A Novel Hypomorphic *PDX1* Mutation Responsible for Permanent Neonatal Diabetes With Subclinical Exocrine Deficiency

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OBJECTIVE—Genes responsible for monogenic forms of diabetes have proven very valuable for understanding key mechanisms involved in β-cell development and function. Genetic study of selected families is a powerful strategy to identify such genes. We studied a consanguineous family with two first cousins affected by neonatal diabetes; their four parents had a common ancestor, suggestive of a fully penetrant recessive mutation.

RESEARCH DESIGN AND METHODS—We performed genetic studies of the family, detailed clinical and biochemical investigations of the patients and the four parents, and biochemical and functional studies of the new mutation.

RESULTS—We found a novel mutation in the pancreatic and duodenal homeobox 1 gene (*PDX1*, *IPF1*) in the two patients, which segregated with diabetes in the homozygous state. The mutation resulted in an E178G substitution in the PDX1 homeodomain. In contrast to other reported *PDX1* mutations leading to neonatal diabetes and pancreas agenesis, homozygosity for the E178G mutation was not associated with clinical signs of exocrine pancreas insufficiency. Further, the four heterozygous parents were not diabetic and displayed normal glucose tolerance. Biochemical studies, however, revealed subclinical exocrine pancreas insufficiency in the patients and slightly reduced insulin secretion in the heterozygous parents. The E178G mutation resulted in reduced Pdx1 transactivation despite normal nuclear localization, expression level, and chromatin occupancy.

CONCLUSIONS—This study broadens the clinical spectrum of *PDX1* mutations and justifies screening of this gene in neonatal diabetic patients even in the absence of exocrine pancreas manifestations. ***Diabetes*** **59:733–740, 2010**

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Although most cases of juvenile-onset insulin-dependent diabetes are represented by type 1 diabetes, in a subset of patients diabetes occurs in the neonatal period or very early. A number of monogenic defects have already been recognized to underlie these rare cases, and several genes have been identified. Neonatal diabetes is permanent in approximately half of the patients and may be caused by mutations affecting genes that play a critical role in β-cell development, survival, or function. Currently, monogenic causes are identified in >50% cases of permanent insulin-dependent diabetes occurring before the age of 6 months (1). Genes responsible for monogenic neonatal diabetes have been identified by candidate gene studies (*PDX1*, *GCK*, *HNF1B*, *KCNJ11*, and *ABCC8*), by linkage and positional gene identification in neonatal diabetes syndromes (*EIF2AK3*, *FOXP3*, *PTF1A*, and *GLIS3*), or by linkage and candidate gene study in nonsyndromic neonatal diabetes (*INS*) (rev. in 1,2). While monogenic inheritance is easily suspected in neonatal diabetes occurring in association with other remarkable clinical features (syndromic diabetes), finding new genes responsible for nonsyndromic monogenic diabetes may be particularly challenging because these patients may be misclassified as type 1 diabetic. The observation that HLA class II alleles in patients with permanent insulin-dependent diabetes presenting before age 6 months was observed to be similar to that of healthy controls (3,4) strongly supports the hypothesis that most cases of neonatal or very early–onset diabetes have a different disease etiology than type 1 diabetes.

Genetic study of highly selected families with monogenic inheritance is a powerful alternative to identify these genes. Here, we studied a single extended family with two related patients affected by neonatal diabetes with no other clinical features. We showed that a novel homozygous mutation in the *PDX1* gene is responsible for diabetes in these patients, and we performed detailed clinical and functional investigations to determine the mechanisms responsible for this unexpected clinical presentation for *PDX1* mutation.

RESEARCH DESIGN AND METHODS

The family was of Moroccan Caucasian origin and was identified through a diabetic child with neonatal insulin-dependent diabetes (subject 8), whose parents were consanguineous. A first cousin of the proband (subject 4) had similar presenting manifestations and consanguineous parents. Initially, eight individuals (subjects 1–8) including the two patients, their parents, and unaffected siblings were studied. Another child (subject 9) was born after the initial genetic study and was genetically diagnosed prenatally, and clinically

confirmed subsequently, as nonaffected. The study was explained to the parents, who agreed to participate in the genetic study and in subsequent clinical and metabolic explorations and signed informed consents. The study protocol was approved by the Hospices Civils de Lyon. Blood samples were obtained on all family members, and DNA was extracted using standard procedures (see Supplementary Methods, available in the online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1284/DC1).

Metabolic investigations. Oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT) procedures were performed on the four parents (subjects 1, 2, 5, and 6) using standard protocols (Supplementary Methods).

Genetic studies: linkage and mutation screening and analyses. A genome-wide SNP scan was performed using the Affymetrix 10K microarray panel. We performed multipoint genetic analyses using Merlin software under a rare disease recessive model (allele frequency: 0.000001) with complete penetrance and no phenocopy.

We performed mutation screening by sequencing genomic DNA of patients and their parents using Big Dye terminator sequencing on an ABI-3730 sequencer (Applied Biosystems). Sequences of primers used for *PDX1* sequencing are shown in supplementary Table 1. Multiple protein sequence alignments in the region of the mutation were generated using Polyphen (5).

Plasmid constructs. Full-length mouse *Pdx1* cDNA was cloned into the pcDNA3 mammalian expression vector, in frame with three hemagglutinin (HA) epitope tags at the NH_2-terminus (HA-Pdx1). The E178G mutation was recapitulated in the mouse sequence by PCR mutagenesis and cloned using the same strategy (HA-Pdx1 E178G). The constructs were confirmed by sequencing.

Immunofluorescence. Baby hamster kidney (BHK) cells were seeded in plastic chamber slides and transfected with Pdx1-expression plasmids using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, cells were fixed with 4% paraformaldehyde and stained with mouse anti-tubulin (Sigma), rabbit anti-HA (Santa Cruz), and DAPI (nuclei).

Reporter assays. BHK cells were transfected with the indicated combinations of Pdx1-expression plasmids, the Pdx1-responsive somatostatin promoter reporter $(TAAT)_5$-65 SMS-CAT (6), and cytomegalovirus (CMV)-β galactosidase (internal control). Chloramphenicol acetyltransferase (CAT) activity was assessed as described previously (7) and normalized to β-galactosidase activity.

Chromatin immunoprecipitation. Min6 insulinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 25.5 mmol/l glucose (Invitrogen) and nucleofected with 2.5 μg Pdx1 expression vectors by AMAXA. After 48 h, cells were harvested in 1× PBS and chromatin immunoprecipitation was performed using rabbit anti-HA or purified rabbit IgG (Santa Cruz), essentially as previously described (8). Chromatin occupancy was assessed by quantitative PCR using previously described primers that amplify the Pdx1 autoregulatory region, the insulin promoter, and the albumin promoter (9). Enrichment is presented as a fraction of input chromatin.

RESULTS

Patient and family description: a single extended family with two patients with neonatal diabetes. The family was identified through a patient with neonatal diabetes and extended to include a first cousin of the patient with similar presenting manifestations. The parents of each patient were consanguineous, and both nuclear families were related, with a common ancestor to all patients' parents (Fig. 1). Patient 4, a boy, was born after 39 weeks of pregnancy and was small for gestational age (birth weight 2,030g [<2 SD] and birth length 45 cm [<2 SD]). His glycemia was normal at birth and until day 15 of life, when he presented with hyperglycemia at 24.2 mmol/l. Patient 8, a girl, was born after 36 weeks of pregnancy and was small for gestational age (birth weight 1,700g [<2 SD] and birth length 45 cm [<1 SD]). On the first day of life, her glycemia was 19.6 mmol/l. Anti-GAD and islet cell and human insulin antibodies were negative in both patients. Both patients were treated with an insulin pump (1.0 units · kg^{-1} · day^{-1}) and became euglycemic with excellent linear growth thereafter. A1C levels were maintained between 7.0 and 9.0% (patient 4) and 7.0 and 8.0% (patient 8). The parents of these two children (subjects 1, 2, 5, and 6) were healthy and nondiabetic.

Based on the family structure (Fig. 1), with parental consanguinity, a common ancestor to the four parents, and the absence of clinical manifestations in the parents, we considered that recessive inheritance of a monogenic defect was the most likely mode of inheritance for neonatal diabetes in this extended family.

Genetic study: a homozygous *PDX1* E178G mutation responsible for neonatal diabetes. Based on the family structure available at the time of genetic study (eight individuals), we estimated that the maximum expected logarithm of odds (LOD) score under linkage would be 3.26. We performed a 10K genome scan and linkage analysis under a fully penetrant recessive model. We identified a single region compatible with linkage (LOD score 3.24) on chromosome 13q12 (Fig. 1). This region extends over 4.4 Mb, between SNPs rs943721 and rs723918, and contains 31 genes referenced in NCBI RefSeq, including the *PDX1* gene, encoding pancreatic and duodenal homeobox 1, also known as insulin promoter factor 1 (*IPF1*). Homozygous or compound heterozygous mutations in *PDX1* have been previously reported in two unrelated patients with neonatal diabetes and exocrine pancreas deficiency due to pancreas agenesis or hypoplasia (10,11), and heterozygous mutations are responsible for maturity-onset diabetes of the young (MODY4) (7,12). Consequently, and despite the absence of exocrine pancreas deficiency in these patients, we considered *PDX1* as a candidate gene for neonatal diabetes segregating in this family. We sequenced all exons of *PDX1*, exon-intron boundaries, and 2 kb of 5′ flanking regions in this family and identified an A641G substitution (RefSeq NM_000209.3) homozygous in both patients, heterozygous in the parents, and segregating with the disease in the homozygous state, resulting in an E178G nonsynonymous change (RefSeq NP_000200.1) (Fig. 2*A*). E178G is located in the second helix of the PDX1 homeodomain, which is an essential and highly conserved region that mediates DNA binding to TAAT-rich motifs in PDX1 target genes (6). The homeodomain also contains a nuclear localization signal (NLS) (13). The E178G substitution was not found in 368 unrelated Caucasian controls. This amino acid is conserved among homologous proteins, including in echinodermates, hemichordates, mollusks, and annelids (Fig. 2*B*).

Clinical investigation of homozygous patients. Following the identification of *PDX1* E178G homozygous mutations in the patients, we performed further clinical and biochemical investigations and abdominal imaging. Both patients' weight and length, as well as bone age, were within normal range at age 47 months (patient 4) and 48 months (patient 8), with treatment consisting only of 1.0 units · kg^{-1} · day^{-1} insulin (supplementary Fig. 1).

Biochemical investigations of endocrine and exocrine pancreas function are summarized in Table 1. C-peptide secretion following a meal was undetectable, consistent with marked β-cell deficiency, and glucagon level was normal or slightly increased, indicating the presence of functional α-cells. Serum lipase levels were low or undetectable, and stool examination revealed slightly increased fecal fat excretion, low chymotrypsin, and low elastase levels, indicating biochemical evidence of some exocrine pancreas deficiency. IGF-1 levels were very low, and vitamins A, D, E, and K levels were at the lower limit of the normal ranges, which is consistent with some degree of malabsorption. This biochemical evidence of exocrine

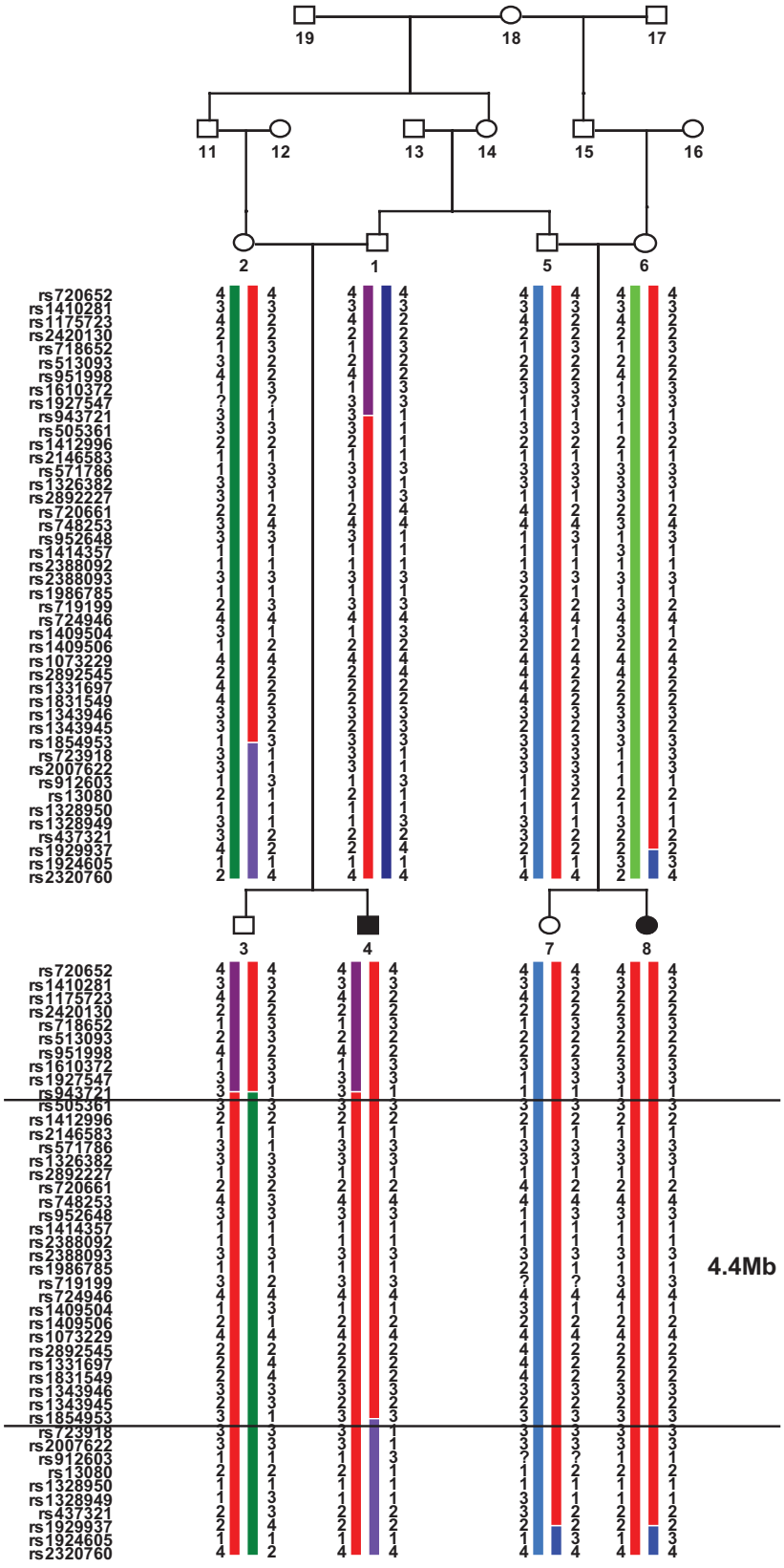

FIG. 1. Neonatal diabetes family tree with linkage analysis. Diabetic patients (neonatal diabetes) are shown in black. A unique region of 4.4 Mb segregates with neonatal diabetes in this family (homozygous red haplotype in patients).

pancreas deficiency contrasts with the absence of detectable clinical signs.

Abdominal ultrasound imaging of patient 4 revealed a normal-sized pancreas, with the presence of a 1.1-cm cyst. For patient 8, ultrasound imaging revealed a well-individualized and homogeneous pancreas head but could not identify the body and the tail (not shown). These results suggest that *PDX1* E178G homozygosity is sufficient for

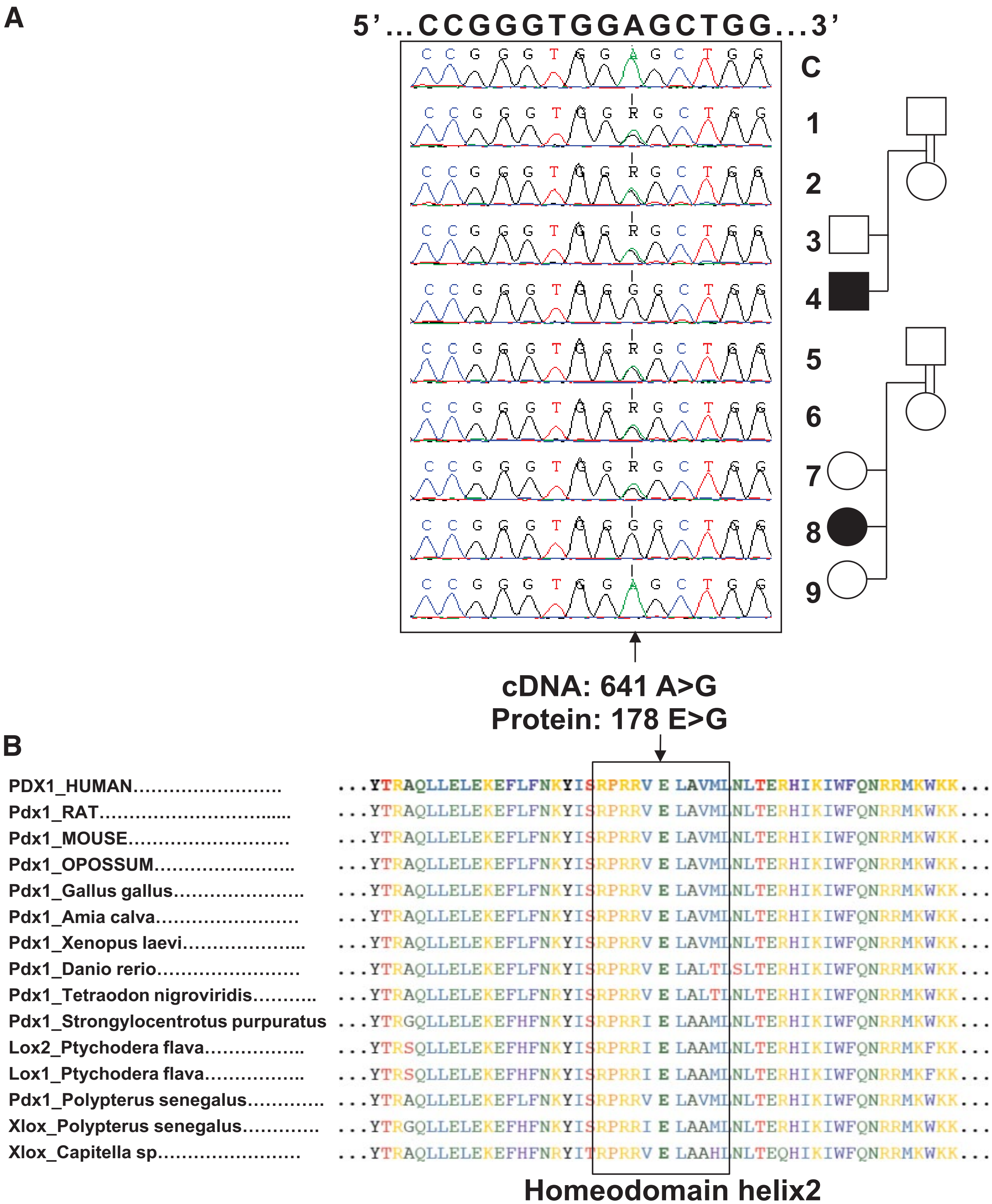

FIG. 2. ***PDX1*** **mutation identification in the neonatal diabetes family.** ***A*****: Sequence of all individuals of the family, including prenatal case (subject 9), showing a homozygous mutation segregating with disease. Positions are given relative to reference sequences NM_000209.3 (cDNA) and NP_000200.1 (protein).** ***B*****: Multiple protein sequence alignment of the region of human PDX1-178E, located with helix 2 of the PDX1 homeodomain, showing PDX1 and homologous proteins from various organisms.**

pancreatic organogenesis. We note that the apparently reduced-sized pancreas observed in patient 8 was correlated with lower levels of serum lipase and stool chymotrypsin and elastase compared with patient 4, although he remained asymptomatic.

Detailed investigation of the four heterozygous carrier parents. Individuals heterozygous for *PDX1* mutations associated with neonatal diabetes and pancreatic agenesis in the homozygous state have been reported to have early-onset type 2 diabetes (MODY4); mean ages at

TABLE 1
Follow-up examination of endocrine and exocrine pancreas function in neonatal diabetic patients

	Patient 4 (male)	Patient 8 (female)	Normal values
Age at follow-up examination	47 months	28 months	
Endocrine pancreas			
C-peptide after meal (μg/l)	<0.1	<0.1	0.8–4.0
A1C (%)	8.3	7.7	4.0–6.0
Serum glucagon (ng/l)	261	591	5–250
Exocrine pancreas			
Fecal fat excretion (g/24h)	3.5	3.2	1–3
Stool chymotrypsin (units/g stools)	5.5	1.5	>8.4
Stool elastase (μg/g stools)	170	20	>200
Serum lipase (units/l)	14	<7	8–78
Vitamin A (μmol/l)	0.7	1.28	0.50–2.40
Vitamin D (nmol/l)	N.D.	28	>25
Vitamin E (μmol/l)	22.1	15.2	12.0–28.0
Vitamin K (ng/l)	222	260	100–1,000
Serum IGF1 (μg/l)	43	32	54–194 (male subjects); 62–125 (female subjects)

onset were 35 years (range 17–67) (12) and 39–50 years (11). In contrast, the four parents in our family, heterozygous for the *PDX1* E178G mutation, were not diabetic at the age of examination (30–38 years old) and family history of type 2 diabetes was unremarkable, based on information provided by the family: the ten brothers and sisters of the four parents were healthy and nondiabetic, and none of the obligate carriers of the *PDX1* mutation (subjects 11, 14, 15, and 18 [Fig. 1]) were reported to be diabetic. These family members were not available for study.

To further explore pancreas function in *PDX1* E178G carrier individuals, we performed OGTT and IVGTT in the four parents of the two patients (Fig. 3). All four parents had normal fasting plasma glucose and normal glucose tolerance, with preserved first-phase but reduced late-phase insulin secretory responses during OGTT (Fig. 3*A*). The first-phase insulin secretory response to IVGTT tended to be low in these parents (25–88 mU/l) and was very low in the two fathers (subjects 1 and 5; ≤1st percentile) (Fig. 3*B*). Ultrasonography of the pancreas was normal in the four parents (not shown). The levels of serum lipase; vitamins A, D, E and K; and IGF1 were within normal ranges (supplementary Table 2).

E178G does not affect Pdx1 localization. To gain insight into the milder phenotypic and clinical manifestations of homozygous and heterozygous individuals for the *PDX1* E178G mutation, compared with previously described homozygous and heterozygous individuals carrying other *PDX1* mutations (10–12), we performed functional investigations of this mutation. The homeodomain of PDX1 (AA 144–206), which is 100% conserved between mice and humans, contains the DNA binding domain and NLS (6,13). The E178G mutation was recreated in the context of the mouse *Pdx1* cDNA sequence. Both wild-type (WT) Pdx1 and the E178G mutant were then cloned in frame with an NH_2-terminal HA epitope tag for overexpression and detection in eukaryotic cell lines (HA-Pdx1 WT and HA-Pdx1 E178G). Upon transfection into BHK cells, both WT and mutant Pdx1 localized to the nucleus, with no evidence of cytoplasmic or membrane staining (Fig. 4). These findings indicate that the E178G mutation does not result in mislocalization of Pdx1, consistent with its location at the NH_2-terminal to the NLS contained within the third helix of the homeodomain.

E178G reduces Pdx1 transactivation. To determine whether E178G disrupted the ability of Pdx1 to transactivate target gene promoters, we assessed its activity using a CAT reporter plasmid harboring the Pdx1-responsive TAAT1 enhancer of the somatostatin promoter (6). BHK cells were transfected with the reporter in combination with HA-Pdx1 WT, HA-Pdx1 E178G, or an empty vector control (Fig. 5*A*). As expected, expression of HA-Pdx1 WT potently induced reporter activation ~12-fold above that of empty vector control. HA-Pdx1 E178G displayed significantly reduced activity compared with the HA-Pdx1 WT protein (sixfold activation over empty vector; $P < 0.01$ compared with HA-Pdx1 WT). This difference in activity was not explained by expression level, as Pdx1 protein level from HA-Pdx1 WT– and HA-Pdx1 E178G–transfected cells were equivalent in this system (Fig. 5*B*).

Pdx1 E178G displays normal chromatin occupancy. Decreased transactivation activity may be explained by the inability of a transcription factor to access chromatin around the promoter or enhancers of its target genes. We addressed this possibility using quantitative chromatin immunoprecipitation (ChIP) in the mouse insulinoma β-cell line Min6 to measure HA-tagged Pdx1 protein occupancy of endogenous target gene promoters. HA-Pdx1 occupied two previously established target genes, the proximal promoter of the insulin gene, and area I of the Pdx1 gene itself but not the albumin promoter, which served as a negative control (Fig. 6). We observed similar specific enrichment for Pdx1 target sequences in immunoprecipitates from cells expressing HA-Pdx1 and HA-Pdx1 E178G, suggesting that the mutation does not disrupt normal chromatin occupancy or DNA binding.

DISCUSSION

PDX1 has been well established as a key factor in pancreas development and function (14,15), with homozygous mutations resulting in pancreas agenesis associated with neonatal diabetes, intrauterine growth retardation, and exocrine pancreas deficiency in humans and mice (10,11,16,17). Only two patients with homozygous or compound heterozygous *PDX1* mutations have been described to date: one with a homozygous frameshift mutation that prevents translation of the homeodomain and C-terminus (Pro63fsdelC) (10) and the other with compound heterozy-

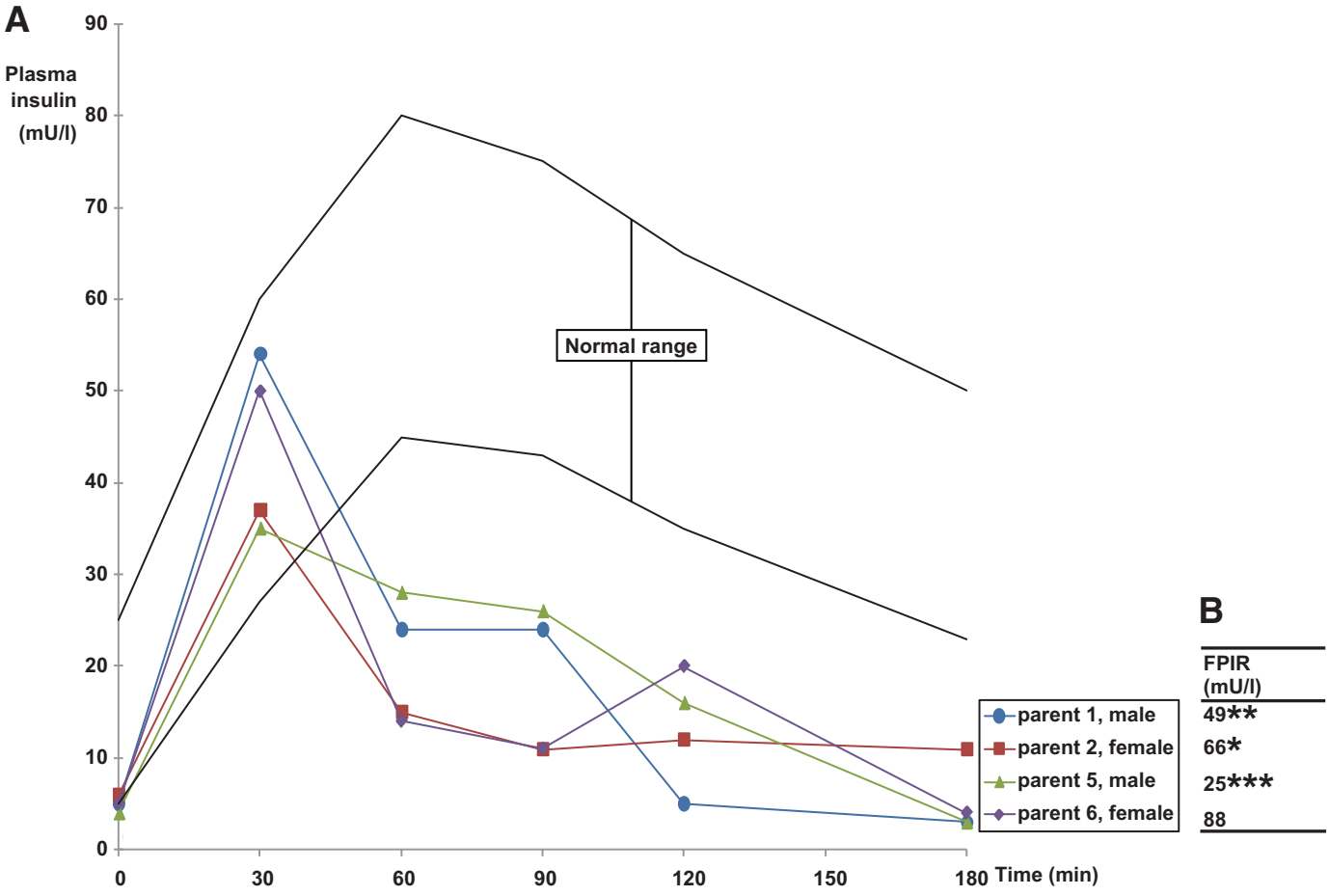

FIG. 3. OGTT and IVGTT in the four heterozygous parents. *A*: Plasma insulin levels during OGTT. Normal ranges (higher–lower values) of plasma insulin levels are shown by the black curves. Plasma glucose levels were all within normal ranges (<6.1 mmol/l at time 0 min and <7.8 mmol/l at time 120 min). Control values for plasma insulin levels were established from 30 normal-weight and nondiabetic Caucasian individuals aged 18–40 years, studied in the same laboratory, using the same procedure. *B*: First-phase insulin response (FPIR) during IVGTT. *5th percentile. **1st percentile. ***Below 1st percentile compared with a nondiabetic reference population (28). Age of parents was 30–38 years and BMI 19.4–23.6 kg/m^2.

gous mutations (E164D and E178K) affecting the homeodomain (11). Here, we identified a novel homozygous *PDX1* mutation, E178G, which results in a milder syndrome, with complete endocrine pancreas deficiency from birth and prenatally (intrauterine growth retardation) but with no clinical manifestation of exocrine pancreas dysfunction despite biochemical evidence of subclinical exocrine pancreas deficiency and structural abnormalities detected by ultrasound scan. In our patients, there was visible pancreatic tissue, although quantitatively reduced in patient 8, and pancreatic α-cells appeared functional, as indicated by glucagon secretion. The pancreatic cyst observation was discordant between the patients and may be coincidental.

Heterozygous carriers of the *PDX1* E178G mutation were asymptomatic and nondiabetic, contrasting with the MODY or early type 2 diabetes phenotype reported in heterozygous carriers of inactivating *PDX1* mutations in humans and in mice (7,12,18–20). The two parents heterozygous for *PDX1* E164D and E178K mutations studied by Schwitzgebel et al. (11) had high-normal fasting glucose at the time of examination, the mother had gestational diabetes mellitus, and the family showed a significant history of early-onset type 2 diabetes in relatives (11). Despite their nondiabetic status and normal glucose tolerance, *PDX1* E178G heterozygous parents showed low insulin secretory response during OGTT, and two of the four parents had very low first-phase insulin response during IVGTT. Interestingly, detailed metabolic explorations performed in heterozygous *PDX1* Pro63fsdelC subjects showed that they had increased insulin sensitivity, in addition to impaired insulin secretion (18). The normal phenotype observed in *PDX1* E178G heterozygotes may be the result of slightly impaired insulin secretion compensated by slightly increased insulin sensitivity. Based on these findings and the unremarkable history of type 2 diabetes in the extended family, it is unlikely that heterozygosity for this mutation predisposes one to type 2 diabetes, unless one is at a late or very late age.

Studies of *PDX1* mutations found in neonatal diabetic patients and heterozygous parents suggest that disease severity is variable and correlates with the nature and functional consequences of the mutation. Several rare *PDX1* variants have also been identified by sequence screening in patients selected from multiplex type 2 diabetic families, some of which were reported to cosegregate with early-onset type 2 diabetes in a dominant mode (21–23). A possible role of rare *PDX1* variants has recently been proposed in ketosis-prone diabetes (24). Remarkably, exocrine pancreas development and function are only affected in the most severe mutations in the homozygous state, indicating a greater sensitivity of the endocrine

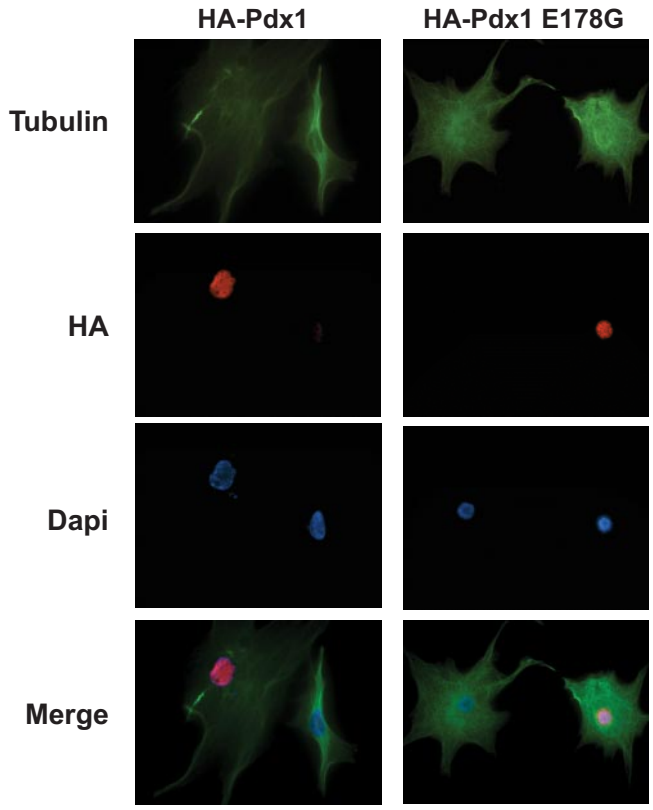

FIG. 4. Pdx1 E178G localizes to the nucleus. Localization of Pdx1 protein in transfected BHK cells stained for HA (Pdx1 overexpression), tubulin, and DAPI (nuclei). Magnification 60×. (A high-quality digital representation of this figure is available in the online issue.)

compared with the exocrine compartment to PDX1 dysfunction. This concept of differential sensitivity to gene dosage has been well illustrated in mouse models carrying various combinations and gradations of *Pdx1* mutations, where homozygosity for hypomorphic *Pdx1* mutations also resulted in a milder phenotype, with development of a normal-sized pancreas and delayed onset of diabetes (25,26).

Our functional data indicate a specific effect of E178G on the transactivation function of PDX1 because neither nuclear localization nor chromatin occupancy was affected by the mutation. In agreement with our findings for the E178G mutation, the E178K mutation studied by Schwitzgebel et al. (11) did not alter nuclear localization nor the ability of Pdx1 to bind to DNA target sequences, as assessed by electrophoretic mobility shift assay. Further, in vitro interactions with NeuroD/Beta2, Foxa2, and Pbx1 were unimpaired. Rather, in functional studies conducted in BHK cells, Pdx1 E178K displayed reduced transactivation activity due to a decrease in Pdx1 steady-state protein levels resulting from impaired protein stability. In our study, Pdx1 steady state levels were not altered by the E178G mutation when expressed in the Min6 β-cell line, which may more closely mimic the situation of primary β-cells. Taken together, the results support a specific effect of the E178G mutation on the transactivation function of Pdx1 independent of subcellular localization, DNA binding, or expression level, suggesting that this mutation may alter Pdx1 interaction with cofactors such as NeuroD, Foxa2, Pbx1, or E47 or a novel factor not yet identified.

Our study extends the phenotypic spectrum of *PDX1* mutations and justifies further mutation screening of this

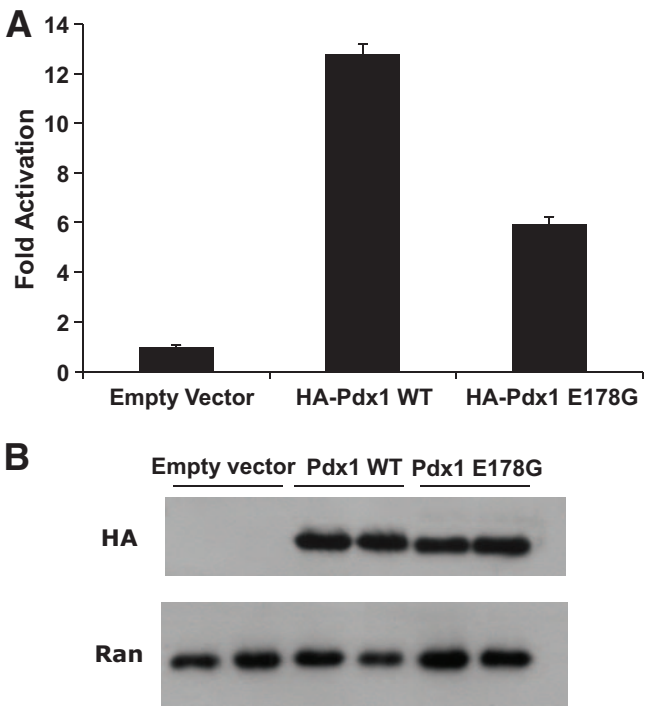

FIG. 5. Pdx1 E178G has reduced transactivation activity. *A*: Activation of Pdx1-responsive CAT reporter in transfected BHK cells, normalized to β-galactosidase activity. $n = 3$; HA-Pdx1 WT vs. HA-Pdx1 E178G, $P < 0.01$. *B*: Pdx1 expression level in transfected BHK cells assessed by Western blot analysis, visualized with anti-HA (HA). Ran GTPase (Ran) was used as control.

gene in nonsyndromic neonatal diabetic patients. Based on previous knowledge, such patients are unlikely to have been tested for *PDX1* mutations, in the absence of the evocative clinical phenotype (1,27), and we recommend

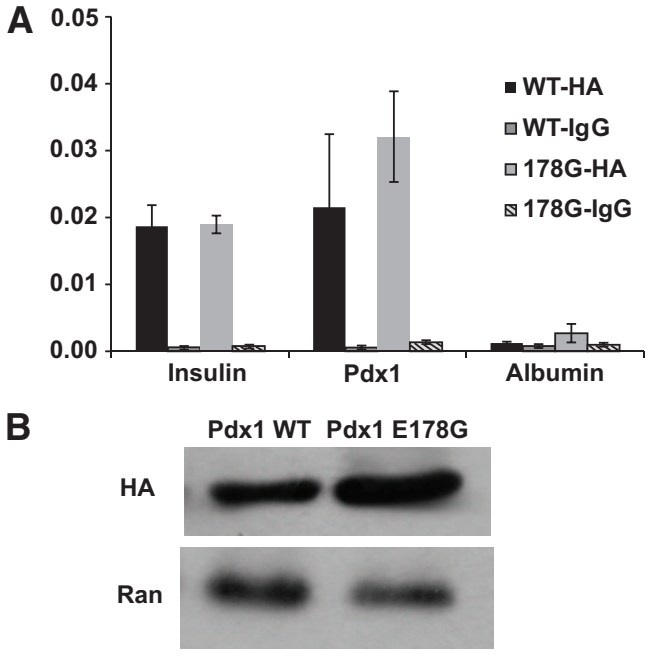

FIG. 6. Pdx1 E178G does not alter Pdx1 chromatin occupancy. *A*: HA-Pdx1 occupancy of the insulin and Pdx1 promoters was measured by quantitative chromatin immunoprecipitation assay in transfected Min6 cells with HA antibody (WT-HA, 178G-HA) or isotype matched control (WT-IgG, 178G-IgG). $n = 4$. *B*: Western blot of protein extracted from transfected Min6 cells. Ran GTPase (Ran) was used as control.

extending the current practice for molecular diagnosis of neonatal diabetes to include *PDX1* screening.

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