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Hyperinsulinism of Infancy: Novel *ABCC8* and *KCNJ11* Mutations and Evidence for Additional Locus Heterogeneity

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Hyperinsulinism of infancy is a genetically heterogeneous disease characterized by dysregulation of insulin secretion resulting in severe hypoglycemia. To date, mutations in five different genes, the sulfonylurea receptor (*SUR1*, *ABCC8*), the inward rectifying potassium channel ($K_{IR}6.2$, *KCNJ11*), glucokinase (*GCK*), glutamate dehydrogenase (*GLUD1*), and short-chain 3-hydroxyacyl-coenzyme A dehydrogenase (*SCHAD*), have been implicated. Previous reports suggest that, in 40% of patients, no mutation can be identified in any of these genes, suggesting additional locus heterogeneity. However, previous studies did not screen all five genes using direct sequencing, the most sensitive technique available for mutation detection. We selected 15 hyperinsulinism of infancy patients and systematically sequenced the promoter and all coding exons and intron/exon boundaries of *ABCC8* and *KCNJ11*. If no mutation was identified, the coding sequence and intron/exon boundaries of *GCK*, *GLUD1*, and *SCHAD* were sequenced. Seven novel mutations were found in the *ABCC8* coding region, one mutation was found in the *KCNJ11* coding region, and one novel mutation was found in each of the two promoter regions screened. Functional studies on β-cells from six patients showed abnormal ATP-sensitive K^+ channel function in five of the patients; the sixth had normal channel activity, and no mutations were found. Photolabeling studies using a reconstituted system showed that all missense mutations altered intracellular trafficking. Each of the promoter mutations decreased expression of a reporter gene by about 60% in a heterologous expression system. In four patients (27%), no mutations were identified. Thus, further genetic heterogeneity is suggested in this disorder. These patients represent a cohort that can be used for searching for mutations in other candidate genes. (*J Clin Endocrinol Metab* 89: 6224–6234, 2004)

HYPERINSULINISM OF INFANCY (HI) is a heterogeneous disease characterized by dysregulation of insulin secretion resulting in severe, often life-threatening hypoglycemia (1–3). Over the last 10 yr, much has been learned about the genetic etiology of this syndrome. About 50% of patients have mutations in one of the two subunits of the β-cell ATP-sensitive K^+ channel (K_{ATP}), *ABCC8* and *KCNJ11* (4–7). The identification of these mutations and the characterization of their effect on channel function have provided important new information regarding the structure and function of this critical channel (8, 9). Approximately 5% of HI patients have dominant mutations in *GLUD1*, which encodes for the mitochondrial enzyme responsible for the reversible conversion of glutamate to 2-oxoglutarate (7, 10). Patients with these mutations typically have relatively mild, diazoxide-responsive hypoglycemia that is associated with asymptomatic hyperammonemia (11). Rare cases of HI caused by activating mutations in the glucokinase gene (*GCK*) have also been reported (12, 13). Recently, mutations in the short-chain hydroxy-L-3-acyl dehydrogenase gene (*SCHAD*) were found in association with HI (14–16). A few patients with a unique syndrome of exercise-induced hypoglycemia were recently described (17, 18), although the genetic etiology is still un-

Abbreviations: ER, Endoplasmic reticulum; HI, hyperinsulinism of infancy; K_{ATP}, ATP-sensitive K^+ channel; SDS, sodium dodecyl sulfate; SNP, single-nucleotide polymorphism; TMD, transmembrane domain; UTR, untranslated region.

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known. However, even when all of these known genetic causes of HI are considered together, in a significant proportion of patients, perhaps as many as 40–45%, no mutation was found in any of the previously mentioned genes. However, previous studies have used techniques to identify mutations that have a limited sensitivity for detecting unknown mutations, such as single-stranded conformational polymorphism. Thus, it is possible or even likely that additional locus heterogeneity is present and that the disease is caused by mutations in other, as yet unidentified genes.

We selected a cohort of HI patients and, using direct sequencing, systematically searched the entire coding and intron/exon boundaries and 5′-upstream regions of *ABCC8* and *KCNJ11*. If no mutation was identified, then the coding region and intron/exon boundaries of the other three previously identified HI candidate genes were sequenced. Twelve *ABCC8* and *KNCJ11* mutations were found in 11 patients. No mutations were identified in four patients.

Patients and Methods

Patient population

Fifteen index patients with the clinical diagnosis of HI were selected for this study. Patients were chosen from a very heterogeneous cohort of more than 150 HI individuals from throughout Israel and Europe (DNA donated by patients and physicians). Selection criteria included clear clinical diagnosis of HI based on accepted criteria, which included inappropriate insulin levels at the time to hypoglycemia and increased glucose requirements to prevent hypoglycemia (19, 20); significant clinical data available to allow correlation between genetic and clinical findings; availability of sufficient DNA to complete the project; and whenever possible, ethnic isolates with multiple cases so that further genetic studies could be accomplished if indicated. The study population was limited to 15 due to technical reasons, and the first 15 patients that met the inclusion criteria were selected; thus, this population cannot be considered representative of the HI population in general. Informed consent was obtained from the parents of all patients, and the study was performed in accordance with the ethical guidelines of participating institutions. The basic clinical characteristics of the index patients are described in Table 1.

Genetic screening

All coding exons and exon/intron boundaries of *ABCC8, KCNJ11*, *GLUD1*, *GCK*, and *SCHAD* were PCR amplified, purified, and sequenced using the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). PCR primers are available from the authors upon request. The regions of the *ABCC8* and *KCNJ11* promoters previously shown to contain major promoter activity were also amplified and sequenced (21). For *ABCC8*, this included the entire 5′-untranslated region (UTR) and 220 bases 5′ to the transcription start site (forward primer, CAC CCC ACT CCC CAT CTT AG; reverse primer, ACC CCC TGG TCC ACC CGG TA). The *KCNJ11* promoter was divided into two amplicons. The first included 70 bases 5′ to the transcription start site and 250 bases of exon 1 (forward primer, GTC TCG AAC TCC TGA CCT AGT; reverse primer, CAG GCG TGT CAG CAC GTA TT). The second segment, which contained nucleotides 712-1020 bases 5′ to the transcription start site, had to be amplified using a nested PCR (first primer set: forward, CTC CAA AGG CCA GGT TGT GAG TCC; reverse, GGT GGC TCA CGC CTG TAA TCC CAG TAC GTT; and nested primers: forward, GTG CGC CCC CCT CCC GCC GTC CTA; reverse, ATC CTG CGT TCT CTG GAG TC). These two segments covered the entire region with known major promoter activity (21).

For each novel variant identified, a control group consisting of at least 50 individuals was tested to exclude the possibility that the variant is a common polymorphism. The control group was either Caucasian or Arab, depending on the ethnic background of the proband. Two hundred ten Ashkenazi Jewish control subjects were screened for the presence of the *ABCC8* promoter variant (−64 c→g, see, *Mutation analysis*).

Electrophysiology

All data were obtained from isolated preparations of viable cells after surgery using the inside-out recording configurations of the patch-clamp technique as described previously (5, 22). The pipette contained a standard NaCl-rich bathing solution containing 140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.13 mM $MgCl_2$, 10 mM HEPES, and 2.5 mM glucose (pH 7.4 with NaOH), and the bath solution contained 140 mM KCl, 10 mM NaCl, 1.13 mM $MgCl_2$, 1 mM EGTA, 2.5 mM glucose, and 10 mM HEPES (pH 7.2 with KOH) for all recordings. In patches with a suitable number of K_{ATP} channel events, open-state probability was assessed as described previously (5, 22).

Studies of promoter function

ABCC8. PCR products of the wild-type and mutant *ABCC8* minimal promoter region and the 5′-UTR were subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and then into the pGL2-Basic vector (TP99; Promega). All clones were sequenced to confirm orientation and the integrity of the sequence. Vector constructs were transiently transfected (calcium phosphate-DNA coprecipitation) into hamster insulinoma tumor cells (HIT-T15) together with cytomegalovirus β-Gal control plasmid as follows. Cells (1×10^6) were plated on a 2-cm^2 plate and incubated overnight at 37 C. The following day, transfections were performed for each plate. The transfection mixture contained 1 μg of test construct or control plasmid (CMV-β-Gal), 125 mM (final concentration) $CaCl_2$ in HEPES-buffered saline (final concentration, 140 mM NaCl, 0.75 mM Na_2HPO_4, and 25 mM HEPES; pH 7.05; total volume, 240 μl). After 5 min in ice, this mixture was added to the cells, which were then incubated for 4 h. Glycerol diluted in 1× PBS (1:5) was then added for 1 min, after which the cells were washed three times in 1× PBS, covered with 2 ml of medium, and incubated for 48 h. Cells were then harvested and assayed for β-Gal (using standard methods) and luciferase activity according to the Luciferase Assay System (Promega).

KCNJ11. The 5′-UTR was amplified using the following primers modified to introduce *Mlu*1 (forward) and *Hin*dIII (reverse) restriction sites (forward, TAA TTA ACG CGT AGG TGG AGA GCC GGC AGG GT; reverse, TTA TAA GCT TCT CGG ACG TGG CCT AGG). This PCR fragment was cut with the appropriate restriction enzymes and inserted directly into the pGL2-Basic vector (TP99; Promega). Afterward, 200 bases were removed from the 5′ end of the insert using the restriction enzymes *Fsp*I and *Mlu*1. Similarly, the second part of the promoter was amplified using primers to introduce new *Mlu*1 (forward) and *Eco*RV (reverse) restriction sites (forward, TAT TAC GCG TTG CGC CCC CCT CCC GCC GTC CTA; reverse, CAT CGA TAT CCT GCG TTC TCT GGA GTC). This amplicon was cut with the appropriate restriction enzymes and ligated to the vector containing the 5′-UTR region. The integrity of the final constructs (wild type and mutant) was confirmed by direct sequencing of the final vectors. The activity of the mutant and wild-type *KCNJ11* promoter construct was assayed as described earlier for the *ABCC8* promoter construct, using the same transfection protocol, except that 1 μg of test plasmid and 0.5 μg of control plasmid were used to transfect 0.5×10^6 INS1r9 cells (kindly provided by Wang and Wollheim, Geneva, Switzerland).

Construction of ABCC8 and KCNJ11 plasmids for in vitro activity studies

Plasmids containing *ABCC8* cDNA plus a *myc*-epitope and *KCNJ11* cDNA were made as previously described (23). Point mutations in *ABCC8* were introduced into hamster cDNA in the pECE vector (24), using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The integrity of the constructs was confirmed by restriction mapping and sequencing. K_{ATP} channels were reconstituted by coelectroporation of 8 μg *ABCC8* and 1 μg *KCNJ11* plasmid into 7×10^6 COSm6 cells, as previously described (23).

Photolabeling and surface expression

After an overnight incubation after electroporation, whole-cell photolabeling (25) and surface expression assays were performed as pre-

TABLE 1. Patient clinical data

Patient no.	Ethnicity	Age at diagnosis	Responsive to diazoxide	Responsive to octreotide	Pancreatectomy	Histology	Electrophysiology	Family history	Mutations identified
1	Bengal	Birth	No	No	Yes	Diffuse	K_{ATP} channelopathy	No	3992-9 g→a (homozyg)
2	Bedouin	Birth	Partial	Partial	Yes	Diffuse	Not done	Yes	R836X (homozyg)
3	India	Birth	No	Partial	Yes	Diffuse	K_{ATP} channelopathy	No	G111R (homozyg)
4	Arab	Birth	No	No	Yes	Diffuse	Not done	No	R1494W (homozyg)
5	Caucasian	Birth	No	Partial	Yes	Diffuse	K_{ATP} channelopathy	No	G70E and R1419H
6	Caucasian	Birth	No	Partial	Yes	Diffuse	K_{ATP} channelopathy	No	2154 +3a→g and G1342E
7	Arab	Birth	No	Partial	Yes	Diffuse	Not done	No	1113 ins T (homozyg)
8	Arab	Birth[a]	No	Partial	Yes	Diffuse	Not done	Yes	P254L (homozyg)
9	Bedouin	2 d	Partial	Yes	No		Not done	Cousin with transient neonatal hypoglycemia	+88 g→t (homozyg)
10	Caucasian	Birth	No	Partial	Yes	Diffuse	K_{ATP} channelopathy	Yes (sister)	2154 + 3a→g (heterozyg)
11	Caucasian	3 d	No	No	Yes	Diffuse	Not done	No	−64 c→g (heterozyg)
12	Ashkenazi Jewish	Birth	No	Partial	No		Not done	No	None
13	Mixed Ashkenazi-Sepharadic Jewish	4–6 months	Responsive	Not tested	No		Not done	No	None
14	Caucasian	4–6 months	No	No	Yes	Diffuse	No defects	No	None
15	Caucasian	Birth	Yes	Not tested	No		Not done	Yes	None

Homozyg, Homozygous; heterozyg, heterozygous.

[a] Prenatal diagnosis was made by determining umbilical blood insulin levels and by ultrasound since previous sibling was affected.

viously described (23). Briefly, for whole-cell photolabeling, cells were incubated for 30 min with 1 nM [^{125}I]-azido-glibenclamide in the presence and absence of 1 μM cold glibenclamide and cross-linked using UV light. Cells were then washed with PBS, solubilized with loading buffer [pH 6.8; 30 mM Tris, 1.25% sodium dodecyl sulfate (SDS), 1 mM EDTA, 50 mM dithiothreitol; 10% glycerol], and resolved in a 7–10% SDS gel.

Surface expression was quantified by using a luminometer-based assay to measure the SUR1c-*myc*-tagged subunit. Transfected COSm6 cells were gently washed in PBS and incubated for 3 h at 4 C using a mouse monoclonal IgG1 c-*myc* antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in DMEM plus 10% fetal bovine serum to a concentration of 0.3 μg/ml. After incubation, the cells were washed three to four times with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubated for 1 h with horseradish peroxidase-conjugated goat antimouse IgG (Sigma Co., St. Louis, MO). Chemiluminescence was then measured using the Wallac 1429 VictorII (Perkin-Elmer, Wellesley, MA) and Luminol (Santa Cruz Biotechnology) as the horseradish peroxidase substrate.

Rubidium efflux

To determine channel activity, K_{ATP} channels were reconstituted into COSm6 cells, and [$^{86}Rb^+$] efflux assays were performed 48 h after the electroporation as previously described (26, 27). Briefly, after overnight incubation with 1 μCi/ml of [$^{86}Rb^+$] at 37 C in DMEM supplemented with 10% fetal bovine serum, the cells were preincubated for 30 min at room temperature in Krebs Ringer bicarbonate buffer containing 1 μCi/ml [$^{86}Rb^+$], the metabolic inhibitor oligomycin (2.5 μg/ml), and 2-deoxyglucose (1 μM) in the presence or absence of 1 μM glibenclamide. The supernatant was aspirated at the end of the preincubation and at the indicated time points and replaced with fresh Krebs Ringer bicarbonate buffer containing oligomycin and 2-deoxyglucose but no [$^{86}Rb^+$]. At the end of the time course, the reaction was stopped with 0.3% SDS. Radioactivity in the cells and supernatants from each time point was determined using a γ-counter, and the results are reported as the cumulative counts in the aspirated solution divided by the total of the counts in the lysate and the solutions.

Results

Mutational analysis

ABCC8 (Table 2). Two splice-site mutations, seven coding sequence mutations, and one promoter mutation were identified in *ABCC8*. None of these were present in any of the control subjects tested.

One of the two splice-site mutations (3992-9 g→a) was previously described in patients from the United States, Germany, and Saudi Arabia and was shown to be responsible for 69% of HI-associated chromosomes in the Ashkenazi Jewish population (4, 28, 29). Previous studies, using intragenic single-nucleotide polymorphism (SNP) haplotype analysis, suggested that the mutation occurred *de novo* in each population (29). Haplotype analysis in patient 1 failed to show any similarity with any of the other reported haplotypes associated with this mutation (data not shown), thus indicating another *de novo* event.

The novel splice mutation in the splice donor site of intron 15 (2154 + 3a→g) was found in two unrelated index patients of Irish origin (patients 6 and 10). Both were heterozygous for the mutation. Patient 6 inherited this mutation on the maternal allele and was found to have a second mutation (G1342E) on the paternal allele (Table 2). Patient 10 has a haploidentical, affected sibling (Table 1). Both inherited this mutation on the paternal allele, and both inherited the same maternal allele on which no mutation was identified.

Two of the seven coding mutations were previously reported. One, R836X, was identified in a proband from a Bedouin family and had previously been found in three families of Mexican origin (30). Haplotype analysis using flanking SNPs revealed that a single founder mutation was

TABLE 2. HI-associated genetic variants

Mutation designation	Exon/intron	Sequence[a]	Mutation type	Patients carrying mutation (no.)
ABCC8				
−64 c→g	Promoter	gcc gcc ccc gGc	Promoter	11
G70E	2	ccc ggg cac gAg	Missense	5
G111R	3	gcc ggg atg Agg	Missense	3
2154 + 3a→g	Intron 15	agg tat ggc tGt	Splice-site	6, 10
R836X	21	cag cga atc Tga	Nonsense	2
1113 ins T	27	ttt ttt gag ttt ttt Tga	Single-base insertion	7
3992-9 g→a	Intron 32	cgc aag cgt aaA	Splice-site	1
G1342E	33	caa ggg aag gAg	Missense	6
R1419H	35	ctg cgc tca cAc	Missense	5
R1494W	37	gcc cgg gcc Tgg	Missense	4
KCBJ11				
+88 g→t	Promoter	gaa gtg agg Ttg	Promoter	9
P254L	Exon 1	gcc cCg ctg cTg	Missense	8

[a] For each mutation, the *upper line* indicates the wt sequence, and the *lower line* indicates the mutant sequence.

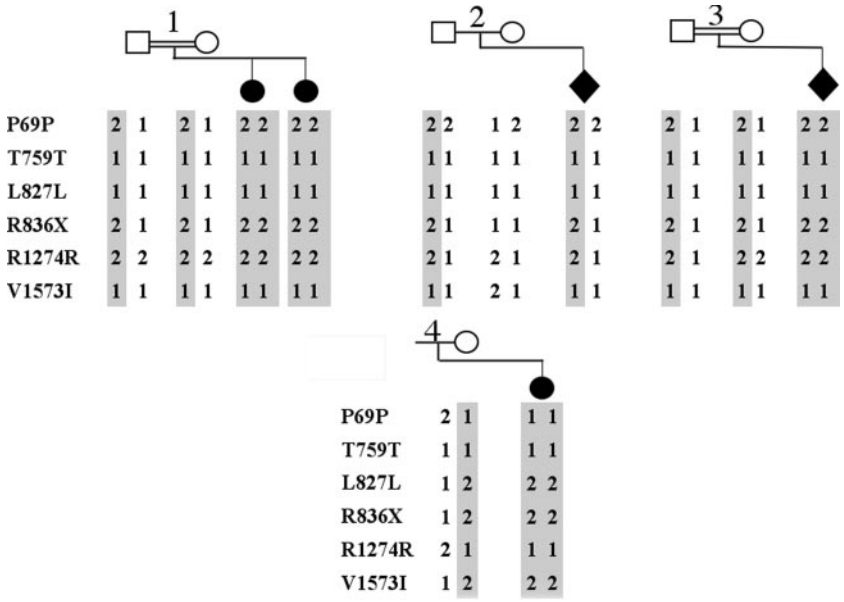

FIG. 1. Haplotype analysis of four families segregating the R836X mutation. Families 1–3 are of Mexican origin, whereas family 4 is of Israeli Bedouin origin. The affected child of family 2 had focal HI and inherited the mutant allele from his father. None of the families are known to be related, although the parents of families 1 and 3 are first cousins, as shown by the *double lines* connecting them. *Squares* indicate males, *circles* indicate females, and *diamonds* indicate that the sex of the individual is not known to us. *Filled symbols* indicate individuals with HI. Five biallelic SNPs in the *ABCC8* gene were studied. For each SNP, *1* indicates the presence of the more common base, and *2* indicates the presence of the less common base. All mutant alleles from the Mexican patients are identical, indicating that this is a founder mutation, but they are different from the mutant allele from the Bedouin patient, excluding a common origin of this mutation in these two populations.

present in all three Mexican probands, two with diffuse HI and one with focal HI. In contrast, in the Bedouin family, the SNP haplotype was entirely different, suggesting that there is no genetic connection between the founder mutations in the two ethnic groups (Fig. 1). After identifying this mutation in the index patient, two other affected members of the same Bedouin tribe were evaluated and found to be homozygous for the same mutation. This mutation was not identified in any of the 100 Israeli Arab control chromosomes tested.

The R1494W mutation, found in one patient of Arab descent, was previously reported in two patients with focal HI (30, 31). The effect of this mutation on channel trafficking is described in *Functional studies of missense mutations.* Another mutation in the same codon (R1494Q) and a third in the adjoining codon (A1493T) were previously described in patients of European Caucasian ancestry (32).

One novel single base insert was identified in an Israeli Arab patient (1113 ins T). This mutation causes a frame shift and a stop codon at position 1113. If translated, the resulting truncated protein would not be expected to be functional.

Four novel missense mutations were identified and characterized *in vitro* (Table 2, and in *Functional studies of missense mutations).* In addition, a novel mutation was identified in the *ABCC8* promoter (−64 g→c) on the paternal allele of a single HI patient of Spanish descent. This variant has not been previously described in any HI or control subjects and was not found in 100 control chromosomes tested.

KCNJ11 (Table 2). Two novel mutations were identified, a missense mutation at codon 254 (P254L) and a mutation in the 5′-UTR, 88 bases 5′ of the transcription start site (+88 g→t). Neither of these mutations was present in any of the control subjects tested. The missense mutation, P254L, was identified in the homozygous state in a single Arab patient, and the 5′-UTR mutation was found in the homozygous state in an Israeli Bedouin proband. The functional significance of each mutation is discussed in *Functional studies of missense mutations* and in *Regulatory variants in ABCC8 and KCNJ11.*

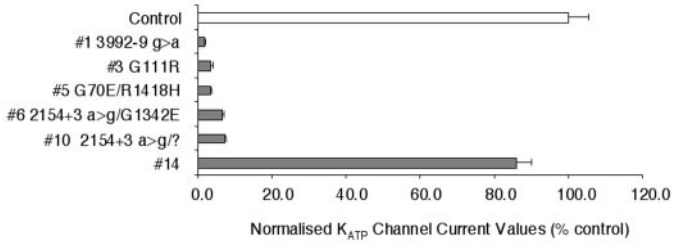

FIG. 2. K_{ATP} channel current values in β-cells isolated from control and HI pancreata. All data were obtained using inside-out membrane patches (see *Patients and Methods*), and values are expressed as percentage of control (human) cells (*open bar*). Individual patients (*filled bars*) are identified by their patient numbers as shown in Table 1.

Neither was identified in 100 Israeli Arab control chromosomes.

GLUD1, GCK, SCHAD. In four patients, no mutations were found in *ABCC8* or in *KCNJ11.* In these four patients, the coding sequences of *GLUD1, GCK,* and *SCHAD* were sequenced, and no mutations were identified.

Functional studies

β-cell electrophysiology studies. Figure 2 summarizes the K_{ATP} channel activity in β-cells obtained from normal human controls (islet transplant donors) and from HI patients for whom appropriate tissue was available. Patients 1, 3, 5, 6, and 10 showed marked defects in K_{ATP} channel activity. The recorded average peak currents were (percentage of control given in parenthesis) 0.4 ± 0.25 pA (n = 8, 1.8%), 0.8 ± 0.45 pA (n = 4, 3.7%), 0.7 ± 0.19 pA (n = 19, 3.5%), 1.4 ± 0.35 pA (n = 4, 6.6%), and 1.6 ± 0.04 pA (n = 5, 7.4%), respectively, compared with control values of 21.2 ± 5.5 pA (n = 134, 100%). Interestingly, in the β-cells from patient 14, in whom no mutation was identified, the level of K_{ATP} channel activity was similar to that found in normal cells, 18.2 ± 4.2 pA (n = 4, 85.9%). In all the other patients, β-cells were not available

either because the patients did not undergo surgery (patients 9, 12, and 13) or the surgical specimen could not be transported to the laboratory in the United Kingdom (patients 2, 4, 7, 8, and 11).

Splice-site mutation. To determine the effect of the novel 2154 + 3a→g mutation on splicing *in vivo*, we studied frozen pancreatic tissue from patient 10 and preserved, cultured islets from both patient 10 and her haploidentical affected sibling. RT-PCR was performed, and all PCR products were sequenced. RT-PCR of cDNA generated from the frozen pancreas spanning the region including exons 14–22 revealed two distinct bands. One of these bands represented the normal expected fragment, whereas the other band was found to contain a fragment with a 410-base deletion that started at codon 703 at the end of exon 15 and ended at codon 840 in the middle of exon 21 (Fig. 3A). RT-PCR of cDNA from cultured islets gave variable results. In one experiment, two bands were seen, one was the normal transcript, whereas the second band contained a 258-base deletion extending from within exon 15 (codon 687) to within exon 19 (codon 773) (Fig. 3B). This variant was found only in the cultured islets obtained from the sibling. When the experiment was repeated, three bands were seen (Fig. 3C). Again, one of the bands was the normal transcript. The third band showed a deletion of all of exon 15, whereas the middle band on that gel contained a mixture of the two splice variants. This pattern was seen on RT-PCR of cultured islets from both patients (patient 10 and her sibling). On other occasions, only the normal transcript was amplified. The same PCR reaction on cDNA from control pancreases and from other HI patients consistently revealed only the normal transcript. It was not possible to determine the expression level of the normal *ABCC8* transcript relative to that of the abnormal splice variants or to the transcript level normally found in healthy β-cells prepared and processed the same way.

Regulatory variants in ABCC8 and KCNJ11. Two variants were identified in the regulatory regions of the K_{ATP} channel genes, −64 c→g in *ABCC8* and +88 g→t in *KCNJ11*. To determine the functional significance of these variants, each was PCR amplified and cloned into a luciferase reporter vector. Each variant significantly reduced reporter gene expression (40 ± 7.8% and 44 ± 5% of the wild type for the *ABCC8* and the *KCNJ11* variants, respectively; Fig. 4).

Functional studies of missense mutations (Fig. 5). To determine the effect of the missense mutations on K_{ATP} channel expression, trafficking, and/or function, mutant *SUR1* and $K_{IR}6.2$ were transiently transfected alone or with their respective wild-type partner in COSm6 cells and analyzed by photolabeling, surface expression of tagged SUR1c-*myc* subunit, and rubidium efflux (30, 33). When the mutations in *ABCC8* (G70E, G111R, R836X, G1343E, R1419H, and R1494W) were expressed alone, all except R836X were photolabeled with the high-affinity sulfonylurea ligand [^{125}I]-azido-glibenclamide (data not shown). This indicated integrity of the glibenclamide-binding site of these mutant constructs. The missense mutation, R836X, caused a truncation that resulted in loss of the binding site for glibenclamide. All *myc*-tagged wild-type and mutant constructs, including the missense truncation mutation, expressed similar protein levels as determined by Western blot analysis (data not shown).

Figure 5A shows photolabeling of the reconstituted mutant and wild-type K_{ATP} channels. Wild-type K_{ATP} channels

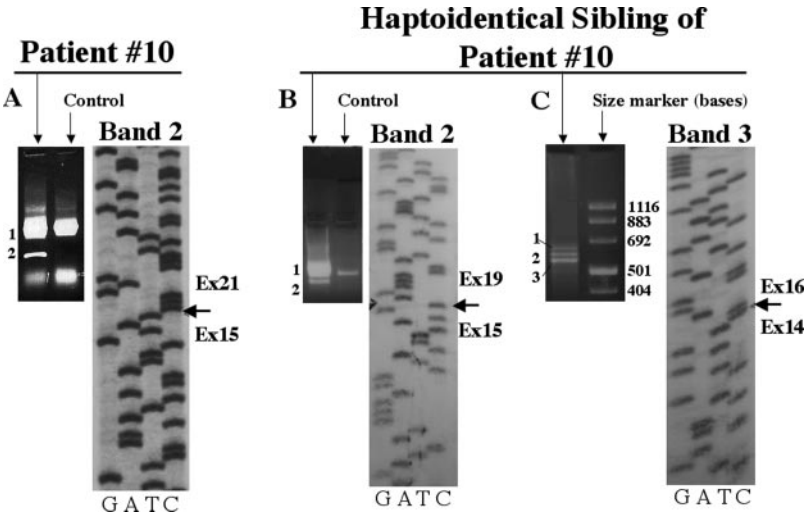

FIG. 3. A, The *left panel* shows agarose gel electrophoresis of the RT-PCR product amplified from RNA extracted from the pancreas of patient 10, showing a dominant band (1) that contains normal *ABCC8*. The smaller band (2) contains a truncated *ABCC8* transcript that is missing 410 bases as demonstrated by direct sequencing (*right panel*). To determine whether this same splice variant was present in patient 10's haploidentical sister, cultured β-cells were obtained, and RNA was extracted. The experiment was repeated several times, and variable results were obtained. In some experiments, only normal transcript was identified, whereas in other experiments, different alternatively spliced products were seen, as shown in B and C. B, The *left panel* shows agarose gel electrophoresis of RT-PCR product demonstrating two product bands (1 and 2). Band 1 is wild-type *SUR1*, whereas band 2 shows the deletion that extends from within exon 15 to within exon 19 (*right panel*). C, In this experiment, the *left panel* shows agarose gel electrophoresis of RT-PCR product showing three different products, labeled 1–3; 1 is wild-type *ABCC8*, 3 had exon 15 deleted, and 2 is a combination of the two bands. The *right panel* shows the sequence of band 3 with a deletion of all of exon 15.

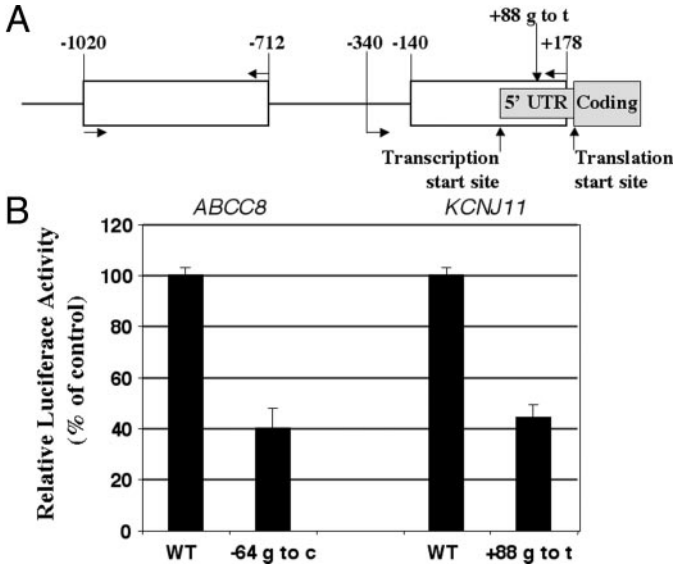

FIG. 4. A, Schematic diagram of KCJN11 promoter construct used for functional studies. *Shaded rectangles* represent the 5′-UTR and the coding region as indicated. *Clear rectangles* represent the two regions that were included in the final construct. The *horizontal line* indicates the portion of DNA that was not included in the final construct. *Horizontal arrows* indicate positions of PCR primers. *Numbers above vertical lines* indicate the nucleotide location relative to the transcription start site. The location of the mutation (+88 g→t) is identified by a *vertical arrow*. B, Relative luciferase activity of mutant *ABCC8* and *KCNJ11* promoter constructs. Each construct was tested in three independent experiments, and results were expressed as the percentage of the wild-type activity in each experiment.

appear at the plasma membrane only if *SUR1* and $K_{IR}6.2$ are coexpressed and if the core protein is glycosylated to produce the mature form of the receptor. The upper two bands represent the core (140 kDa) and mature (150–170 kDa) form of the receptor, and the lower band corresponds to the inward rectifier (38–40 kDa), which is co-photolabeled when both subunits are coexpressed. Although this is not a quantitative assay, the photolabeling of the core form of the wild-type and mutant receptors appears to be very similar. In contrast, the mature form, which is indicative of channels trafficking at least to the Golgi, was present only in the G70E homozygote, in the G70E/R1419H compound heterozygote, and to a lesser extent, in G111R. In mutants G1343E, R1419H, and R1494W in *ABCC8* and P254L of *KCNJ11*, only the core band was detected, indicating that these mutant channels are retained in the endoplasmic reticulum (ER). As shown in the lower part of Fig. 5A, all the mutants co-photolabeled the inward rectifier, indicating that although not present in the plasma membrane, both subunits do associate.

Figure 5B shows the results of the surface expression studies. This experiment clearly shows that there is a parallel correlation between the loss of the mature band and the level of channels that are expressed at the cell surface. The first three *SUR1* mutant channels, G70E homozygote, G70E/R1419H compound heterozygote, and G111R, were expressed at the plasma membrane, albeit at much lower levels than the wild-type channel. The remaining mutants, G1343E, R1419H, R1494W in *ABCC8* and P254L in *KCNJ11*, did not reach the cell surface at all, confirming the retention pattern observed by photolabeling. We then assayed for recombinant channel activity by analyzing glibenclamide-inhibitable [$^{86}Rb^+$] efflux (Fig. 5C). We confirmed that channel activity of the G70E and G111R mutants was decreased when compared with wild-type controls. No channel activity was detected from any of the mutant subunits retained in the ER. Patient 5 was a compound heterozygote for mutations G70E and R1419H. Coexpression of an equimolar mixture of the two mutants resulted in a proportional reduction in channel activity, as determined by [$^{86}Rb^+$] efflux and surface expression, compared with the control.

Patients in whom no mutant allele was identified

In four patients, no mutation was identified in *ABCC8*, *KCNJ11*, *GCK*, *GLUD1*, or *SCHAD*. Two of these patients (patients 12 and 14) had typical severe HI. Patient 12 presented both clinically and biochemically with a classical phenotype of severe HI and was unresponsive to diazoxide and only partially responsive to octreotide. Due to the difficulty in preventing recurrent hypoglycemia using medical therapy, near total pancreatectomy was recommended but was refused by the parents. The patient was successfully managed using an intensive medical treatment protocol that included continuous sc infusion of octreotide and glucagon, along with frequent feedings and continuous enteral feeding during the night. At the age of 20 months, another trial of diazoxide treatment was performed, and a partial response was obtained. Currently, at the age of 3.5 yr, the patient is developmentally normal, but he still requires treatment with continuous sc infusions of both glucagon (4 μg/kg·h) and octreotide (12 μg/kg·d), frequent feedings during the day, and continuous feeding during the night.

We were also not able to identify a mutation in patient 14. His presentation was also severe, drug unresponsive, and required two partial pancreatectomies to prevent hypoglycemia. β-cells from this patient were isolated and shown to have normal K_{ATP} channel function. We also found that cytosolic Ca^{2+} concentrations in isolated β-cells were not significantly elevated when compared with normal values (data not shown).

Patient 13 had a relatively mild form of the disease, which presented with general convulsions associated with hypoglycemia at 6 months of age. He showed good response to diazoxide and has been well controlled with treatment ever since.

Finally, patient 15 had neonatal-onset HI associated with intermittent, mild hyperammonemia that was responsive to diazoxide. The patient's father was diagnosed with HI at the age of 30 yr, although in retrospect, he appears to have had episodes of mild, episodic, nonfasting hypoglycemia since birth. Despite the fact that the clinical presentation of diazoxide-responsive hyperinsulinism with hyperammonemia suggests the presence of a *GLUD1* mutation, no sequence alteration was identified. At the age of 9.5 yr, patient 15 is treated with diazoxide (1.5 mg/kg·d). No hypoglycemic event has been documented in the past 4 yr.

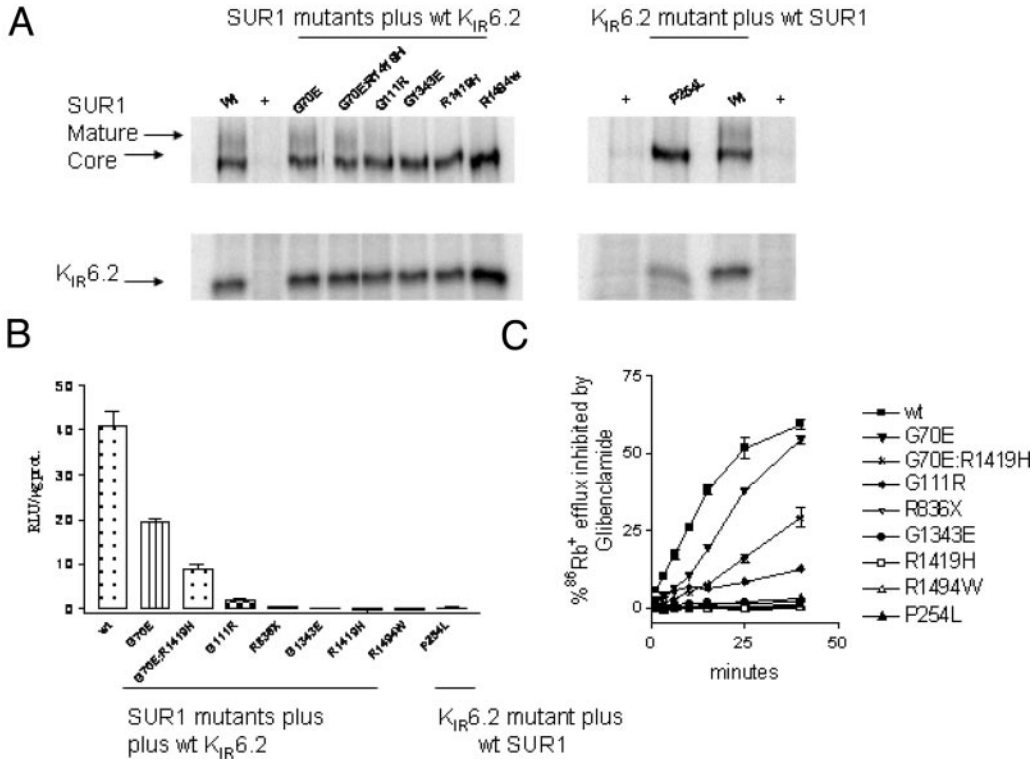

FIG. 5. A, Whole-cell photolabeling of reconstituted K_{ATP} channels with [^{125}I]-iodoazido-glibenclamide as described in *Patients and Methods*. COS cells coexpress wild-type (wt) $K_{IR}6.2$ with *SUR1* mutations (G70E, G70E/R1419H, G111R, G1343E, R1419H, and R1494W) or coexpress wt *SUR1* with $K_{IR}6.2$ mutation (P254L). Reconstituted wt channel-labeled bands show *SUR1* core glycosylated protein (140 kDa), mature or complex glycosylated protein (150–170 kDa) (*top*), and $K_{IR}6.2$ protein (40 kDa) (*bottom*). Bands were specifically displaced with 1 μM of cold glibenclamide (+). Appearance of the mature form indicates the transit through the secretory pathway to the Golgi-plasma membrane, whereas the absence of mature form indicates retention in the ER. B, Surface expression of reconstituted mutant K_{ATP} channels shown previously was assessed using a luminometer-based immunoassay, as described in *Patients and Methods*. All mutants analyzed showed defective trafficking. Homozygous expression of G70E and G111R had reduced surface expression, whereas mutations R836X, G1343E, R1419H, R1494W, and P254L did not reach the plasma membrane at all, although they did associate with their respective wt partner as shown in Fig. 5A (*lower panel*). Compound heterozygous expression for mutation G70E/R1419H (4 μg/4 μg) showed reduced surface expression when compared with mutation G70E (8 μg) expressed alone. The data are expressed as relative light units (RLU) per microgram of protein. The *error bars* are SDs. Measurements were done in triplicate. C, Relative K_{ATP} channel activity was measured by [$^{86}Rb^+$] efflux in the presence of the opener diazoxide (0.3 mM), as described in *Patients and Methods*. The wt channels showed robust efflux upon diazoxide incubation. Channels with *SUR1* and $K_{IR}6.2$ mutations that did not reach the plasma membrane also did not show efflux. Only G70E (8 μg), G111R (8 μg), and compound heterozygote G70E/R1419H (4 μg/4 μg) reconstituted with wt $K_{IR}6.2$ (1 μg) show reduced efflux. All *SUR1* and $K_{IR}6.2$ mutants analyzed show reduced trafficking of the channel to the plasma membrane, which in turn affects the channel activity.

Discussion

We screened 15 HI probands for mutations in five genes (*ABCC8*, *KCNJ11*, *GCK*, *GLUD1*, and *SCHAD*). Nine novel and three previously described mutations were identified; 10 were identified in *ABCC8*, and two were identified in *KCNJ11*. Three mutations (3992-9 g→a, R836X, and R1494W) were previously reported in other ethnic groups. In the first two cases, intragenic SNP haplotype analysis excluded the possibility of founder mutation affecting multiple ethnic groups, whereas the third case was located in a region where two other mutations were previously described (30–32). Thus, these three regions of *ABCC8* appear to be mutation hot spots.

Eleven of the 15 patients studied required pancreatectomy to alleviate hypoglycemia. Channel function was studied in the β-cells from six of these patients, and in five, all with *ABCC8* gene mutations, a marked decrease in the expression and activity of K_{ATP} channel was demonstrated. In contrast, the sixth patient had normal channel function, and no *ABCC8* or *KCNJ11* mutation was identified. These findings confirm the correlation between *ABCC8* and *KCNJ11* mutations and defects in the β-cell K_{ATP} channel.

One novel splice-site mutation was identified in two patients. By studying mRNA extracted from one patient's pancreas and from cultured β-cells from the two patients, we demonstrated that this mutation results in the production of at least three different splice variants as well as the normal transcript. Based on known characteristics of the mammalian splice mechanism, the specific splice variants that we observed cannot be readily explained. To exclude mutations on the second *ABCC8* allele, exons 15–21 and their intron-exon boundaries were PCR amplified and sequenced from genomic DNA, and no evidence of additional mutations or deletions was discovered. Therefore, we conclude that this single-base change in the exon 15/intron 15 splice junction may have caused major changes in the splicing process, resulting in several alternative splice variants in the two probands. Apparently normal *ABCC8* transcript was also

consistently identified by PCR amplification of pancreas and β-cell mRNA from these patients. However, it was not possible to determine the amount of normal *ABCC8* transcript present, and thus it was not possible to compare it to the amount present in normal β-cells prepared the same way. Similarly, it was not possible to determine whether this apparently normal mRNA was translated into any functional protein. However, because this mutation was associated with a complete loss of K_{ATP} channel function in patients 6 and 10 (22), we conclude that either very little normal *ABCC8* transcript was produced or it was not correctly translated into functional protein.

Two novel mutations were identified in regulatory regions, one in the promoter of *ABCC8*, −64 c→g, and the other in the 5′-UTR of *KCNJ11*, +88 g→t. This is the first time that mutations in the regulatory region of these genes have been associated with HI. Functional analysis of these sequence changes revealed that each mutation decreased transcriptional activity in the model system by approximately 55–60%. Patient 9 was homozygous for the *KCNJ11* promoter mutation. A decrease in transcriptional activity of only 55–60% may not be sufficient to cause disease; however, this activity was evaluated using a small fragment of the promoter region in a cell line derived from rodent insulinoma. In the human β-cell, the degree of suppression of transcriptional activity may be quite different. Neither mutation was identified in 100 normal chromosomes or in other HI-associated chromosomes, indicating that these are not common polymorphisms; however, additional studies are needed to prove unequivocally that these mutations are, in fact, the cause for HI in these patients.

In patient 11, the mutation −64 c→g was located on the paternal allele, and no mutation was identified on the maternal allele. This mutation is located in a putative SP1/AP2 binding site. Further studies are needed to determine how it affects binding to these, and perhaps other, transcription factors. It remains unclear how a single mutation with only a partial effect on transcription efficiency can cause HI because heterozygosity for mutations that completely eliminate channel function is not associated with clinical disease (34). This discrepancy may be partially explained by the limitations of the *in vitro* method used. Thus, it is possible that this mutation has a much more dramatic effect in the normal human β-cell. Alternatively, the patient may have focal HI, although this seems unlikely because he underwent three surgeries, and greater than 95% of the pancreas was removed. No focal lesion was identified, and the histology was reported to be consistent with diffuse HI. However, if the focal lesion is not removed and identified, the histological differentiation between normal neonatal pancreas outside a focal lesion and diffuse HI, although possible, can be quite difficult (35). Unfortunately, the pathological specimen is not available for reevaluation. A third possibility, which can never be entirely excluded, is that the maternal allele contains a mutation outside the regions tested or carries a deletion of one or more exons.

We used photolabeling in combination with surface expression studies and rubidium efflux assays to determine the effect of missense mutations on protein expression, post-translational processing, and cell surface expression. In the pancreatic β-cell, the K_{ATP} channel is a hetero-octameric complex, $[(SUR1{:}K_{IR}6.2)]_4$, formed by four inward rectifiers and four sulfonylurea receptors that physically associate in a 1:1 stoichiometry (26). This requirement is tightly regulated by the presence of ER retention (36) and exit (23) signals that ensure that only properly assembled, full-length K_{ATP} channels reach the plasma membrane. All of the six novel missense mutations on *ABCC8* and *KCNJ11* exhibit altered trafficking of the K_{ATP} channel to the plasma membrane, suggesting that this is a common mechanism by which missense mutations result in altered or lack of channel activity.

The first five transmembrane domains (TMD) in the N-terminal region of *SUR1* (TMD0) include the region that specifically associates with $K_{IR}6.2$, enhancing surface expression and forming a mini K_{ATP} channel capable of bursting similar to native channels (37). Two HI-associated TMD0 missense mutations have been previously studied and have shown to cause a severe trafficking defect. Chan *et al.* (38) demonstrated that these mutations prevent the association of *SUR1* with $K_{IR}6.2$; however, this finding was not confirmed by others (39). In the current study, we describe two different missense mutations in the same domain, G70E and G111R. Although both have reduced trafficking through the Golgi, neither appears to affect association with $K_{IR}6.2$. Thus, the specific mechanism that causes this functional defect is still unknown. Our recombinant approach did not allow us to quantitate the number of channels at the plasma membrane or to define functionality. However, in β-cells derived from these patients at surgery, 44% of all experiments failed to identify any channel activity, and in the remaining 56%, only brief single-channel events were seen that were unresponsive to diazoxide or the intracellular addition of nucleotides. This confirms that few channels actually traffic to the plasma membrane and those that do are functionally abnormal.

In contrast, the three other *ABCC8* missense mutations, G1343E, R1419H, and R1494W, cluster in the second nucleotide-binding fold. These mutations exert a profound effect by confining the channel to the ER. It is interesting that none of these mutations affect the association of *SUR1* with $K_{IR}6.2$, as shown in the photoaffinity labeling studies. Other mutations, such as a single amino acid deletion (40) or missense mutations (41–43), with altered trafficking cluster in the second nucleotide-binding fold and C terminus of *SUR1*. Several mechanisms have been proposed to explain the trafficking defect, including increased degradation (44), unmasking of the retention signal (42), or retention in the trans-Golgi network (41). At the present time, we cannot ascertain which mechanism accounts for the phenotype observed with our mutants. The *KCNJ11* mutation, P254L, which is 137 amino acids from the C terminus, is located in the cytoplasmic part of the protein. It interacts with *SUR1* but cannot exit from the ER. The mechanism is not yet understood.

Defects in intracellular trafficking appear to be common mechanisms by which missense mutations cause recessive HI. This can be explained by the hetero-octomeric structure of the mature channel because, if mutant proteins would be readily incorporated into the channels and transported to the plasma membrane, then in the heterozygous state, 15 of 16 membrane-bound channels would be expected to contain mutant SUR1. In this situation, a dominant-negative effect

would be expected, and dominant or codominant inheritance would be observed (45).

In nine probands, two mutant alleles were identified, thus fully explaining the disease in these probands. In two probands, only a single mutant allele was found. In patient 10, the mutation 2154 + 3a→g was found on the paternal allele, suggesting that the patient may have had focal HI; however, histological analysis revealed diffuse disease that was confirmed by studies of ion channel electrophysiology (22). Furthermore, the proband had a haploidentical sibling with similar clinical, histological, and functional findings. A defect in splicing was observed in the proband and the sister. A second proband (patient 6), who was heterozygous for the same mutation on the maternal allele, had a different mutation on the paternal allele, confirming that this is a recessive mutation. Dominant inheritance of disease was further excluded by the fact that the carrier parent of each proband was clinically healthy. The possibility that this mutation can express a phenotype in the heterozygous state (autosomal dominant with variable penetrance) cannot be rigorously excluded; however, for this patient, the most likely conclusion is that there is a mutation present on the maternal allele that failed to be detected, despite direct sequencing of the entire gene.

In four patients, no mutation was identified in any of the five genes. These patients may be the most interesting patients of the group because some or all of them may have disease caused by mutations in other genes. Identifying the genetic cause of disease in these patients will be particularly challenging because they do not have affected relatives, and thus, linkage analysis is not possible. Mutation analysis of all genes that could potentially be responsible for unregulated insulin release is not feasible because the candidate list is too long. None of the patients had unique clinical or biochemical features that might direct the search to a particular candidate gene or signaling pathway. Patients 12 and 14 are phenotypically similar to most patients with K_{ATP} channel mutations; they have severe HI that failed to respond to diazoxide treatment. Patient 14 required pancreatectomies, whereas patient 12 responded to very intensive medical management, including continuous sc infusion of octreotide and glucagon as well as strict dietary management. β-cells isolated from the resected pancreas of patient 14 had normal K_{ATP} channels, confirming our genetic findings but, unfortunately, not providing evidence for any specific alternative mechanism causing hyperinsulinism.

Patients 13 and 15 have clinically milder disease that was responsive to diazoxide. Patient 15 had mild hyperammonemia. The father has a similar, albeit milder, clinical picture, suggesting autosomal dominant inheritance. This syndrome has previously been associated with mutations in *GLUD1*; however, no mutation was detected in this patient. MacMullen *et al.* (46) reported finding *GLUD1* mutations in 50 of 65 probands with HI/hyperammonemia, suggesting that mutations in other genes can cause this apparently discrete clinical syndrome. Therefore, it is likely that mutations in another gene, perhaps one coding for another enzyme in the same metabolic pathway as *GLUD1*, could be responsible for this syndrome.

In conclusion, we performed sequence analysis on the entire coding region of *ABCC8* and *KCNJ11* in a cohort of 15 HI patients. In patients in whom no mutation was identified in these two genes, the three other genes known to be associated with hyperinsulinemic hypoglycemia were similarly sequenced. This is the first study to report mutation screening, using direct sequencing, of all five genes (*ABCC8*, *KCNJ11*, *GCK*, *GLUD1*, and *SCHAD*) in the same patient cohort. Nine novel mutations were identified, including two in regulatory regions, one in *ABCC8*, and the others in *KCNJ11*, demonstrating for the first time mutations in the regulatory regions of these genes associated with HI. Mutations were found in K_{ATP} channels in 11 of 15 patients. Four patients with no apparent mutations in any of the five genes tested were thus identified. These patients represent a new cohort of patients that will be studied to identify new genes responsible for this disease.

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