Novel Mutations and a Mutational Hotspot in the MODY3 Gene

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Maturity-onset diabetes of the young 3 (MODY3) is a type of NIDDM caused by mutations in the transcription factor hepatocyte nuclear factor-1α (HNF-1α) located on chromosome 12q. We have identified four novel HNF-1α missense mutations in MODY3 families. In four additional and unrelated families, we observed an identical insertion mutation that had occurred in a polycytidine tract in exon 4. Among those families, one exhibited a de novo mutation at this location. We propose that instability of this sequence represents a general mutational mechanism in MODY3. We observed no HNF-1α mutations among 86 unrelated late-onset diabetic patients with relative insulin deficiency. Hence mutations in this gene appear to be most strongly associated with early-onset diabetes. *Diabetes* 46:1081–1086, 1997

Maturity-onset diabetes of the young (MODY) is a form of NIDDM with a strong genetic basis. Diagnostic criteria for MODY include *1*) age of onset <25 years, *2*) correction of fasting hyperglycemia without insulin for at least 2 years, *3*) nonketotic disease, and *4*) autosomal dominant mode of inheritance (1). It is therefore expected that a significant proportion of MODY is caused by single-gene defects. To date, linkage analysis has shown that three different, dominant-acting loci can cause MODY. The MODY1 locus, on chromosome 20q, was shown to be linked in a single extended family (2). MODY1 was recently shown to be caused by mutations in the transcription factor hepatocyte nuclear factor-1α (HNF-4α) (3). MODY2, located on chromosome 7p (4), is caused by mutations in the glucokinase gene (5). MODY3 was mapped to chromosome 12q (6) and is known to be caused by mutations in another transcription factor, HNF-1α (7). Although the relative prevalence of these MODY subtypes varies regionally (8–10), families linked to chromosome 12q are the most frequently described. The glucokinase defects in MODY2 interfere with glucose uptake, resulting in hyperglycemia (5). Although the mechanism of hyperglycemia in MODY1 and MODY3 is not yet understood, phenotypic characterization of these families has shown a deficient insulin secretion response to glucose (11–13).

We report here the characterization of the MODY3 gene region and the results of mutation analysis in eight different MODY3 families. We show that MODY3 can be caused by a number of different mutations in HNF-1α including a mutational hotspot.

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Received for publication 12 February 1997 and accepted in revised form 10 March 1997.

M.A.G., O.T., L.B., J.C.P., Y.W., W.-J.N., L.F., K.D.M., B.M.Y., J.L.W., G.M.D., and J.D.T. hold stock in Millenium Pharmaceuticals, a company engaged in diabetes research and drug development, which has a corporate partnership with Hoffman-La Roche. Millenium Pharmaceuticals is providing funds to the laboratory of A.S.K. to identify MODY families and search for mutations in the HNF-1 gene.

BAC, bacterial artificial chromosome; HNF-1α, hepatocyte nuclear factor-1α; MODY, maturity-onset diabetes of the young; PAC, P1 artificial chromosome; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

RESEARCH DESIGN AND METHODS

Family ascertainment and linkage analysis. Families were ascertained based on the criteria described by Lehto et al. (13). Genomic DNA was isolated from peripheral blood lymphocytes using standard protocols. Genotyping was performed using standard fluorescent and radioactive techniques for microsatellite analysis. Haplotypes were determined manually and confirmed using the haplotyping function of the Genehunter program (14).

Microsatellite screening. Small insert libraries were generated by restricting bacterial artificial chromosome (BAC) and P1 artificial chromosome (PAC) clones with *Sau*3AI and ligating these fragments into the Bluescript vector (Life Technologies). Approximately 2,000 clones from each BAC were screened by hybridization for each class of microsatellite repeat as described by Chen et al. (15).

Sequencing of the exon-intron boundaries of the HNF1 gene. The BAC clones (92N15, 167I24) containing the HNF-1α gene were isolated from a human BAC library obtained from Research Genetics (Huntsville, AL). Random sheared libraries were made from these BAC clones and were sequenced to eightfold redundancy. Exon-intron boundaries were determined by comparing the genomic sequence with that of the published HNF-1α cDNA (accession number M57732).

Mutation detection by sequencing. Patient DNAs were screened for mutations by direct sequencing of polymerase chain reaction (PCR) fragments amplified from each of the 10 coding exons in HNF-1α. PCR primers were designed to hybridize to intronic sequences and were tailed with sequences complementary to standard M13 sequencing primers. Primer sequences used in this study are available on the World Wide Web at http://www.diabetes.org. PCR amplifications were carried out in 1× PCR buffer (Perkin Elmer), 250 μmol/l dNTPs, 0.8 μmol/l forward/reverse primers, 0.05 U/μl AmpliTaq DNA polymerase and 20 ng of genomic DNA. Thermocycling parameters were as follows: 95°C for 1 min and 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 45 s, and finally 72°C for 10 min. Before fluorescent sequencing, primer-dimer products were removed by adding 0.2 U/μl Exonuclease I plus 0.04 U/μl shrimp alkaline phosphatase in 1× PCR buffer (Perkin Elmer)

and incubating at 37°C for 30 min, and 0.5–1 µg of the remaining DNA product was submitted for sequence analysis. Sequencing was performed with fluorescently labeled M13 primers and was analyzed on ABI373 and ABI377 DNA Sequencers, according to the recommendations of the manufacturer (Applied Biosystems). Each exon was sequenced three times on each strand. Sequence data was analyzed using the Sequencher software package.

Mutation analysis. Patient DNA was amplified by PCR in a 20-µl amplification mixture containing 10–20 ng DNA, 200 µmol/l dNTPs (Pharmacia Biotech), 20 µmol/l primers, and 0.05 U/µl AmpliTaq (Perkin Elmer) and 1× PCR buffer (Perkin Elmer). The cycling parameters were as above. PCR product (2 µl) was mixed with 5 µl of loading dye (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 20 mmol/l EDTA), denatured at 97°C for 5 min, and chilled immediately in an ice water bath. The samples were electrophoresed on 10% acrylamide (50:1) and 10% glycerol gels and run in 0.5× TBE buffer in a SA-32 Gibco BRL sequence apparatus at 4 W and 4°C, for 18–24 h. After electrophoretic separation, the gels were stained with 1× SYBR Green I (Molecular Probes), according to the manufacturer's instructions, and scanned at a photo-multiplier tube voltage of 700 in a Molecular Dynamics FluorImager 575.

Exon 4 Pro291insC mutations were also assayed by sizing radioactively labeled PCR amplification products on denaturing gels. A total of 25 ng of patient DNA, 16 mmol/l ammonium sulphate, 67 mmol/l Tris-HCl pH 8.8, 0.01% Tween 20, 1.5 mmol/l $MgCl_2$, 200 µmol/l dNTP, 3 pmol of [^{32}P]γ-dATP (Amersham), 3 pmol each of forward and reverse primer, and 0.5 U AmpliTaq (Perkin Elmer) were mixed in a final volume of 20 µl. Samples were denatured at 94°C for 30 s followed by 35 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s and finished with a final extension at 72 °C for 10 min. PCR products were mixed with an equal volume of loading buffer (see above), denatured for 5 min at 90°C, and then submitted to denaturing electrophoresis on 6% acrylamide/bisacrylamide 19:1, 7 mol/l urea, 1× TBE gels run with constant power (50 W for 160 min) followed by autoradiography. The primers chosen (5′-CAAGCTGGCCATGGACACGTAC and 5′-AGAGGGCAGGTGGAGGCAGG) generated a 101-bp fragment in nonmutated samples and a 102-bp fragment from mutant alleles.

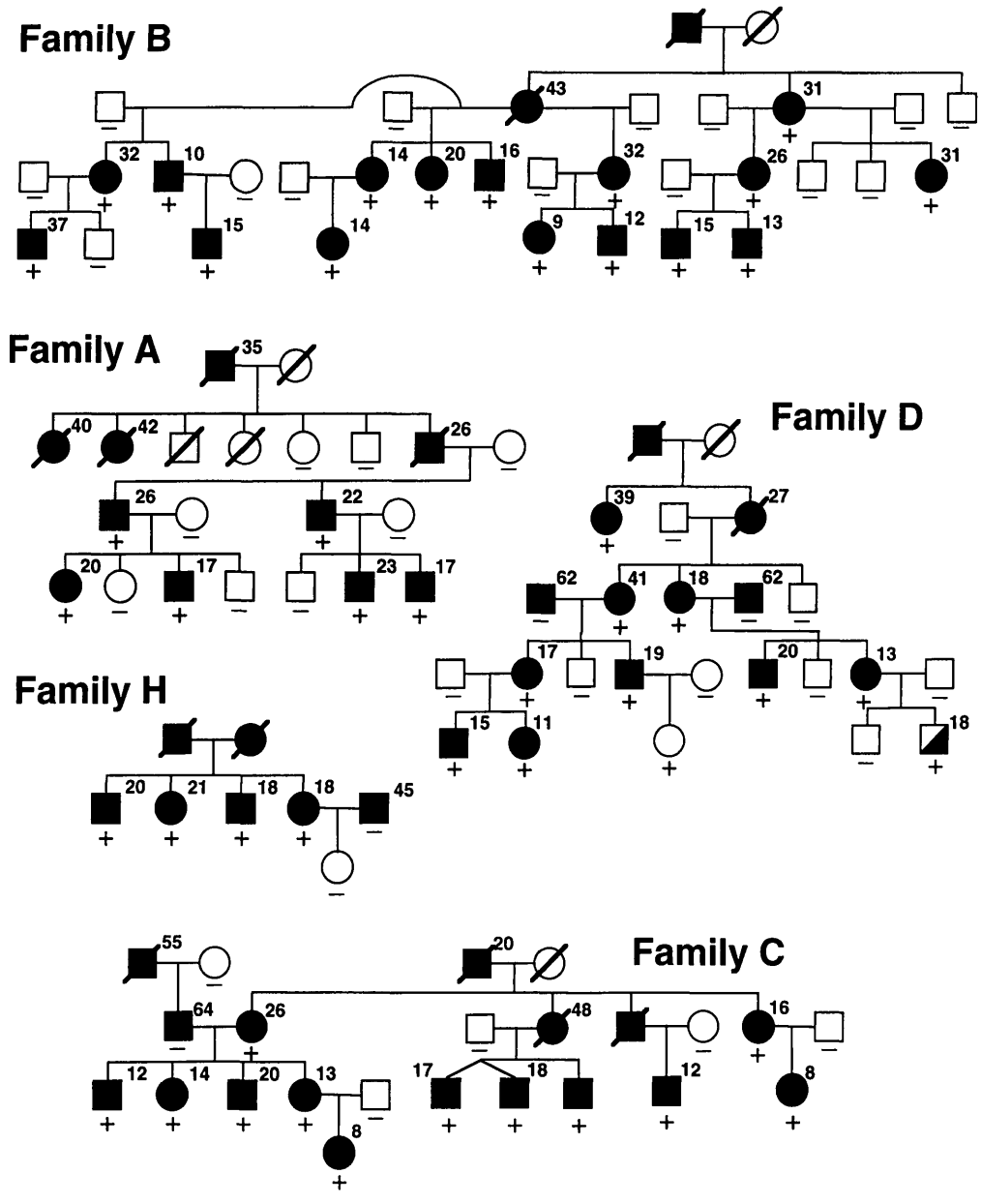

FIG. 1. MODY3 families: individuals with NIDDM (■ and ●); unaffected individuals (□ and ○); individuals with impaired glucose tolerance (◪ and ◒). Heterozygous HNF-1α mutation carriers are denoted + and noncarriers – under each square or circle, when known (see Table 1 for information on specific mutations). Numerals indicate age at diagnosis of diabetes when known. Pedigrees for families E-F-G and J are shown in Lehto et al. (13) and family K is shown in Fig. 2.

RESULTS

Linkage analysis. Of 35 families screened by linkage analysis, we identified four North American (A, B, C, D) and five Finnish MODY3 families (E, F, G, H, J) (13). Linkage to the MODY3 region of chromosome 12q was determined by microsatellite genotyping and haplotype analysis (Fig. 1). Three of the Finnish MODY3 families (E, F, G) were found to have a common haplotype and thereby a common ancestor with MODY3 (13).

Map construction and mutational analysis. To facilitate genetic localization of the MODY3 gene, a high density BAC and PAC map was created in the region of chromosome 12q spanning markers D12S86 and D12S342. Clones spanning this interval were screened for the presence of microsatellites, permitting the precise ordering of both previously published and newly identified genetic markers. A high-resolution "sequence ready" physical map covering the same interval was generated in parallel. A combination of sample sequencing, exon-trapping, and cDNA selection was used to identify over 100 transcripts.

Single-strand conformation polymorphism (SSCP) assays and nucleotide sequence analyses were performed to scan for mutations among known and novel transcripts in the critical region. Single affected individuals, one from each of five linked families (A, B, C, D, and E-F-G), were used for initial mutation screening. Fifteen transcript sequences were screened. Of the transcript sequences analyzed, only HNF-1α sequences were found to bear mutations in representative members of several MODY3 families (Table 1). A single member from each of 12 other MODY families, too small for linkage analysis, were then screened. From those, families H and K were also found to bear HNF-1α mutations. Sequence analysis in all family members confirmed that HNF-1α mutations segregated with early-onset NIDDM.

A commonly occurring mutation in exon 4. We observed five different mutations among these seven families (Table 1). One mutation, the insertion of a C nucleotide in exon 4 (Pro291insC), was observed in three independent families (C, H, and the E-F-G superfamily). Two of these families are Scandinavian (E-F-G and H), whereas the third (family C) is of Lebanese origin. This same mutation was observed by Yamagata et al. (7) in the "Edinburgh" family. This insertion (Pro291insC) is found after a series of eight consecutive cytidine residues. The propensity for insertion or deletion of additional nucleotides in simple sequence repeats during DNA replication is well documented (17). These observations led us to consider the possibility that the observed mutations have occurred independently and that this site represents a mutational hotspot.

To determine whether these families could be related by distant ancestry, we looked specifically for evidence of haplotype sharing within the HNF-1α gene by analyzing intragenic sequence polymorphisms (Table 2). The results confirm that families C, E-F-G, and H bear distinct haplotypes, consistent with the hypothesis that these identical mutations occurred independently.

We sought additional evidence that this is a mutational hotspot by conducting SSCP and denaturing fragment size analysis of exon 4 in 304 diabetic subjects from 207 Finnish and Swedish families. These patients had been diagnosed with IDDM or NIDDM before age 41 (mean age at diagnosis was 28 ± 11 years). One of these families, family K, did indeed harbor the exon 4 mutation (Table 1). Interestingly, of the three generations of diabetic patients available for study, only the latter two exhibited mutations (Fig. 2). Haplotype analysis (Fig. 2) showed that the maternal grandmother (I-b) transmitted the mutation-bearing chromosome to her daughter (II-e). Phenotypic features were consistent with the results of mutational analysis; I-b has diet-treated impaired glucose tolerance diagnosed at age 76, while II-e and her son (III-f) are both insulin-treated and were diagnosed at ages 18 and 11, respectively. These results are consistent with the presence of a de novo mutation in subject II-e.

Screen for HNF-1α mutations in late-onset diabetic patients. Given prior observations that MODY3 diabetes is associated with defects in insulin secretion (11–13), we set out to determine whether mutations in HNF-1α could account for late-onset NIDDM by testing for intragenic mutations among late-onset diabetic patients exhibiting evidence of relative insulin deficiency. We screened 86 unrelated diabetic patients from Sweden and Finland (the mean age at NIDDM diagnosis in this cohort was 57 ± 10 years. The patients had insulin levels at the 30-min time point of the oral glucose tolerance test (OGTT), which were in the lowest quartile of diabetic patients screened that were not on insulin therapy.

We screened all HNF-1α exons by SSCP analysis and then submitted all SSCP variants to sequence analysis. We

TABLE 1
HNF-1α mutations in MODY3 families

Family	Location	Amino acid change	Codon	Type	Nucleotide change	Nucleotide position
A	Exon 4	Arg→His	272	Missense	G→A	838
B	Exon 1	Leu→Arg	107	Missense	T→G	343
C	Exon 4	Frameshift	291	Chain terminating	C insertion	895
D	Exon 2	Arg→Trp	131	Missense	C→T	414
E-F-G	Exon 4	Frameshift	291	Chain terminating	C insertion	895
H	Exon 4	Frameshift	291	Chain terminating	C insertion	895
J	Exon 4	Thr→Met	260	Missense	C→T	877
K	Exon 4	Frameshift	291	Chain terminating	C insertion	895

Nucleotide and codon positions correspond to those in GenBank accession number M57732. Families G, E, F, and J correspond to families B, C, D, and A, respectively, in Lehto et al. (13), where these mutations were previously reported.

TABLE 2
Comparison of intragenic HNF-1α haplotypes in MODY3 families bearing the Pro291insC insertion

Polymorphism	Location	Codon	Nucleotide position	Sequence variation	Restriction enzyme	Family			
						C	E-F-G	H	K
HNF-1α (a)	Exon 1	17	74	CTC/ctg	*Xho* I	NA	2	1	1
HNF-1α (b)	Exon 1	27	102	ATC/ctc	*Sau*3AI	NA	2	2	2
HNF-1α (c)	Exon 7	459	1398	CTG/ttg	*Pst* I	1	2	2	2
HNF-1α (d)	Exon 7	487	1483	AGC/aac	*Ban* II	1	2	2	2
HNF-1α (e)	Intron 7		7 nt 3′ exon 6	G/a	*Hae* II	1	2	1	2
HNF-1α (f)	Exon 8	515	1568	acg/ACA	*Stu* 1	2	2	1	2
HNF-1α (g)	Intron 9		24 nt 5′ exon 10	T/c	*Nhe* I	NA	2	1	2

Families were screened by sequence analysis and/or PCR-RFLP analysis. Sequence variants were determined by direct sequence analysis. Nucleotide positions are as described in Table 2. Several individuals from each family were typed, permitting assignment of phase: 1, site not susceptible to restriction by the enzyme specified; 2, allele is susceptible; NA, not available; sequence variants indicated by capital letters are susceptible to the restriction enzyme.

observed no coding variants other than previously described polymorphisms (7). Hence we found no evidence that mutations in HNF-1α account for late-onset NIDDM in this Swedish and Finnish cohort.

DISCUSSION

HNF-1α mutations in MODY3. In total, we observed five HNF-1α mutations among eight MODY3 families. Four of these mutations are previously unreported. Of the eight MODY3 families described here, four were shown to harbor an identical insertion mutation (Pro291insC) suggesting that the polycytidine sequence in exon 4 of HNF-1α is a mutational hotspot. Several observations support this hypothesis. First, homopolymeric sequences are known to be relatively susceptible to insertion and deletion mutations (17). Second, we have observed this mutation in several families not known to be related by ancestry and which do not appear to share a common haplotype of intragenic polymorphisms. Finally, we observed a de novo mutation at this location. Of note, the (Pro291insC) mutation in exon 4 is easily assayed (see METHODS).

Population screening for HNF-1α mutations will allow more precise estimates of the prevalence of MODY3; however, available data suggest that mutations in this gene are rare. Overall, HNF-1α mutations accounted for 17% of MODY families in this study. We observed no coding sequence mutations in a cohort of late-onset diabetic patients with evidence of impaired insulin secretion. The cohort studied did not include any of the previously described NIDDM2 families from the Botnia region of Finland (18).

Putative structure/function relationships of the HNF-1α gene mutations. HNF-1α is an important transcriptional activator of many hepatic genes (19). It is also expressed in nonhepatic tissues, including the pancreas, and is presumed to stimulate transcription of genes in those tissues as well. HNF-1α is composed of three of the following functional domains: the dimerization domain, encoded by exon 1; the DNA-binding domain, encoded by exons 2, 3, and 4; and the transcriptional activating domain encoded by exon 5 through 10 (20). We observed both point and chain-terminating mutations in HNF-1α, all of which were located in the dimerization or the DNA-binding domain. Yamagata et al. (7) observed frameshift mutations in the transcriptional activating domain

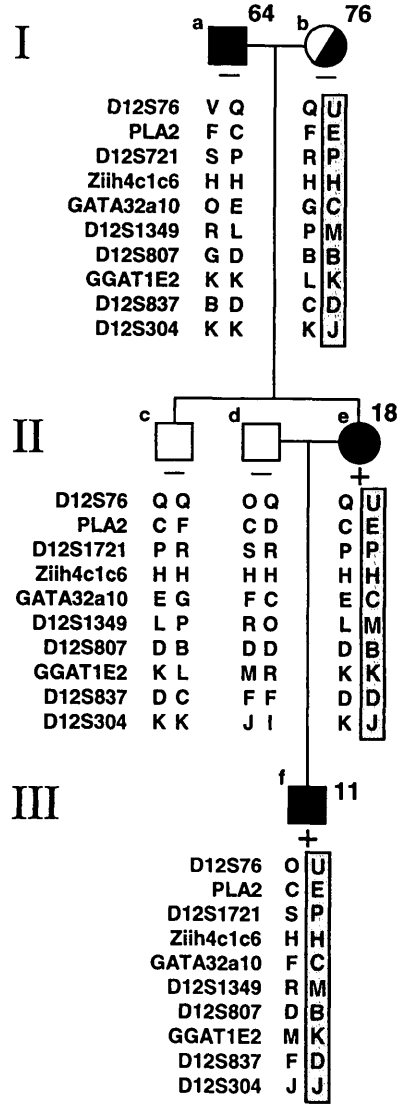

FIG. 2. Haplotype and mutation analysis of family K. Squares and circles are as in Fig. 1. Heterozygous Pro291insC HNF-1α mutations were observed in individuals II-e and III-f, but not in individual I-b, suggesting the occurrence of a de novo mutation. HNF-1α is located between GATA32a10 and D12S1349. Marker Ziih4c1c6 was identified and characterized by this group.

as well. Hence disruption of any of the functional domains of this gene can cause MODY.

Mutations in a gene such as this could result in diminished amounts of functional transcription factor by either haploinsufficiency or dominant-negative mechanisms. Since HNF-1α homodimers form the active transcription factor, mutated or truncated HNF-1α protein may bind to wild-type HNF-1α protein to form nonfunctional dimers. Presuming stoichiometric expression of normal and mutated protein, the amount of functional HNF-1α could be reduced by two thirds. Should HNF-1α mutations preclude the expression of the gene product altogether, for example, by rendering the mutant polypeptide susceptible to premature degradation, as little as one-half of the normal HNF-1α may be present. Either model could account for the dominant nature of HNF-1α mutations. That MODY 1 is caused by mutations in HNF-4α (3) supports the hypothesis that HNF-1α deficiency is responsible for MODY, since HNF-4α is known to activate transcription of HNF-1α (20). HNF-1α can also dimerize with the closely related transcription factor, variant HNF1 (vHNF1) (21). Multiple isoforms of these genes, generated by alternative splicing, can form admixed heterodimers (22).

HNF-1α knockout mice exhibited hepatic dysfunction, phenylketonuria, and Fanconi's syndrome including renal glycosuria, but did not exhibit hyperglycemia (23). Renal glycosuria was also reported in two of the original MODY families described by Tattersall (1) and was a common feature among Finnish MODY3 families examined (M.L. and L.G., unpublished observations). However, the MODY3 phenotype is significantly less severe than that seen when HNF-1α is disrupted in mice.

The HNF-1α transcription factor regulates the expression of a variety of genes known to be important in glucose metabolism. One report suggests that HNF-1α directly regulates the expression of insulin via its promoter (24). The promoters of the glucose transporter (GLUT2) and glucokinase, required in islet cells for efficient insulin secretion, both contain HNF-1α binding sites (25). Several glycolytic and gluconeogenic enzymes are also known to be regulated by or contain binding sites for HNF-1α, including glucokinase (25), pyruvate kinase (26), and phosphoenolpyruvate carboxykinase (27). Transcriptional activation of glycolytic genes in hepatocytes is dependent on the action of glucose and insulin (28), so HNF-1α might also directly influence liver glucose metabolism. As evidenced by gene knockout studies, HNF-1α deficiency disrupts normal hepatic and renal function (23). HNF-1α might similarly alter the function of pancreatic β-cells resulting in impaired insulin secretion.

Understanding the precise mechanism by which HNF-1α deficiency causes diabetes will require extensive investigation. The elucidation of mutations in the HNF-1α gene linked to NIDDM should help focus such efforts.

ACKNOWLEDGMENTS

This work was funded by Hoffmann-La Roche (Nutley, NJ), and by grants from the Sigrid Juselius Foundation, the Swedish Medical Research Council, and the Påhlssons Foundation.

We thank James Brayer, Gayun Chan, Feng Lin, Juliet Bryce, Lewis Wogan, K. Randy Yang, Fiona Hawkins, and Stuart Yang for their superb technical work. We gratefully acknowledge the assistance of Cheryl McMenimen, Jill Goranson, Susan Scotti, Michael Wantman, and the Botnia, Helsinki, and Malmö Research Teams for their efforts in family studies.

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