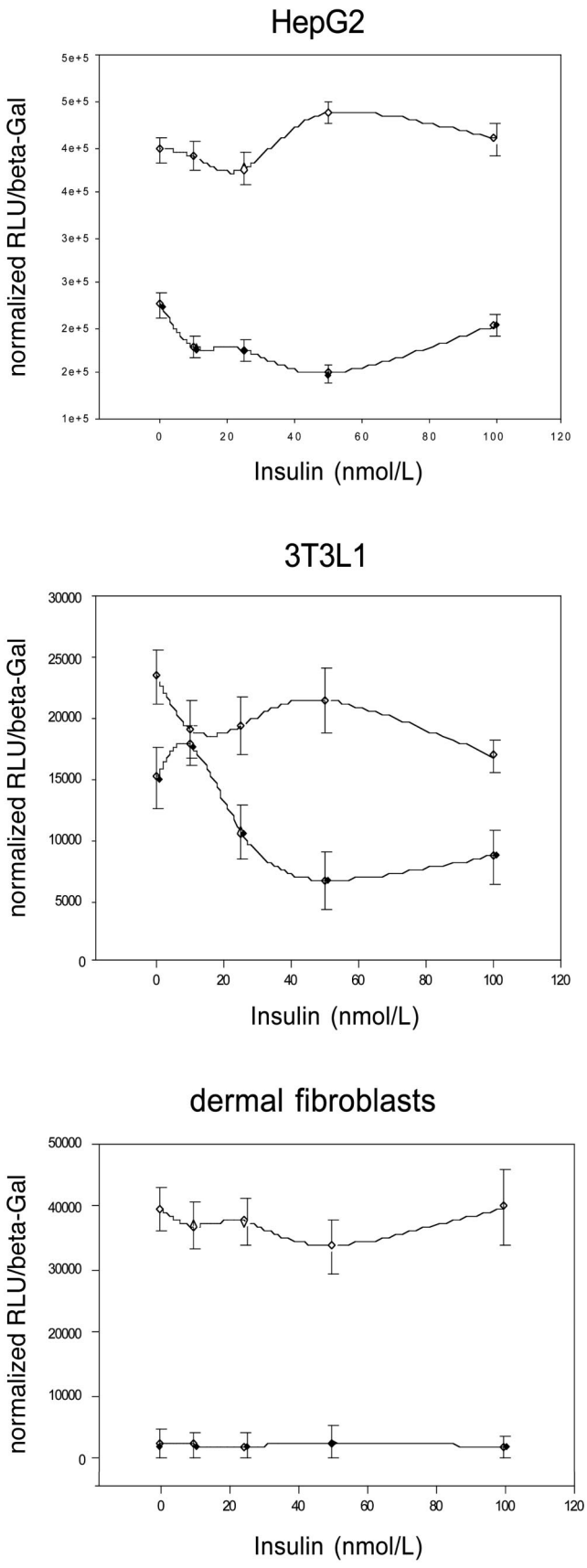
[a] $P < 0.05$.

TABLE 6. *PCK1* −232C→G SNP genotype and allele frequencies in Caucasian and Oji-Cree samples

	Total	Genotype no. and frequencies			*P* value	Allele frequencies −232G	*P* value
		−232C/C	−232G/C	−232G/G			
Oji-Cree type 2 DM	170	27	82	61		0.600	
		0.159	0.482	0.359			
					0.0065		0.0014
Oji-Cree controls	392	102	191	99		0.496	
		0.260	0.487	0.253			
Caucasian type 2 DM	115	23	68	24		0.504	
		0.200	0.591	0.209			
					0.0002		0.0003
Caucasian controls	260	107	117	36			
		0.411	0.450	0.139		0.364	

pression through an insulin response sequence (IRS) between nt −395 and −386, which is downstream of the IRS in the rat promoter (14, 20). No naturally occurring human variants have been found in this region (4). Our results demonstrate that a common *PCK1* promoter variant remote from the IRS might alter normal down-regulation by insulin *in vitro*, although the mechanisms are unclear. Despite the obvious *in vitro* consequences, it is not clear that the differences observed in transcription will necessarily result in important physiological differences leading to type 2 DM due to such factors as redundancy of pathways *in vivo*. Independent replication of both the association and the transcriptional findings would be required using different populations and *in vitro* model systems, respectively.

As with any association study, a positive result may not necessarily be due to the variant in question being a direct determinant of risk, but might arise from population stratification or LD with a nearby unmeasured risk determining



polymorphism. The results summarized in Tables 3–5 suggest that there are distinctive LD relationships between *PCK1* SNPs in different ethnic groups. For instance, of all pairwise LD estimates, approximately one sixth were significant in African and Caucasian subjects, about one third were significant in East Indian and Chinese subjects, and about half were significant in aboriginal (Oji-Cree and Inuit) populations, strongly suggesting interethnic differences in the genetic architecture of *PCK1*. Furthermore, the block of alleles most commonly associated with −232C→G differed between ethnic groups; only 583A→G showed significant LD with −232C→G in all groups. These varying LD relationships taken together with the clear *in vitro* functional impact of −232C→G increase the likelihood that this marker is itself a contributor to the observed genetic associations.

The −232C→G SNP occurs close to a region analogous to the P3(I) domain of the rat *PCK1* promoter (14). This domain is *trans*-activated when CCAAT/enhancer-binding protein (C/EBP) isoforms C/EBPα and C/EBPβ form heterodimers and bind. C/EBP is centrally involved in regulating the multiple hormone response of *PCK1* gene expression. Engagement of the C/EBPα and C/EBPβ complex to the P3(I) regulatory element is required along with thyroid hormone (T_3) and the retinoid X receptor binding in a heterodimer to the thyroid response element for T_3 stimulation of *PCK1* (21). cAMP stimulation of *PCK1* transcription requires both cAMP response elements to bind cAMP response element-binding protein or C/EBP (with similar affinity) and C/EBP to bind to the P3(I) regulatory element (22). Alterations of such protein-DNA interactions can now be studied using *PCK1* promoter −232C- and −232G-containing constructs.

How might the −232G→C SNP alter insulin suppression of *PCK1* expression? In transgenic mice, mutation of the accessory factor 2-binding region in *PCK1* (between nt −451 and −353) abolished glucocorticoid-dependent hepatic transcription, with no inhibition of expression by insulin (23). Furthermore, the

FIG. 1. Results of *in vitro* expression analysis of luciferase reporter constructs containing either *PCK1* −232C (●) or −232G (○). The *upper*, *middle*, and *lower panels* show results for transfections in HepG2 cells, 3T3L1 cells, and dermal fibroblasts, respectively. Concentrations of insulin are shown on the *abscissa*. Normalized RLU per unit of β-galactosidase activity are shown on the *ordinate*. The mean ± SE of reporter activity for each insulin concentration is shown for three sets of triplicate experiments performed on 2 separate days. The normalized basal (insulin concentration, 0 nmol/liter) luciferase activity was significantly higher for HepG2 cells, 3T3L1 cells, and dermal fibroblasts transfected with the −232G-containing construct than in those transfected with the −232C-containing construct ($P = 0.02$, $P = 0.05$, and $P < 0.0001$, respectively). Repeated measures analysis of reporter activity across the range of insulin concentrations showed significant differences between the −232G- and −232C-containing constructs transfected in HepG2 ($P = 0.0003$), 3T3L1 ($P = 0.0008$), and dermal fibroblast ($P < 0.0001$) cell lines. Paired *t* tests showed that for an insulin concentration of 50 nmol/liter, reporter activity was significantly reduced from baseline for the −232C-containing construct transfected in both HepG2 ($P = 0.0003$) and 3T3L1 ($P = 0.02$) cell lines, but not in fibroblasts. Paired *t* tests showed that for an insulin concentration of 100 nmol/liter, reporter activity was significantly reduced from baseline for the −232C-containing construct transfected in both HepG2 ($P = 0.05$) and 3T3L1 ($P = 0.02$) cell lines, but not in fibroblasts. In contrast, these differences were not significant for the −232G-containing construct in any cell line (all $P > 0.30$).

induced mutation had opposite effects in liver and kidney, indicating that tissue specificity compounds the complexity of *PCK1* expression and demonstrating that insulin does not exert its negative effect on the *PCK1* promoter through accessory factor 2 (23). Full *PCK1* promoter activation also requires the coactivator PPARγ coactivator-1 (PGC-1) (24). The dependence of insulin suppression of *PCK1* expression on PGC-1 was also suggested by induction of PGC-1 expression in both type 2 DM and mice with a disrupted insulin receptor gene (25).

The region of chromosome 20q in proximity to *PCK1* was linked with type 2 diabetes in at least two genome-wide scans (26, 27), implicating *PCK1* as a candidate gene based on both chromosomal position and function. Although earlier analysis of the *PCK1* promoter using SSCP found no genomic variants in type 2 DM (4), a more recent study using SSCP identified *PCK1* −232C→G and found it to be associated with type 2 diabetes in Japanese subjects (28). Thus, SSCP might have variable sensitivity for detecting certain SNPs. There have been no systematic studies comparing the sensitivity and specificity of SSCP with direct DNA sequencing to detect mutations in different genomic regions using different protocols. In our facility, the increment in cost for screening by direct genomic DNA sequencing compared with SSCP is about 2.5-fold. Discoveries of diabetes-causing gene mutations using sequencing as a screening method (5–8) have lowered our threshold for using this technology. Given the importance of minimizing false negative results when screening for DNA variants with potential pathogenic implications, it may be advantageous to include direct genomic sequencing in a screening strategy despite the increment in cost.

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References

1. **Hanson R, Garber A** 1972 Phosphoenolpyruvate carboxykinase: its role in gluconeogenesis. Am J Clin Nutr 25:1010–1021
2. **Rongstad R** 1979 Rate-limiting step in metabolic pathway. J Biol Chem 245: 1875–1878
3. **Granner DK, O'Brien RM** 1992 Molecular physiology and genetics of NIDDM: importance of metabolic staging. Diabetes Care 15:369–395
4. **Ludwig DS, Vidal-Puig A, O'Brien RM, Printz RL, Granner DK, Moller DE, Flier JS** 1996 Examination of the phosphoenolpyruvate carboxykinase gene promoter in patients with noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 81:503–506
5. **Hegele RA, Cao H, Harris SB, Hanley AJ, Zinman B** 1999 The hepatic nuclear factor-1α G319S variant is associated with early-onset type 2 diabetes in Canadian Oji-Cree. J Clin Endocrinol Metab 84:1077–1082
6. **Hegele RA, Cao H, Frankowski C, Mathews ST, Leff T** 2002 *PPARG* F388L, a transactivation-deficient mutant, in familial partial lipodystrophy. Diabetes 51:3586–3590
7. **Cao H, Hegele RA** 2000 Nuclear lamin A/C R482Q mutation in Canadian kindreds with Dunnigan-type familial partial lipodystrophy. Hum Mol Genet 9:109–112
8. **Cao H, Shorey S, Robinson J, Metzger DL, Stewart L, Cummings E, Hegele RA** 2002 *GCK* and *HNF1A* mutations in Canadian families with maturity onset diabetes of the young (MODY). Hum Mutat 20:478–479
9. **Harris SB, Gittelsohn J, Hanley A, Barnie A, Wolever TM, Gao J, Logan A, Zinman B** 1997 The prevalence of NIDDM and associated risk factors in native Canadians. Diabetes Care 20:185–187
10. **Triggs-Raine BL, Kirkpatrick RD, Kelly SL, Norquay LD, Cattini PA, Yamagata K, Hanley AJ, Zinman B, Harris SB, Barrett PH, Hegele RA** 2002 HNF-1α G319S, a transactivation-deficient mutant, is associated with altered dynamics of diabetes onset in an Oji-Cree community. Proc Natl Acad Sci USA 99:4614–4619
11. **Rocnik EF, van der Veer E, Cao H, Hegele RA, Pickering JG** 2002 Functional linkage between the endoplasmic reticulum protein Hsp47 and procollagen expression in human vascular smooth muscle cells. J Biol Chem 277:38571–38578
12. **Hegele RA, Plaetke R, Lalouel JM** 1990 Linkage disequilibrium between DNA markers at the low-density lipoprotein receptor gene. Genet Epidemiol 7:69–81
13. **Hill WG, Robertson A** 1968 Linkage disequilibrium in finite populations. Theor Appl Genet 38:226–231
14. **O'Brien RM, Printz RL, Halmi N, Tiesinga JJ, Granner DK** 1995 Structural and functional analysis of the human phosphoenolpyruvate carboxykinase gene promoter. Biochim Biophys Acta 1264:284–228
15. **Granner D, Andreone T, Sasaki K, Beale E** 1993 Inhibition of transcription of phosphoenolpyruvate carboxykinase gene by insulin. Nature 305:549–551
16. **Hanson RW, Reshef L** 1997 Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66:581–611
17. **Wynshaw-Boris A, Short JM, Loose DS, Hanson RW** 1986 Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region: multiple hormone regulatory elements and the effects of enhancers. J Biol Chem 261:9714–9720
18. **O'Brien RM, Lucas PC, Forest CD, Magnuson MA, Granner DK** 1990 Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. Science 249:533–537
19. **Liu J, Hanson RW** 1991 Regulation of phosphoenolpyruvate carboxykinase (GTP) gene transcription. Mol Cell Biochem 104:89–100
20. **O'Brien RM, Bonovich MT, Forest CD, Granner DK** 1991 Signal transduction convergence: phorbol esters and insulin inhibit phosphoenolpyruvate carboxykinase gene transcription through the same 10-base-pair sequence. Proc Natl Acad Sci USA 88:6580–6584
21. **Park EA, Song S, Olive M, Roesler WJ** 1997 CCAAT-enhancer-binding protein α (C/EBPα) is required for the thyroid hormone but not the retinoic acid induction of phosphoenolpyruvate carboxykinase (PEPCK) gene transcription. Biochem J 322:343–349
22. **Roesler WJ** 2002 At the cutting edge: what is a cAMP response unit? Mol Cell Endocrinol 162:1–7
23. **Lechner PS, Croniger CM, Hakimi P, Millward C, Fekter C, Yun JS, Hanson RW** 2001 The use of transgenic mice to analyze the role of accessory factor two in the regulation of phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. J Biol Chem 276:22675–22679
24. **Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman BM** 2001 Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413:131–138
25. **Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR** 2002 Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol Cell 6:87–97
26. **Zouali H, Hani EH, Philippi A, Vionnet N, Beckmann JS, Demenais F, Froguel P** 1997 A susceptibility locus for early-onset non-insulin dependent (type 2) diabetes mellitus maps to chromosome 20q, proximal to the phosphoenolpyruvate carboxykinase gene. Hum Mol Genet 6:1401–1408
27. **Ghosh S, Watanabe RM, Valle TT, Hauser ER, Magnuson VL, Langefeld CD, Ally DS, Mohlke KL, Silander K, Kohtamaki K, Chines P, Balow Jr J, Birznieks G, Chang J, Eldridge W, Erdos MR, Karanjawala ZE, Knapp JI, Kudelko K, Martin C, Morales-Mena A, Musick A, Musick T, Pfahl C, Porter R, Rayman JB** 2000 The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study. I. An autosomal genome scan for genes that predispose to type 2 diabetes. Am J Hum Genet 67:1174–1185
28. **Horikawa Y, Yamasaki T, Nakajima H, Shingu R, Yoshiuchi I, Miyagawa J, Namba M, Hanafusa T, Matsuzawa Y** 2003 Identification of a novel variant in the phosphoenolpyruvate carboxykinase gene promoter in Japanese patients with type 2 diabetes. Horm Metab Res 35:308–312