

Identification of a Novel β -Cell Glucokinase (*GCK*) Promoter Mutation ($-71G>C$) That Modulates *GCK* Gene Expression Through Loss of Allele-Specific Sp1 Binding Causing Mild Fasting Hyperglycemia in Humans

Daniela Gašperíková,¹ Nicolas D. Tribble,² Juraj Staník,^{1,3} Miroslava Hučková,¹ Nadežda Mišovicová,⁴ Martijn van de Bunt,² Lucia Valentínová,¹ Beryl A. Barrow,^{2,5} L'ubomir Barák,³ Radoslav Dobránsky,⁶ Eva Bereczková,⁷ Jozef Michálek,⁸ Kate Wicks,⁹ Kevin Colclough,¹⁰ Julian C. Knight,⁹ Sian Ellard,^{10,11} Iwar Klimeš,¹ and Anna L. Gloyn^{2,5}

OBJECTIVE—Inactivating mutations in glucokinase (*GCK*) cause mild fasting hyperglycemia. Identification of a *GCK* mutation has implications for treatment and prognosis; therefore, it is important to identify these individuals. A significant number of patients have a phenotype suggesting a defect in glucokinase but no abnormality of *GCK*. We hypothesized that the *GCK* β -cell promoter region, which currently is not routinely screened, could contain pathogenic mutations; therefore, we sequenced this region in 60 such probands.

RESEARCH DESIGN AND METHODS—The β -cell *GCK* promoter was sequenced in patient DNA. The effect of the identified novel mutation on *GCK* promoter activity was assessed using a luciferase reporter gene expression system. Electrophoretic mobility shift assays (EMSAs) were used to determine the impact of the mutation on Sp1 binding.

RESULTS—A novel $-71G>C$ mutation was identified in a nonconserved region of the human promoter sequence in six apparently unrelated probands. Family testing established cosegregation with fasting hyperglycemia (≥ 5.5 mmol/l) in 39 affected individuals. Haplotype analysis in the U.K. family and four of the Slovakian families demonstrated that the mutation had arisen independently. The mutation maps to a potential transcriptional activator binding site for Sp1. Reporter assays demonstrated that the mutation reduces promoter activity by up to fourfold. EMSAs

demonstrated a dramatic reduction in Sp1 binding to the promoter sequence corresponding to the mutant allele.

CONCLUSIONS—A novel β -cell *GCK* promoter mutation was identified that significantly reduces gene expression in vitro through loss of regulation by Sp1. To ensure correct diagnosis of potential *GCK*-MODY (maturity-onset diabetes of the young) cases, analysis of the β -cell *GCK* promoter should be included. *Diabetes* 58:1929–1935, 2009

Diagnostic molecular genetic testing is available for many different monogenic forms of diabetes (1). One of the remaining clinical and scientific challenges, however, are the patients who clearly have a monogenic subtype of diabetes but are negative on screening using existing genetic tests (2,3). Genetic linkage analysis can be used to demonstrate or exclude linkage to known genes if there are sufficient family members to reach statistical significance (4).

However, many patients presenting with apparent monogenic forms of diabetes do not have large extended families to facilitate this process (4). Traditionally, the coding region and exon-intron boundaries of the gene of interest have been screened for mutations; in some cases, high levels of conservation between species have been used to identify putative regulatory regions such as promoters and enhancers for additional mutational screening (5,6).

Mutations in the gene encoding the key regulatory enzyme glucokinase (*GCK*) are one cause of maturity-onset diabetes of the young (MODY) subtype *GCK* (*GCK*-MODY) previously termed MODY2, which is an autosomal dominantly inherited form of non-insulin-dependent diabetes classified by the gene that is mutated (7). The phenotype of *GCK*-MODY has been well characterized and is distinct from other subtypes of MODY as a result of mutations in genes encoding transcription factors (8).

The heterozygous inactivating mutations seen in patients with *GCK*-MODY shift the set point for glucose-stimulated insulin secretion from ~ 5 to ~ 7 mmol/l, resulting in elevated fasting plasma glucose (FPG) levels (5.5–8.0 mmol/l) but normal 2-h plasma glucose increments (typically < 4.6 mmol/l) after a 75-g oral glucose tolerance test (8). There are a number of patients fulfilling these criteria who have no abnormality of the *GCK* coding region (9). Tissue-specific expression of *GCK* is

From the ¹DIABGENE and Diabetes Laboratory, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic; the ²Diabetes Research Laboratories, Oxford Centre for Diabetes, Endocrinology & Metabolism, University of Oxford, Oxford, U.K.; the ³Children Diabetes Center at the First Pediatric Department, Comenius University School of Medicine, Bratislava, Slovak Republic; the ⁴Department of Clinical Genetics, Jessenius School of Medicine, Martin, Slovak Republic; the ⁵Oxford NIHR (National Institute for Health Research) Biomedical Research Centre, Churchill Hospital, Oxford, U.K.; the ⁶Diabetology Outpatient Department, Reimanus Hospital, Presov, Slovak Republic; the ⁷Children Endocrinology Outpatient Department, Dunajska Streda, Slovak Republic; the ⁸National Institute of Endocrinology and Diabetology, Lubochna, Slovak Republic; the ⁹Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, U.K.; the ¹⁰Department of Molecular Genetics, Royal Devon and Exeter NHS Foundation Trust, Exeter, U.K.; and the ¹¹Institute of Biomedical and Clinical Science, Peninsula Medical School, Exeter, U.K.

Corresponding author: Anna L. Gloyn, anna.gloyn@drf.ox.ac.uk.

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D.G. and N.D.T. contributed equally to this study.

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A SK R78

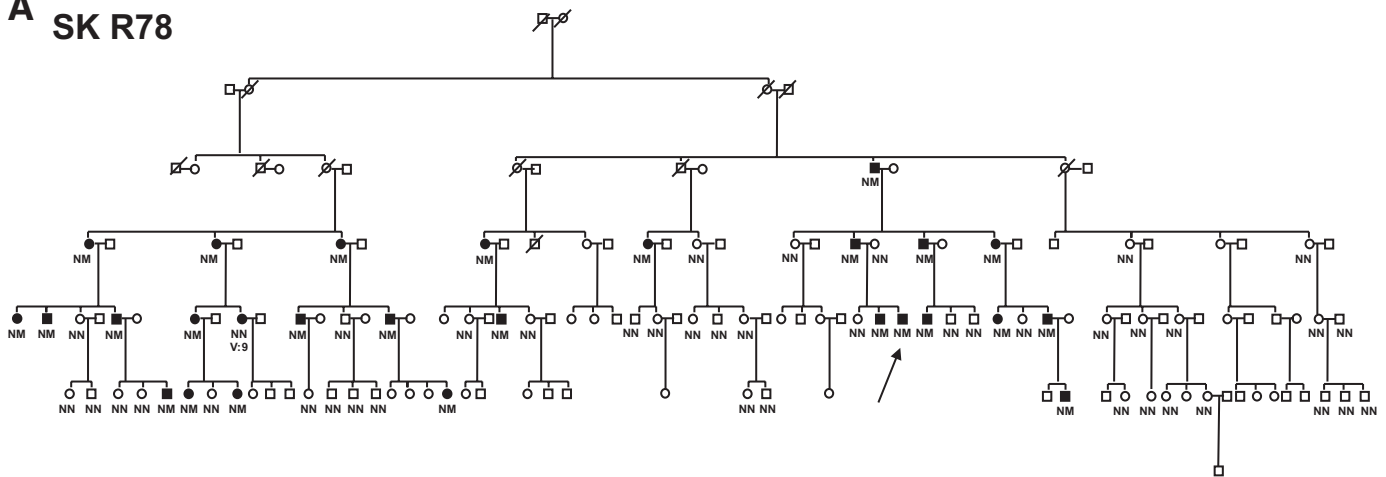
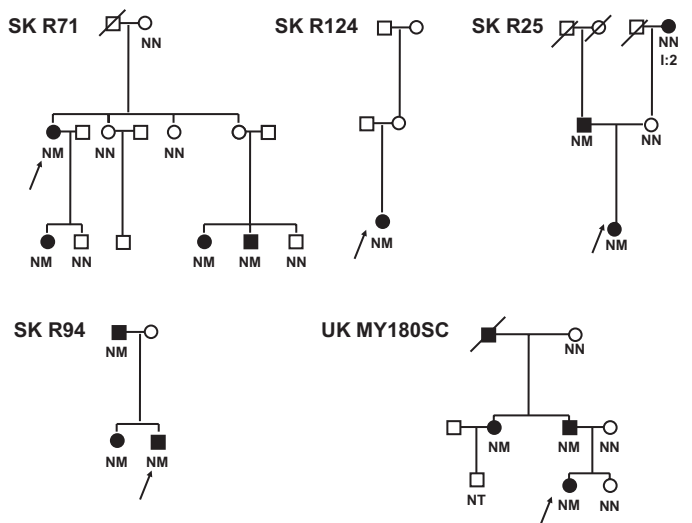


FIG. 1. A: Extended pedigrees of the six probands with the novel $-71G>C$ *GCK* promoter mutation showing five Slovakian (SK R78, SK R25, SK R71, SK R94, and SK R124) and one U.K. (UK MY180SC) family. Arrows indicate the probands. Filled icons indicate individuals with fasting hyperglycemia (≥ 5.5 mmol/l); open icons indicate normoglycemic subjects. The *GCK* mutation status is shown under each symbol. Individuals V:9 (SK R78 family) and I:2 (SK R25 family) are diabetic subjects (phenocopies) with the NN genotype (see RESULTS). NM, heterozygous for the $-71G>C$ *GCK* promoter mutation; NN, wild type. **B:** Haplotype analysis performed in five of the six families with the novel *GCK* promoter mutation. Squares denote male subjects, circles denote female subjects, and solid symbols show individuals with fasting hyperglycemia (≥ 5.5 mmol/l). Genotype is shown underneath each symbol. Solid bars indicate the haplotype cosegregating with fasting hyperglycemia on which the *GCK* c. $-71G>C$ mutation has arisen. The marker order, location of the *GCK* gene, and location on chromosome 7 are shown in the box. NM, heterozygous mutation; NN, mutation not present.



governed by two promoters, initially described as specific for pancreatic β -cells and hepatocytes but now recognized to regulate expression in a wider range of tissues (10). The rodent hepatic *GCK* promoter has been extensively characterized, but there are relatively few data on the transcriptional regulation of the human β -cell promoter (11).

The aim of this study was to extend our mutational screen in probands with a phenotype consistent with an abnormality of *GCK* that have no abnormality of the coding sequence to the β -cell *GCK* promoter to identify mutations that could affect *GCK* expression.

RESEARCH DESIGN AND METHODS

Unrelated probands of 60 families (30 from Slovakia and 30 from the U.K.) with a clinical phenotype suggesting a defect in *GCK* but without a mutation in the *GCK* coding region were included in the study. Partial or entire deletions of the *GCK* gene were previously excluded by multiplex ligation-dependent probe amplification (MLPA) analysis in all U.K. probands (9). Selection criteria included FPG levels ≥ 5.5 mmol/l, treatment by diet, oral hypoglycemic agents, or very low doses of insulin (mean dose consistently < 0.3 IU \cdot kg $^{-1}$ \cdot day $^{-1}$) and detectable C-peptide levels. Eighty-five blood relatives of the six probands with the *GCK* pancreatic promoter mutation were subsequently contacted and invited for blood sampling and mutation testing. This study was performed with full approval of the ethics committees in Bratislava and Lubochna (Slovakia) and in the U.K., and all subjects gave informed consent.

Genetic analysis of the human β -cell *GCK* promoter. DNA was isolated from peripheral blood using standard protocols. 324bp of the human pancreatic islet *GCK* promoter was amplified by PCR (primers available upon request). The promoter region was examined by direct sequencing on an ABI 3130 Capillary Sequencer (Applied Biosystems, Warrington, U.K.). Sequences were compared with the published sequence (NM_000162.2), using either SeqScape (version 2.1.1; Applied Biosystems) or Mutation Surveyor software (version 3.0; Softgenetics, Cambridge, U.K.). Haplotypes were constructed using microsatellites D7S3043, D7S691, D7S2428, GCK1, GCK2, D7S667, and D7S2506 that were run on an ABI 3130 analyzer. MLPA analysis was performed in the five Slovakian probands with the *GCK* promoter mutation using the SALSA MLPA kit P241-Bi MODY/MRC-Holland (Amsterdam, the Netherlands). Results were analyzed using GeneMarker (version 1.75; Softgenetics). To circumvent computational constraints as a result of the pedigree size, the pedigree SK R78 was divided into 40 two-generation small pods using a facility in MEGA2 (<http://portal.litbio.org/registered/help/mega2/index/html>) and the analyses were run in Merlin (12). The logarithm of odds (LOD) score for family SK R78 was performed applying a rare autosomal dominant inheritance model using a disease allele frequency of 0.001 and a frequency of phenocopies of 0.1%.

Biochemistry. Fasting venous blood samples for glucose and hormonal analyses were collected into EDTA tubes (Sarstedt, Nümbrecht, Germany). Plasma glucose concentrations were measured with the glucose oxidase method (Hitachi 911, Hitachinaka, Japan). Insulin and C-peptide were determined using the Elecsys (Roche, Switzerland) chemiluminescence automatic analyzer.

β -Cell *GCK* promoter analysis. Alignment of the human β -cell *GCK* promoter was performed using the University of California Santa Cruz (USCS) bioinformatics BLAT tool. The human β -cell promoter was analyzed for

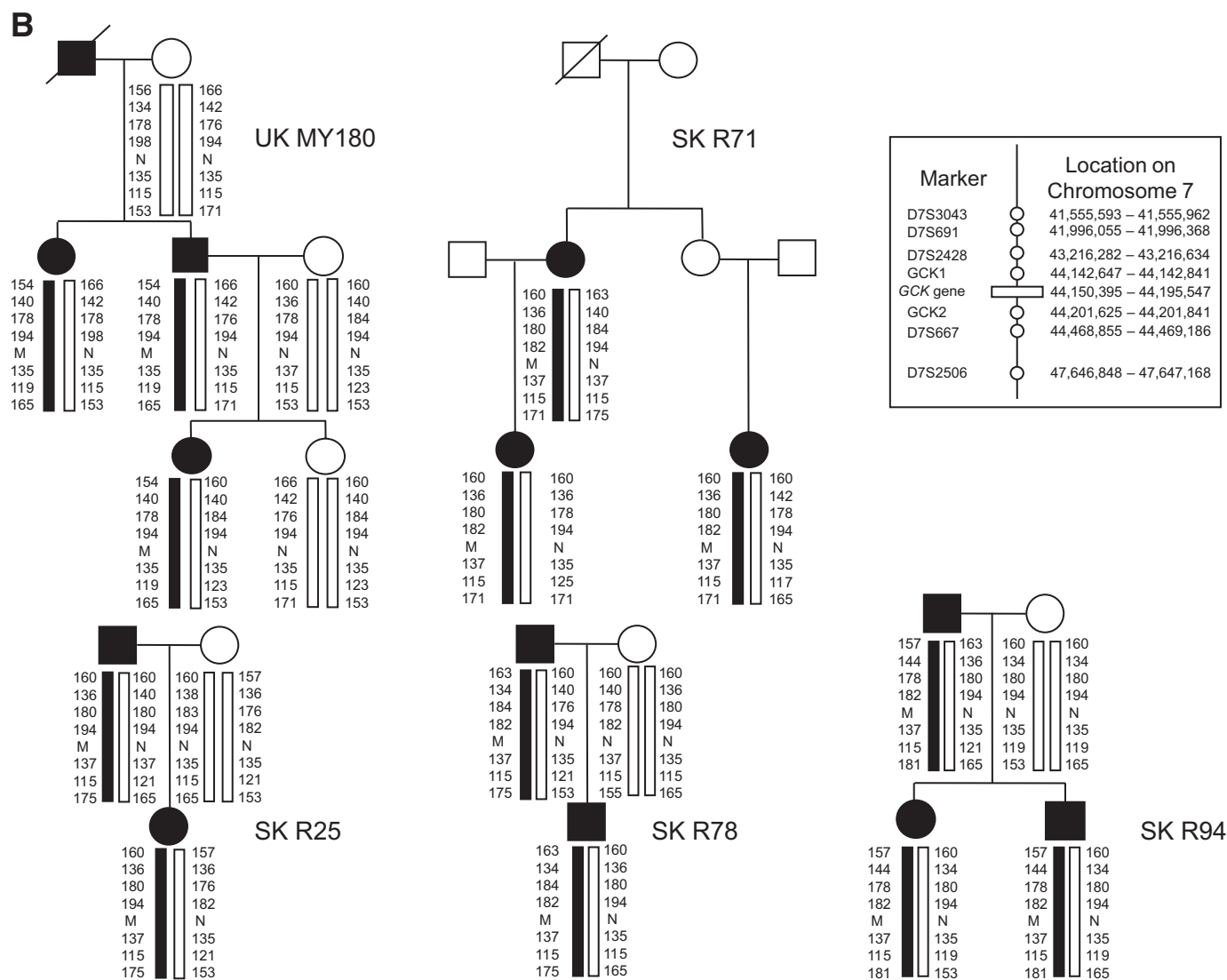


FIG. 1. Continued.

potential transcription factor binding sites by running a Matrix Search for Transcription Factor Binding Sites (MATCH) using TRANSFAC Professional (version 12.1).

Transfection constructs. PCR primers were designed to generate five different lengths of the human *GCK* upstream promoter sequence (supplementary tables and figures, available in an online appendix at <http://diabetes.diabetesjournal.org/cgi/content/full/db09-0070/DC1>). The promoter fragments were amplified from human genomic DNA and subcloned into the pCR 2.1 TOPO vector (Invitrogen, Paisley, U.K.). Prior to cloning, DNA sequencing was performed to ensure that the β -cell *GCK* promoter haplotype associated with variation in FPG levels was not included (13). The fragments were subcloned into a predigested pGL3-basic luciferase reporter vector (Promega, Southampton, U.K.). The $-71G>C$ mutation was introduced using a Quick change mutagenesis kit (Stratagene, La Jolla, CA) (supplementary Table 2). All mutations were confirmed by bidirectional sequencing.

Luciferase assay. INS-1 cells were cultured as previously described to a density of 1×10^4 per well in 24-well tissue culture dishes 24 h before transfection (14). Cotransfection was performed using lipofectamine reagent (Invitrogen) with 580 ng pGL3 promoter construct DNA plus 20 ng pRL-TK per well, according to the manufacturer's protocol. Transfected cells were harvested after 24 h and assayed for luciferase activity in a Veritas microplate luminometer using the Dual Luciferase Reporter Assay System (Promega). The *Renilla* luciferase (pRL-TK) was used as a recovery marker for data normalization. Promoter data are reported as the ratio of the firefly/*Renilla* arbitrary units for each sample minus the value gained by the promoterless pGL3-basic vector. Each transfection experiment was carried out in triplicate on at least three independent occasions. Statistical significance was determined by two-tailed Student's *t* test.

Electrophoretic mobility shift assays. Both wild-type and -71 mutant *GCK* promoter oligonucleotides were generated spanning the potential Sp1 binding region predicted from TRANSFAC analysis and corresponding to sequences -53 to -88 bp from the transcriptional start site. An additional oligonucleotide corresponding to a known Sp1 binding site was also used as a positive control for Sp1 binding (Promega). The oligonucleotide probe sequences are provided in supplementary Table 3. Binding reactions and electrophoretic mobility shift assays were conducted as previously described (15,16). Briefly, 200 ng Sp1 protein (Promega) was incubated with 0.2–0.5 ng of $\alpha^{32}P$ -labeled probe ($1-5 \times 10^4$ cpm) at room temperature for 30 min. For supershift or competition analysis, the reaction mixture was preincubated with 1 μ g Sp1 or USF1 antiserum (Santa Cruz Biotechnology, Heidelberg, Germany) or with 7.5 pmol (100X) unlabeled oligonucleotide probe at room temperature for 20 min prior to addition of the labeled probe.

RESULTS

Identification of a novel $-71G>C$ *GCK* β -cell promoter mutation. Screening of 60 probands with a GCK-MODY phenotype and no abnormality of the *GCK* coding sequence identified six (five from Slovakia and one from the U.K.) apparently unrelated individuals with a novel $-71G>C$ mutation. This mutation was not identified in >400 ethnically matched normal chromosomes. Family testing demonstrated that the mutation cosegregated with

TABLE 1
Clinical characteristics of individuals with the novel $-71G>C$ mutation

	<i>GCK</i> promoter mutation carriers	Nonmutation carriers	Published data on <i>GCK</i> mutation carriers (8)
<i>n</i>	39	52	245
Sex (male/female)	19/20	17/35	125/120
Age (years)	37.0 (8–86)	31.0 (1–80)	26.7 (2–79)
BMI (kg/m ²)	24.6 (18.2–40.8)	25.3 (19.3–36.9)	21.1 (13.8–40.9)
FPG (mmol/l)	7.0 ± 1.0	5.1 ± 0.5	6.8 ± 0.8

Data are means (range) or means ± SD unless otherwise indicated.

fasting hyperglycemia (≥ 5.5 mmol/l) in a total of 39 affected individuals and 52 unaffected individuals across the six pedigrees (Fig. 1A). Using a rare autosomal dominant model, the LOD score in family SK R78 alone was 5.00 that is well above the proposed guidelines for claiming statistically significant linkage with a LOD score of >3.0 (17). MLPA analysis excluded partial or entire *GCK* gene deletions in all six probands. Haplotype analysis in the U.K. family and in four of the Slovakian families demonstrated that the mutation had arisen independently and there was no evidence for a founder effect (Fig. 1B). The β -cell $-71G>C$ promoter mutation is responsible for $\sim 30\%$ (5 of 17 families) of known cases of *GCK*-MODY in Slovakia (D.G., J.S., and I.K., unpublished data), whereas in the U.K. only one family has been identified and over 160 *GCK*-MODY cases with coding mutations have been identified (K.C., A.T. Hattersley, and S.E., unpublished data).

Clinical characteristics of patients with the novel promoter mutation. The clinical characteristics of the individuals with the novel promoter mutation are summarized in Table 1. In line with other cases of *GCK*-MODY, all patients had fasting hyperglycemia (8). The age at diagnosis ranged from 6 to 81 years; 15 case subjects were identified following the detection of the *GCK* mutation in one of the six probands. In affected individuals, BMI ranged from 18.2 to 40.8 kg/m² with a mean ± SD value of 24.6 ± 5.3 kg/m². The majority of patients (23 of 39 [59%]) with the mutation are registered with and seen by a diabetologist.

Of these, 83% (19 of 23) are managed by diet alone, with three patients on oral hypoglycemic agents (one receiving metformin [1,000 mg/day] and two receiving sulfonylureas [Diaprel MR, twice daily]) and one patient on insulin treatment (NPH insulin [0.22 units · kg⁻¹ · day⁻¹]). The decision to treat these four patients was made by their referring physicians. None of these patients have had A1C values $>7\%$, whereas A1C values on treatment are all $<6.5\%$. FPG levels in these patients are independent of insulin dose and/or oral hypoglycemic agents used.

There were two individuals without the novel promoter mutation with diabetes. One patient, a female aged 50 years (pedigree SK R78, subject V:9) (Fig. 1A) with a BMI 28.0 kg/m² has several features consistent with the metabolic syndrome including hypertension (blood pressure 140/90 mmHg) and dyslipidemia (total cholesterol 5.79 mmol/l). She was diagnosed with hyperglycemia at the age of 47 years and is currently treated with metformin. The second female patient (SK R25, I:2) (Fig. 1A) was diagnosed with diabetes at the age of 49 years and is insulin treated. No further clinical or laboratory data are available on this subject because following blood withdrawal for genetic testing the patient refused to cooperate further in

the study. Based on these clinical characteristics, both of these patients have a phenotype that is consistent with classical type 2 diabetes and can be considered as phenocopies.

Activity of β -cell *GCK* promoter in INS-1 cells and effects of the $-71G>C$ mutation. To establish the appropriate promoter construct in which to study the effect of the $-71G>C$ mutation, reporter gene constructs containing different *GCK* promoter fragment lengths were prepared ranging from -263 bp to -1031 bp with respect to the ATG start site. Luciferase activity was detectable with all *GCK* promoter constructs (Fig. 2A).

Decreased expression levels were seen with fragments -618 bp and -826 bp suggesting the presence of repressor elements within this region. The effect of the $-71G>C$ mutation was then analyzed using the -430 bp promoter fragment that had near-maximal reporter gene expression, indicating that necessary binding sites for expression were contained in this fragment; site-directed mutagenesis was also carried out for the full length -1031 bp fragment. When these constructs were analyzed, the *GCK* $-71G>C$ promoter variant caused a dramatic reduction in promoter activity in the presence of the C versus G allele for both promoter lengths ($P = 3.6 \times 10^{-6}$ and 1.9×10^{-6} for the -430 bp and -1031 bp fragments, respectively) (Fig. 2B). This provides robust evidence that the point mutation has a repressive effect on gene expression, suggesting either allele-specific recruitment of a transcriptional repressor or loss of activator binding.

Identification of potential transcription factor binding sites in the β -cell *GCK* promoter. Using the USCS BLAT tool, no sequences could be identified corresponding to the -64 bp to -89 bp region of the human *GCK* promoter in the mouse or rat *GCK* promoters as a result of either a deletion in the rodent or an insertion in the human DNA (supplementary Fig. 1). MATCH analysis conducted on the wild-type *GCK* promoter identified a number of potential activator and repressor regions within the -71 bp region of interest (supplementary Table 4). Identical MATCH analysis conducted on the -71 variant *GCK* promoter demonstrated the potential loss of the majority of these transcription factor binding sites including the transcriptional activator Sp1 (supplementary Table 4).

Sp1 binding is reduced due to the $-71G>C$ mutation. To demonstrate that Sp1 can indeed bind to the putative binding site located at the -71 bp region of the wild-type promoter, gel shift experiments were performed. Using the wild-type oligonucleotide, two clear bands that were specific on competition with molar excess of unlabeled self or an unlabeled probe corresponding to a consensus Sp1 binding site were observed (Fig. 3A).

The mobility of these bands matched those seen when

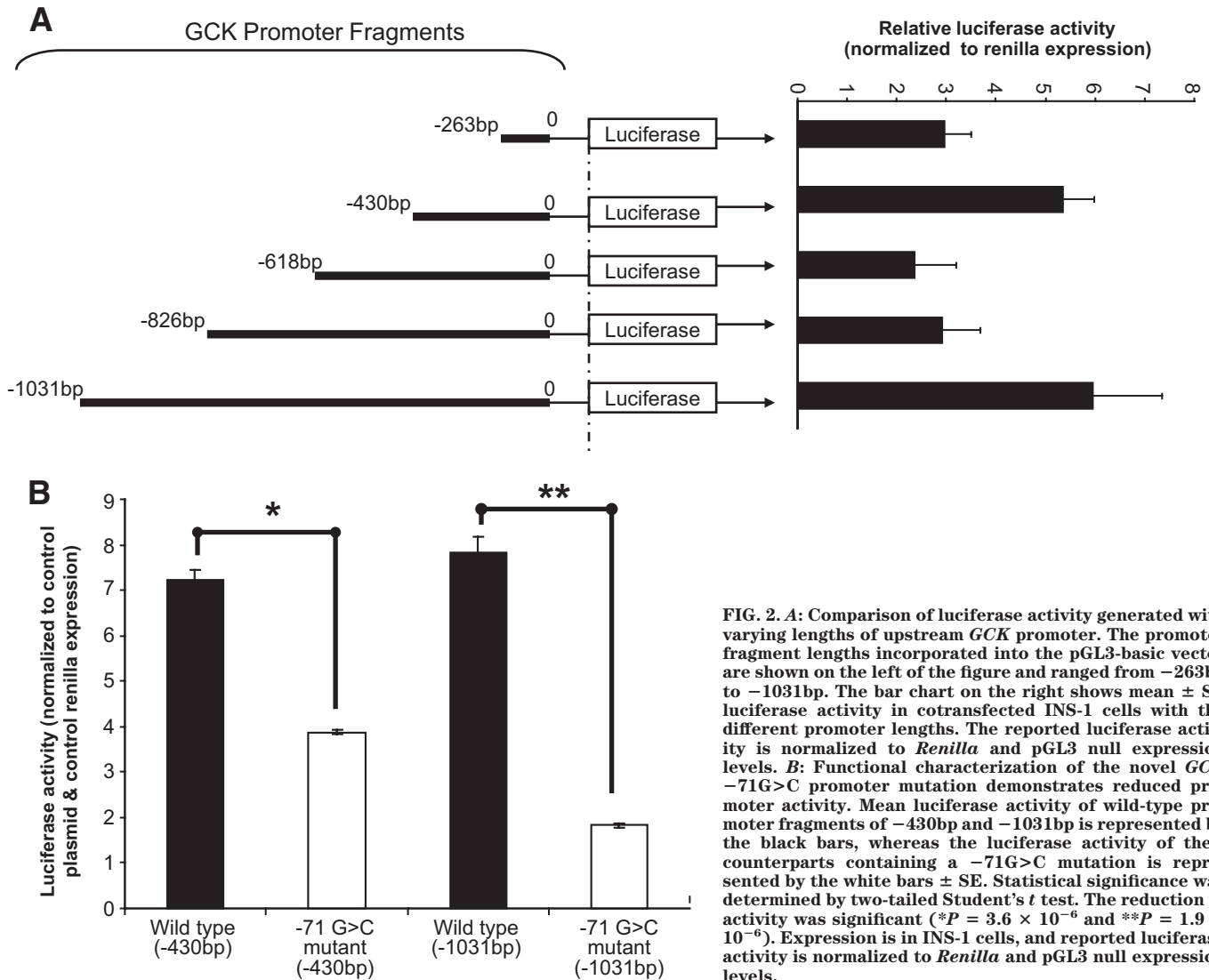


FIG. 2. A: Comparison of luciferase activity generated with varying lengths of upstream *GCK* promoter. The promoter fragment lengths incorporated into the pGL3-basic vector are shown on the left of the figure and ranged from -263bp to -1031bp . The bar chart on the right shows mean \pm SE luciferase activity in cotransfected INS-1 cells with the different promoter lengths. The reported luciferase activity is normalized to *Renilla* and pGL3 null expression levels. **B:** Functional characterization of the novel *GCK* $-71\text{G}>\text{C}$ promoter mutation demonstrates reduced promoter activity. Mean luciferase activity of wild-type promoter fragments of -430bp and -1031bp is represented by the black bars, whereas the luciferase activity of their counterparts containing a $-71\text{G}>\text{C}$ mutation is represented by the white bars \pm SE. Statistical significance was determined by two-tailed Student's *t* test. The reduction in activity was significant ($*P = 3.6 \times 10^{-6}$ and $**P = 1.9 \times 10^{-6}$). Expression is in INS-1 cells, and reported luciferase activity is normalized to *Renilla* and pGL3 null expression levels.

the consensus Sp1 binding site probe was radiolabeled and used in the binding reaction (Fig. 3B). The complexes were lost when a radiolabeled probe corresponding to the C-allele of the point mutation was used in the binding reaction; this probe also competed much less effectively when used as an unlabeled competitor (Fig. 3A). Recruitment of Sp1 was confirmed by supershift experiments for both the Sp1 consensus probe and the -71 wild-type probe bearing the G-allele (Fig. 3B).

DISCUSSION

In this study, we have identified the first mutation in the *GCK* β -cell promoter that causes elevated glucose levels as a result of decreased *GCK* expression in six probands: five from Slovakia and one from the U.K. The mutation cosegregated with fasting hyperglycemia in all six families with a total of 39 affected individuals (Fig. 1A). Haplotype analysis demonstrated that the mutation had arisen independently between the U.K. and Slovakian families, with no evidence for a founder effect in the Slovakian families (Fig. 1B). The clinical phenotype of patients with GCK-MODY attributable to a promoter mutation is indistinguishable from that caused by a mutation in the coding region of the gene (Table 1 and ref. 8). This is entirely

expected as compensation will be provided by the wild-type allele (18,19).

Functional characterization of the *GCK* $-71\text{G}>\text{C}$ promoter variant clearly demonstrates that the *GCK* $-71\text{G}>\text{C}$ promoter variant causes a dramatic reduction in promoter activity. Bioinformatic analysis predicted that this mutation could potentially result in the loss of transcription factor binding within the -71bp region. Allele-specific modulation of Sp1 binding was then demonstrated by the electrophoretic mobility shift assay. Sp1 has previously been demonstrated to enhance *GCK* transcription in gilthead sea bream (*Sparus aurata*) (20). In rodent studies, Sp1 has also been shown to play an integral part in the mechanisms for the transcription of several glycolytic enzymes, such as pyruvate kinase, aldolase A, and acetyl-CoA (21–23). The results clearly show an allele-specific difference in Sp1 binding with the $-71\text{G}>\text{C}$ mutation losing Sp1 binding capacity. This provides a clear explanation of the reduced gene expression levels associated with this novel mutation and the first evidence in humans for an essential role of Sp1 regulation in *GCK* expression.

The significance of this finding is that routine molecular diagnostic testing for GCK-MODY does not currently include the promoter region. Given the implications for prognosis, treatment decisions, and inheritance, it is im-

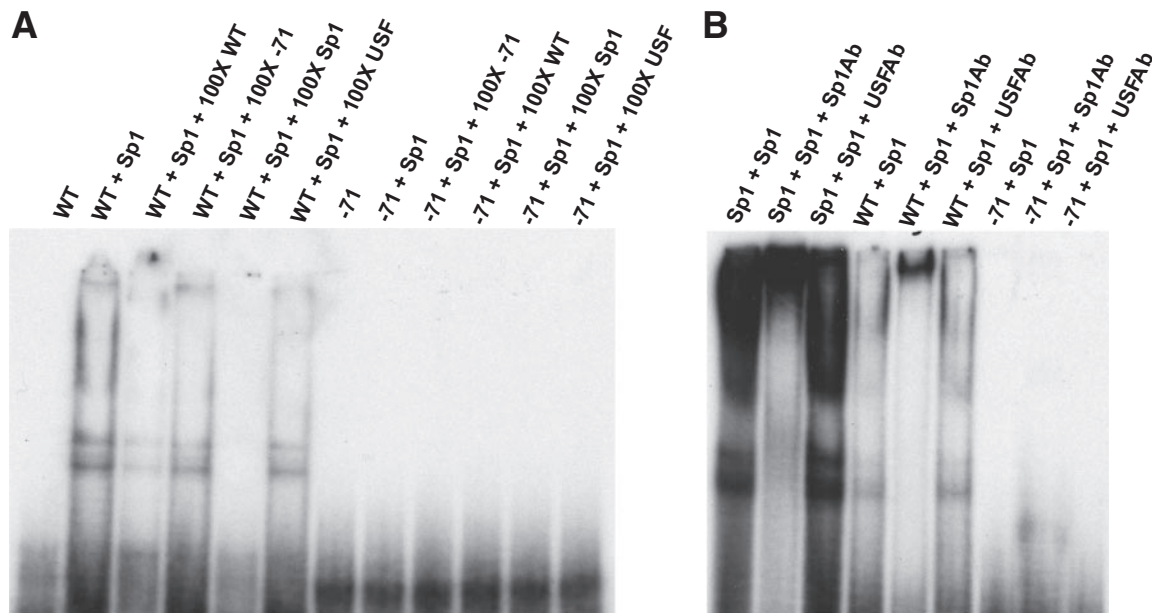


FIG. 3. DNA mobility shift assay with recombinant human Sp1 nuclear protein. Sp1 binds both the wild-type and Sp1 oligonucleotides, whereas the $-71G>C$ mutation causes a dramatic reduction in Sp1 binding. **A:** Labeled *GCK* promoter (-53 to -88) and Sp1 consensus oligonucleotides were incubated in the presence or absence of Sp1 nuclear protein. Competition experiments were conducted using 100-fold molar excess of unlabeled oligonucleotides. **B:** Specific Sp1 and nonspecific USF antibodies were used to confirm the specificity of Sp1 binding that was visualized as a supershifted band of reduced mobility.

portant to identify these individuals (24). Interestingly, a comparison of the promoter sequences across species demonstrated that the sequence containing the novel mutation was absent from both the rat and mouse β -cell promoters. This is unusual because normally pathogenic mutations occur in regions that are conserved (and therefore supposed to be functionally important) across species. This observation has implications for the design of future studies evaluating the role of noncoding regions in the pathogenesis of both monogenic and multifactorial diabetes.

We have identified the first mutation in the *GCK* β -cell promoter in a total of 39 individuals with mild fasting hyperglycemia. This mutation has been demonstrated at the genetic and functional level to cause GCK-MODY. Our results support the inclusion of the *GCK* β -cell promoter region in routine diagnostic testing for GCK-MODY.

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No potential conflicts of interest relevant to this article were reported.

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