A Missense Mutation in Hepatocyte Nuclear Factor-4α, Resulting in a Reduced Transactivation Activity, in Human Late-onset Non–insulin-dependent Diabetes Mellitus

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Abstract

Non–insulin-dependent diabetes mellitus (NIDDM) is a heterogeneous disorder characterized by hyperglycemia resulting from defects in insulin secretion and action. Recent studies have found mutations in the hepatocyte nuclear factor-4 alpha gene (HNF-4α) in families with maturity-onset diabetes of the young (MODY), an autosomal dominant form of diabetes characterized by early age at onset and a defect in glucose-stimulated insulin secretion. During the course of our search for susceptibility genes contributing to the more common late-onset NIDDM forms, we observed nominal evidence for linkage between NIDDM and markers in the region of the HNF-4α/MODY1 locus in a subset of French families with NIDDM diagnosed before 45 yr of age. Thus, we screened these families for mutations in the HNF-4α gene. We found a missense mutation, resulting in a valine-to-isoleucine substitution at codon 393 in a single family. This mutation cosegregated with diabetes and impaired insulin secretion, and was not present in 119 control subjects. Expression studies showed that this conservative substitution is associated with a marked reduction of transactivation activity, a result consistent with this mutation contributing to the insulin secretory defect observed in this family. (J. Clin. Invest. 1998. 101:521–526.) Key words: mutation • late-onset non–insulin-dependent diabetes mellitus • MODY • hepatocyte nuclear factor • gene expression

Introduction

Non–insulin-dependent (type 2) diabetes mellitus (NIDDM)[1] is a phenotypically and genetically heterogeneous disorder affecting more than 100 million people worldwide. It usually appears in the fourth and fifth decades of life and results from defects in insulin secretion as well as in action of insulin on its target tissues, muscle, fat, and liver (1). However, little is known about the molecular mechanisms involved in the development of NIDDM and investigators have begun to use genetic approaches in order to gain a better understanding of why and how NIDDM develops. Such studies have led to the localization of NIDDM susceptibility genes in the Mexican American population of Starr County, Texas (NIDDM1), and in the Swedish-speaking population of Botnia, Finland (NIDDM2) (2, 3).

Genetic studies of families with a monogenic form of NIDDM, termed maturity-onset diabetes of the young (MODY), have also provided new insights into the etiology of diabetes. Accounting for about 5–10% of NIDDM cases, MODY is an autosomal dominant form of NIDDM characterized by early-onset (usually ≤ 25 yr) and a primary defect in insulin secretion (4). The identification of three genes associated with MODY has provided a better understanding of the factors regulating blood glucose levels. These genes include the hepatocyte nuclear factor-4 alpha (HNF-4α)/MODY1 gene on chromosome 20q, the glucokinase/MODY2 gene on chromosome 7p, and the HNF-1α/MODY3 gene on chromosome 12q (5–7). HNF-1α and -4α are transcription factors first identified in the context of their role in regulating liver-specific gene expression although they are also found in other tissues including the insulin-secreting cells of the pancreas (8). Mutations in both of these genes are associated with specific defects in the normal pattern of glucose-stimulated insulin secretion, and their effect on pancreatic β cell function leads to diabetes (5, 7). Glucokinase is a glycolytic enzyme that plays a key role in glucose-

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1. *Abbreviations used in this paper: HNF-1α*, hepatocyte nuclear factor-1 alpha gene; *HNF-4α*, hepatocyte nuclear factor-4 alpha gene; MLS, multipoint-likelihood score; MODY, maturity-onset diabetes of the young; NIDDM, non–insulin-dependent (type 2) diabetes mellitus; TK, thymidine kinase.

sensing by the pancreatic β cell and integration of hepatic glucose metabolism (9). Deficiency of this enzyme leads to defects in insulin secretion in the pancreatic β cells, and glycogen synthesis in the liver, both of which cause hyperglycemia (6).

Although mutations in *HNF-1α* and glucokinase are a major cause of MODY accounting for 35–75% of MODY (6, 7, 10, and 11), they do not appear to be a significant contributory factor to genetic susceptibility for the common late-onset form of NIDDM in White subjects of French ancestry (12, 13, and Vionnet, N., unpublished data). By contrast, we observed nominal evidence for linkage of markers in the region of the *HNF-4α/MODY1* gene with NIDDM in a subset of French families with age at onset ≤ 45 years: two-point multipoint-likelihood score (MLS) equal to 2.84 (*P* = 0.0003) and multipoint-MLS equal to 1.51 (*P* = 0.007) (14). Similar results have been recently obtained in White NIDDM sib-pairs from the United States (15, 16). This prompted us to screen *HNF-4α/MODY1* in 19 French NIDDM families potentially contributing to the observed linkage to assess the role of *HNF-4α/MODY1* in these families.

In this report, we describe a valine-to-isoleucine substitution (V393I) in *HNF-4α* which cosegregates with typical late-onset NIDDM. We also show that this mutation is closely associated with a progressive alteration in insulin secretion, a result compatible with functional expression data, where we demonstrate that the V393I mutation causes a significant decrease of HNF-4α transcription activation of specific target genes including the *HNF-1α* gene.

Methods

Subjects. We investigated 19 NIDDM families for mutations in the coding exons of the *HNF-4α* gene. These families belong to a previously well-described collection of French Caucasian late-onset NIDDM pedigrees (13, 14). There was no evidence of linkage between markers for glucokinase*/MODY2* and *HNF-1α/MODY3* loci and NIDDM in these families (12, 13). Furthermore, direct screening revealed no mutations in the *HNF-1α/MODY3* gene (Vionnet, N., unpublished data). Genotyping polymorphic markers on chromosome 20q in this sample showed nominal evidence of linkage with NIDDM diagnosed before the age of 45 yr (14). The two-point MLS being 2.84 (*P* = 0.0003) and the multipoint MLS reached 1.51 (*P* = 0.007) in the *MODY1* region. 19 families positively contributing to these MLSs were selected for mutational screening. We screened one affected subject from each family. The clinical features of the 19 probands (12 men and 7 women) are: present age, 63±11 years (mean±SD); age at diagnosis, 34±8 years; present body mass index, 27.0±4.1 kg/m²; present fasting plasma glucose and insulin levels, 9.8±3.8 mmol/liter and 14±10 mU/liter, respectively; and current treatment, oral hypoglycemic agents, 11; insulin, 4; oral hypoglycemic agents plus insulin, 4. NIDDM was diagnosed based on the WHO criteria (17), and all control subjects underwent a standard oral glucose tolerance test.

HNF-4α gene screening. The 10 exons and the adjacent introns of the *HNF-4α* gene were amplified by polymerase chain reaction (PCR) (5). The PCR products were purified using G-sephadex P60 gel matrix in 96-well Millipore plate filters, before sequencing reactions. Sequencing was performed on both strands using an AmpliTaq FS Dye terminator Cycle Sequencing Kit (Perkin-Elmer, Corp., Norwalk, CT; Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. Sequencing reactions were purified using G-sephadex P10 gel matrix in 96-well Millipore plate filters before electrophoresis on an ABI Prism 377 DNA sequencer.

PCR-RFLP analysis of exon 9. Genotyping of the V393I mutation was examined by a restriction cleavage assay using a nested forward oligonucleotide with two mismatches at the third and fourth

nucleotides from the 3′ end (5′-TGAGTGGGCATTGTGTTGGCA-ACG TCGA-3′); the reverse primer corresponded to the wild-type sequence (5′-GCATCCCAGACTCTCCATCC-3′). The two mismatches in the forward primer introduce a SalI polymorphic restriction site, depending on the presence or absence of the exon 9 G-to-A transition. Specificity of this screening protocol was controlled by direct sequencing on both strands of the yielded PCR product (data not shown). The amplified product (146 bp) was digested with 5 U of SalI at 37°C, before loading onto 4% High Resolution Blend 3:1 agarose gel. The normal allele is digested into 117 bp and 29-bp fragments, whereas mutant allele is uncut.

Expression vectors and reporter constructs. The mutant V393I HNF-4α2 was generated from the wild-type human HNF-4α2 isoform (18) cloned in the expression vector pSG5 using the Stratagene ExSite™ PCR-based site-directed mutagenesis kit according to the manufacturer's instructions. The sense primer 5′-ATGGGTACCAACATC-ATCGTTGC-3′ contained a G-to-A mutation at position 13 to generate the V393I substitution and a translationally silent T-to-A mutation at position six introducing a RsaI restriction site to aid in screening for clones with V393I mutation. The antisense primer 5′-ATGTTC-CTGCATCAGGTGAGGGTG-3′ corresponded to the wild type sequence. The human apolipoprotein CIII (*apo CIII*) promoter (EcoRI −1400/PvuII +21) and the mouse hepatocyte nuclear factor-1α (*HNF-1α*) promoter (PstI −1300/NheI +157) were cloned in the pBLCAT6 reporter vector to generate the *apo CIII-CAT* and the *HNF-1α-CAT* vectors, respectively. The *CIIIB TK* corresponded to the minimal thymidine kinase (TK) promoter (−105/+51) under the control of the HNF-4 response element B of the *apo CIII* gene promoter (CIIIB), inserted upstream of the *CAT* reporter gene.

Transient transfection assays. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS) and containing 4.5 g/liter glucose. Cells were transfected by the Polyethyleneimine method as described (19), using 300 ng of the *CAT* reporter plasmid and 75 ng of pSG5 expression vector either without cDNA insert or with HNF-4α cDNA insert (wild-type or V393I mutant HNF-4α2). To normalize for differences in transfection efficiencies a CMV-driven β-galactosidase expression plasmid was transfected simultaneously. CAT assays were performed as previously described (20).

Protein expression. Wild-type and V393I mutant HNF-4α proteins were transiently expressed in COS-1 cells. Nuclei were isolated and nuclear extracts were prepared as previously described (21, 22). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's recommendations. Nuclear proteins were separated by SDS-10% PAGE and then transferred to a Hybond-C extra membrane (Amersham Life Sciences-USB, Arlington Heights, IL). The membrane was preincubated overnight at 4°C in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (vol/vol) Tween 20 (TBST) in the presence of 5% (wt/vol) nonfat dried milk. After four washes in TBST, the membrane was incubated for 2 h with a 1:2,500 dilution of the specific anti–HNF-4 α445 antiserum (23) in TBST containing 2% nonfat dried milk. The blot was then washed in TBST and incubated for 1 h with the secondary antibody, a peroxidase-conjugated anti-rabbit at a 1:1,000 dilution in TBST containing 2% nonfat dried milk. The blot was washed again in TBST and the peroxidase activity was revealed with the ECL enhanced chemiluminescence system (Amersham Life Sciences-USB).

Results

One diabetic subject was selected from each family and the 10 exons of the *HNF-4α* gene were amplified and sequenced on both strands (5). The sequences were identical to each other and to the published sequence except for four differences (Table I). Three out of these four substitutions occurred in introns. The fourth substitution in exon 9 (Fig. 1 *A*), a G-to-A transition in codon 393 resulting in a valine to an isoleucine

Table I. HNF-4-α Gene Substitutions in French Late-onset NIDDM Families

Exon/intron	Codon/nucleotide	Nucleotide change
Exon 9	codon 393	GTC(Val)→ATC(Ile)
Intron 1A	nt + 33	G→A
Intron 2	nt-5	C→T
Intron 9	nt + 61	A→T

nt, nucleotide.

mutation, was found in only one subject, a member of the pedigree F-40.

Since the V393I substitution does not result in the gain or loss of a site for a restriction endonuclease, we developed a simplified protocol that would allow us to use a PCR-RFLP assay to test for its presence (Fig. 1 *B*). Using this method, we found that the V393I mutation cosegregated with symptomatic NIDDM in the F-40 pedigree (Fig. 2), and was not found in any of 119 normal healthy control subjects; we did not detect this mutation in any of 191 other French NIDDM families. In pedigree F-40, six non-diabetic subjects were also found to carry the V393I mutation. Four of them are younger than 40 yr (32–39) and, thus, are under the average age at onset of diabetes in this family which is 45 yr (range: 40–55). The other two subjects are aged 48 and 50 yr, respectively. All these subjects have normal glucose tolerance, except subject 21, aged 50 yr, who recently presented with impaired glucose tolerance during an oral glucose test. Oral glucose tolerance tests were performed in five of these subjects (subjects 17–22, Fig. 2) in 1992 and again in 1997. They all had normal glucose tolerance in 1992, including subject 21. However, examination of the insulin/glucose ratio at 2 h after the oral glucose load, which is a reflection of insulin secretory function, showed a decrease of this parameter in the last 5 yr (6.35±3.30 versus 2.67±2.97 mU/mmol; $P = 0.043$ paired Wilcoxon signed-rank test; individual data are shown in Fig. 3). These data support a significant and progressive impairment of insulin secretion in the non-diabetic subjects who have inherited the V393I mutation. Clinical and biological data of diabetic and non-diabetic family members who carry the mutation are summarized in Table II. Two family members who did not inherit the V393I mutation, subjects 10 and 27, also had mild impaired glucose tolerance. Subject 27 is obese (BMI = 31.1 kg/m^2), a condition that is often associated with abnormalities in glucose homeostasis. In addition, both of her parents have diabetes, with her mother carrying the mutation V393I (subject 24). Subject 10 is 75 yr of age, and has only recently developed mild impaired glucose tolerance associated with high insulin values. This is likely a reflection of the glucose intolerance that is present in a significant fraction (10–25%) of elderly individuals (24).

The effect of the V393I mutation on *HNF-4α* function was evaluated using normal and mutant form of HNF-4α2, the most abundant HNF-4α isoform present in liver, intestine, kidney and pancreatic islets tissues, and three different HNF-4α targets: the *apo CIII* promoter, *HNF-1α* promoter, and the *TK* promoter under the control of the HNF-4α response element CIIIB (*CIIIB TK*). The activities of these three promoters in the presence of wild-type or mutant HNF-4α2, compared with their basal activities in COS-1 cells lacking endogenous HNF-4α

activity are presented in Fig. 4 *A*. Wild-type HNF-4α2 activated expression of *apo CIII*, *HNF-1α*, and *CIIIB TK* promoters by 140, 185, and 60%, respectively. In contrast, V393I HNF4-α2 had lower activity, increasing expression by only 70, 90, and 20%, respectively. Thus, the transactivation activity of the V393I mutant HNF-4α2 was reduced at least by half, when compared with that of the wild-type HNF-4α2 protein. This decrease was not due to a reduced expression of the mutant HNF-4α2 protein, as ascertained by Western blot analysis of nuclear extracts of transfected COS-1 cells (Fig. 4 *B*).

Discussion

These genetic and biochemical data suggest that the V393I mutation is a diabetogenic mutation rather than a rare variant of no functional consequence. The mutation was present in all NIDDM subjects from pedigree F-40 and in a glucose intolerant family member, but was not detected in 119 healthy non-
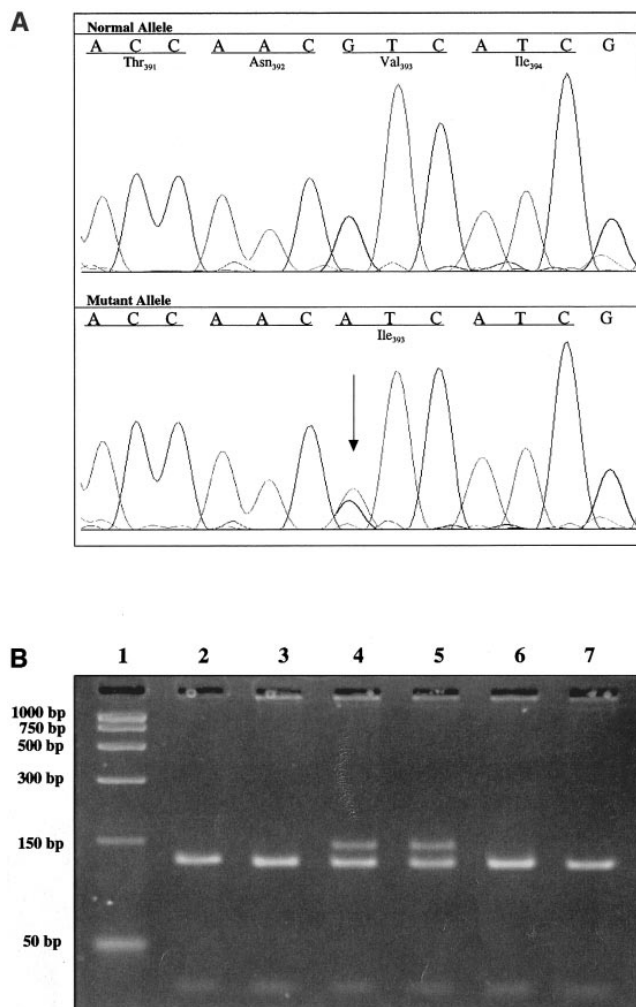
Figure 1. (*A*) Partial sequence of exon 9 of *HNF-4α* gene. The sequences of the normal and mutant alleles are shown. The arrow indicates the G-to-A substitution at codon 393. (*B*) PCR-RFLP analysis of exon 9 V393I mutation in *HNF-4α* gene. Lane *1* contains 50 ng of standard PCR size marker (Promega Corp., Madison, WI). Lanes *2*, *3*, *6*, and *7* show the normal band pattern. Lanes *4* and *5* show the band pattern for V393I mutation heterozygous carriers, where the mutant allele is uncut.
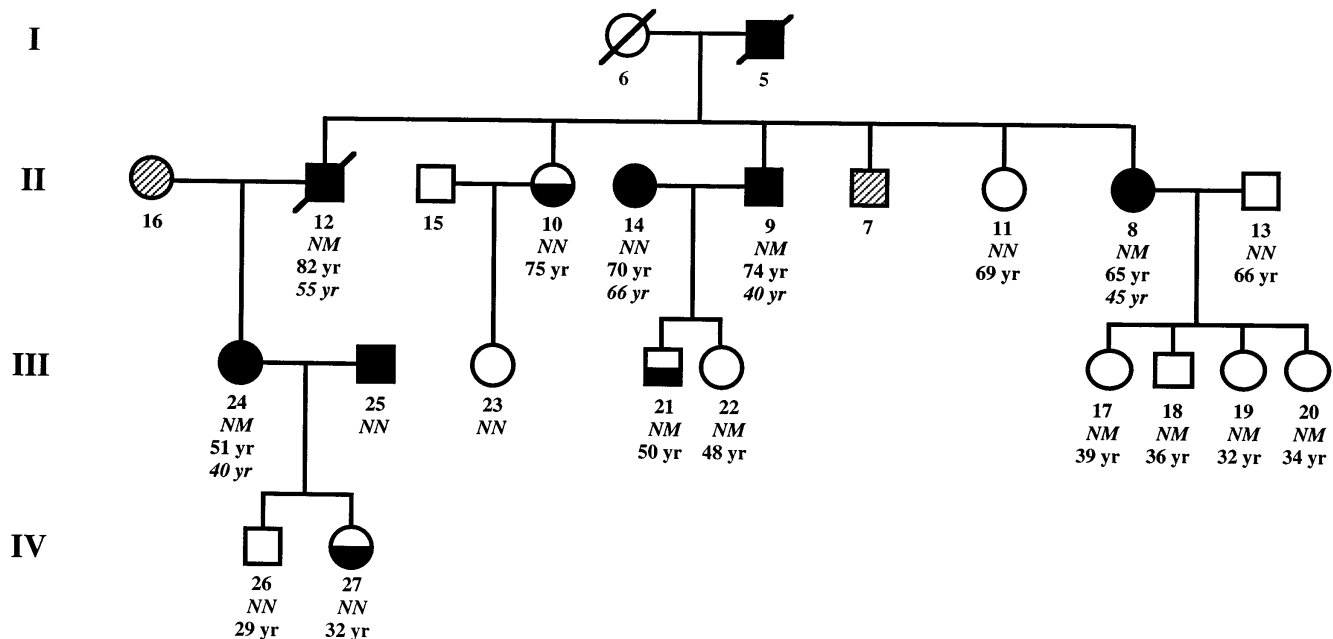
Figure 2. Segregation of the V393I mutation in Pedigree F-40. Closed, half-closed, and open symbols represent subjects with NIDDM, impaired glucose tolerance and normal glucose tolerance, respectively, while hatched symbols represent untested subjects. The first line under the symbols is the identification number. The second line shows the genotype at codon 393 of HNF-4α (*italics*): *N*, normal allele (Val393); *M*, Mutant allele (Ile393). The third and fourth lines show, respectively, the current age of subjects and the age at diagnosis of diabetes (*italics*), when appropriate. The proband is subject No. 12, who is now deceased.

diabetic control subjects. Moreover, five of the six middle-aged unaffected subjects in family F-40 carrying the V393I mutation exhibited a decline in insulin secretion and as a consequence are likely to be at increased risk of developing NIDDM. In this regard, the insulin secretory response observed in these five

subjects is very similar to that seen in the prediabetic members of the MODY pedigree with the Q268X mutation in *HNF-4α* as they have normal insulin secretion rates at lower glucose levels but lower than normal rates at high glucose levels (25). The dominant-like transmission of diabetes in family F-40 may evoke undiagnosed MODY where several subjects may be non-penetrant. However, the large number of non-diabetic middle-aged subjects with the V393I mutation together with the observed decline in insulin/glucose ratio with age in these subjects, suggests that the inheritance of diabetes in family
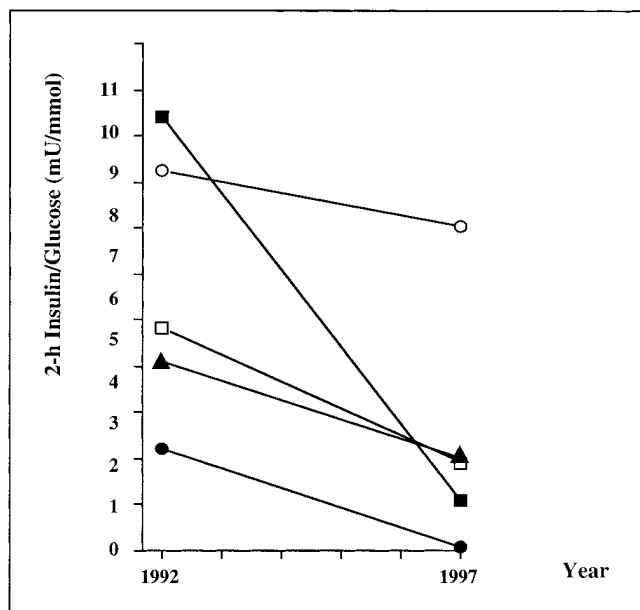

Figure 3. Five year evolution of insulin secretion in five non-diabetic family members who have inherited the V393I mutation. Data show the insulin/glucose ratio at 2 h during an oral glucose tolerance test. Family members are subjects 17 (*open square*), 18 (*closed circle*), 20 (*closed square*), 21 (*open circle*), and 22 (*closed triangle*).

Table II. Clinical and Biological Profile of Diabetic and Non-diabetic Carriers of the V393I Mutation in Family F-40

	Diabetic subjects	Non-diabetic subjects	P
Sex: male/female	2/2	2/4	–
Age (yr)	68±13	36±7	0.01
BMI (kg/m2)	28.1±3.0	23.3±2.5	0.05
Age at diagnosis (yr)	45±7	–	–
Systolic pressure (mmHg)	137±15	130±7	0.44
Diastolic pressure (mmHg)	80±10	81±3	0.86
Fasting glucose (mmol/liter)	9.4±3.9	5.3±0.7	0.02
Fasting insulin (mU/liter)	7±5	6±4	0.95
Triglycerides (mmol/liter)	1.50±0.44	1.06±0.47	0.20
Total cholesterol (mmol/liter)	5.31±1.16	6.19±1.32	0.52
HDL cholesterol (mmol/liter)	1.54±0.28	1.64±0.36	0.67
Creatinine (μmol/liter)	85±21	66±12	0.13
Treatment: diet/OHA (n)	1/3	–	–

Data expressed as mean±SD. Statistics are Kruskall-Wallis test. *BMI*, body mass index; *OHA*, oral hypoglycemic agents.

A

B
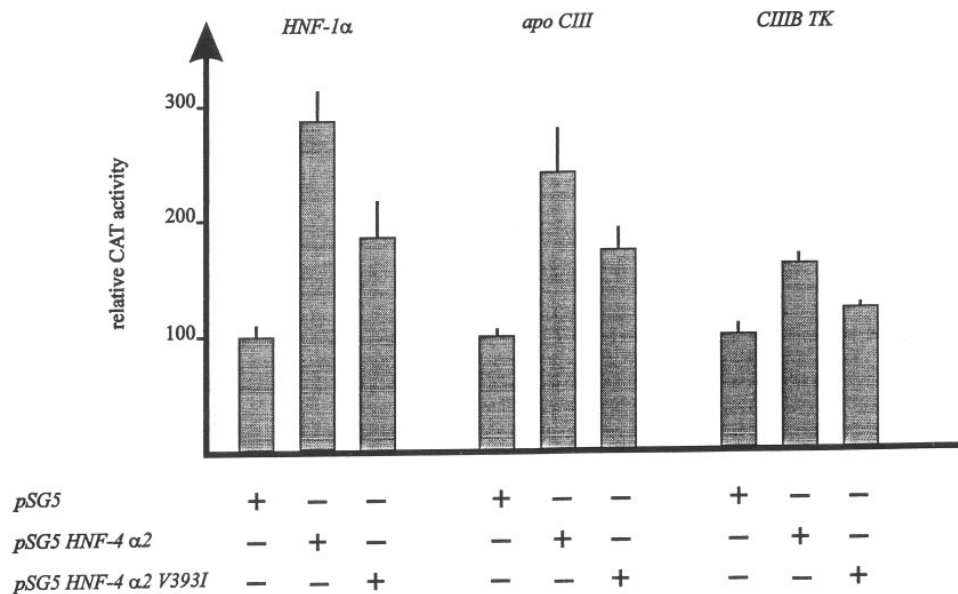
Figure 4. The effect of the V393I mutation on HNF-4α transactivation activity. (*A*) Transactivation activity. The mutation effect was analyzed on three promoters: the human *apo CIII* (*apo CIII*), the mouse *HNF-1α* (*HNF-1α*), and the thymidine kinase promoter under the control of the HNF-4α response element of the *apo CIII* gene promoter (*CIIIB TK*). 100% corresponds to the basal activity of each promoter without cotransfected HNF-4α. The error bars indicate the standard deviation of β-galactosidase normalized CAT activities from three experiments performed in triplicate. (*B*) Expression of wild-type and V393I mutant HNF-4 proteins. The amounts of wild-type (lane *1*) and V393I (lane *2*) HNF-4 proteins present in nuclear extracts of transfected COS-1 cells were determined by Western blotting using the specific anti-HNF-4 a445 antiserum. 5 μg of nuclear proteins was loaded in each lane.
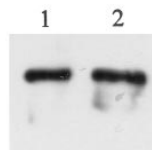
F-40 is multifactorial with the V393I mutation in HNF-4α being one of the genetic factors involved.

The V393I substitution is located in the F domain of HNF-4α, a region which in other members of the nuclear receptor superfamily is involved in modulating transactivation activity (26, 27). This domain exhibits a modulatory effect on the transactivation activity of HNF-4α (28; Suaud, L., and B. Laine, unpublished data). The F domain is also adjacent to the activation function-2 activation domain (AF-2 AD) which represents a crucial module in the activation function-2 (AF-2) (28). In this regard, it has been reported for several nuclear receptors that the AF-2 AD module is involved in protein–protein interactions with coactivators or corepressors that are required for nuclear receptor transcription activity (for review see reference 29).

Genetic studies are beginning to provide a better understanding of the molecular basis of NIDDM. The identification of mutations in *HNF-4α* in families with classical MODY (5, 30, 31) and in those believed to have the more common polygenic late-onset form of NIDDM (this report and reference 32) underscores the difficulties associated with the clinical classification of this disorder. The studies presented here suggest that the V393I mutation in *HNF-4α* is probably the major genetic factor contributing to the development of NIDDM in the

F-40 pedigree. The later age at diagnosis of NIDDM observed in this family compared with a typical MODY family is likely a reflection of relative differences in the effects of various mutations on HNF-4α activity. Severe mutations present as typical MODY and mild mutations as late-onset NIDDM. Interestingly, the natural history of the development of diabetes due to inheritance of either a severe or mild *HNF-4α* mutation is quite similar in that there is a progression from normal to impaired to diabetic glucose tolerance. What distinguishes the two types of mutations is the age at onset of diabetes which is also presumably determined by nongenetic factors. For MODY, we believe the major factor triggering the development of diabetes is the insulin resistance associated with puberty. The factor(s) that triggers the development of diabetes in families such as F-40 is unknown; however, it may be similar to those that contribute to the development of late-onset polygenic NIDDM. Prospective studies of prediabetic subjects who have inherited the V393I mutation may lead to the identification of these factors and to a better understanding of all forms of late-onset NIDDM.

The *HNF-4α/MODY1* V393I mutation described here is not sufficient to explain the observed evidence for linkage of NIDDM with the *MODY1* locus in our families, since it was found in only a single pedigree. However, we cannot exclude

that a second diabetes-predisposing locus lies in this chromosomal region. In summary, the present data are consistent with the role of HNF-4α as a crucial regulatory factor in controlling integration of intracellular metabolic signals (33). Moreover, a relatively small impairment of its transactivation activity is associated with progressive alteration of glucose homeostasis. As HNF-4α is an orphan receptor, it represents a potential target for specific drugs that could increase HNF-4α activity, and thereby enhance expression of downstream genes, such as *HNF-1α/MODY1*, in order to correct or prevent diabetes.

Note added in proof: While this paper was submitted, we have used the luciferase reporter instead of the CAT reporter. In these conditions, the *apo CIII* and *HNF-1α* promoters were activated 18- and 8-fold by the wild-type HNF-4α2, respectively, versus 11- and 4-fold obtained with the V393I mutant HNF-4α2, respectively. Therefore, the reduction of the transactivation activity of the V393I mutant HNF-4α2 compared to that of the wild-type HNF-4α2 remains consistent and similar to that indicated in the manuscript.

Acknowledgments

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