Differential Effects of HNF-1α Mutations Associated with Familial Young-Onset Diabetes on Target Gene Regulation

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Hepatocyte nuclear factor 1-α (HNF-1α) is a homeodomain transcription factor expressed in a variety of tissues (including liver and pancreas) that regulates a wide range of genes. Heterozygous mutations in the gene encoding HNF-1α (*HNF1A*) cause familial young-onset diabetes, also known as maturity-onset diabetes of the young, type 3 (MODY3). The variability of the MODY3 clinical phenotype can be due to environmental and genetic factors as well as to the type and position of mutations. Thus, functional characterization of *HNF1A* mutations might provide insight into the molecular defects explaining the variability of the MODY3 phenotype. We have functionally characterized six *HNF1A* mutations identified in diabetic patients: two novel ones, p.Glu235Gly and c-57-64delCACGCGGT;c-55G>C; and four previously described, p.Val133Met, p.Thr196Ala, p.Arg271Trp and p.Pro379Arg. The effects of mutations on transcriptional activity have been measured by reporter assays on a subset of HNF-1α target promoters in Cos7 and Min6 cells. Target DNA binding affinities have been quantified by electrophoretic mobility shift assay using bacterially expressed glutathione-S-transferase (GST)-HNF-1α fusion proteins and nuclear extracts of transfected Cos7 cells. Our functional studies revealed that mutation c-57-64delCACGCGGT;c-55G>C reduces *HNF1A* promoter activity in Min6 cells and that missense mutations have variable effects. Mutation p.Arg271Trp impairs HNF-1α activity in all conditions tested, whereas mutations p.Val133Met, p.Glu235Gly and p.Pro379Arg exert differential effects depending on the target promoter. In contrast, substitution p.Thr196Ala does not appear to alter HNF-1α function. Our results suggest that *HNF1A* mutations may have differential effects on the regulation of specific target genes, which could contribute to the variability of the MODY3 clinical phenotype.

INTRODUCTION

Heterozygous mutations in the gene encoding hepatocyte nuclear factor 1-α (HNF-1α) are the cause of familial young-onset diabetes, also known as maturity-onset diabetes of the young (MODY)-3 (1). This monogenic β-cell diabetes is characterized by autosomal-dominant inheritance, early age of onset and a progressive β-cell failure resulting in increasing hyperglycemia. Patients with mutations in HNF-1α show also a decreased renal threshold for glucose reabsorption and increased sensitivity to sulfonylureas.

HNF-1α is a homeodomain-containing transcription factor expressed in liver, kidney, pancreas and gut (2). It regulates a large number of liver-specific genes such as those encoding albumin, α1-antitrypsin and β-fibrinogen, as well as pancreatic genes involved in glucose metabolism and transport, including those encoding pyruvate kinase and the glucose transporter GLUT2, and also regulates the insulin gene expression (3,4). Expression of other islet-enriched transcription factors such as HNF-4α, Pdx-1 and NeuroD1/Beta2 is altered in HNF-1α knockout mice, suggesting a complex interrelationship and hierarchical network of transcriptional elements in pancreatic β cells (4). Thus, in mouse and human pancreas, HNF-1α is a major regulator of HNF-4α, acting directly through a distinct essential *cis* element in the HNF-4α P2 promoter (5,6). HNF-1α contains three functional domains (Figure 1A): the N-terminal dimerization domain (residues 1–32), a C-terminal transactivation domain (residues 281–628) and the DNA binding
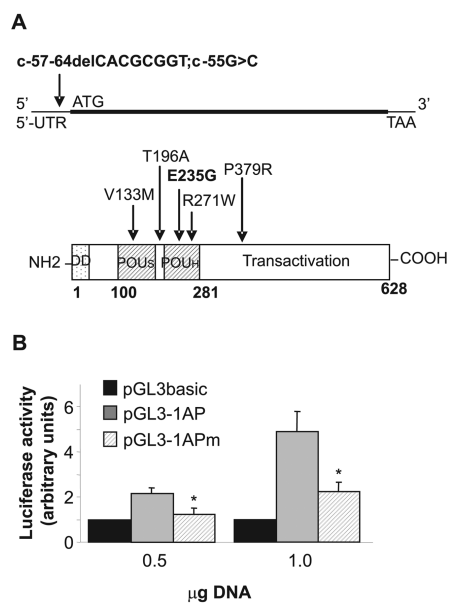
A

c-57-64delCACGCGGT;c-55G>C

B

Figure 1. Characterization of mutation c-57-64delCACGCGGT;c-55G>C in the 5' UTR of *HNF1A*. (A) Location of mutation c-57-64delCACGCGGT;c-55G>C in the 5'-UTR of the *HNF1A* gene and missense mutations in the HNF-1α protein. The *HNF1A* cDNA (*upper panel*) and the functional domains of HNF-1α protein (*lower panel*) are schematically represented. In the protein, amino acids 1–33 contain the dimerization domain (DD). DNA binding domain, including POU$_S$ and POU$_H$ homeodomains, is located between amino acids 100–281. The transactivation domain is spanning the C-terminal half part of the protein. The positions of the mutations analyzed are indicated. (B) Transcription of *HNF1A* is impaired by the c-57-64delCACGCGGT;c-55G>C mutation. Min6 cells, grown on six-well culture dishes, were cotransfected with the indicated amount of plasmids pGL3basic, pGL3-1AP and pGL3-1APm and 250 ng pCMVβ. Cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activities. Luciferase activities, normalized to β-galactosidase, were relative to pGL3basic activity (arbitrarily given as 1). Data represent means ± SEM of four experiments done in duplicate. *$P \leq 0.05$.

domain, spanning residues 98–280. It binds to DNA as a homodimer or heterodimer with the structurally related HNF-1β transcription factor (7). More than 300 different MODY3-causing muta-

tions have been found in both the coding sequence and promoter of *HNF1A*. These mutations include gene deletion, frame shift, missense, nonsense and splice site mutations in more than 700 families (8,9). Functional studies of a relatively small number of HNF-1α mutations, usually focused on their effects on expression of a single target gene, have shown that diabetes can result from loss-of-function or dominant-negative effects (8). The clinical phenotype of MODY3 is variable from one family to another and heterogeneous within each family (10). This variability has been explained by environmental and/or additional genetic factors (11,12). Additionally, the type and/or position of the mutation appear to modulate the age of diagnosis (9,13).

In this work, we analyze the functional effect of six HNF-1α variants located at different gene positions that we have identified in an HNF-1α mutation screening among a group of patients with familial, young-onset diabetes. Two mutations are considered novel: c-57-64delCACGCGGT;c-55G>C, located in the 5' untranslated region (5'UTR) of *HNF1A*, and p.Glu235Gly, located in the DNA binding domain. Other mutations, located in the DNA binding and transactivation domains, have been previously reported (p.Val133Met, p.Thr196Ala, p.Arg271Trp and p.Pro379Arg) (8,9). Here, we report the effect of the 5'UTR mutation on *HNF1A* expression as well as the differential effects of the missense mutations on the ability of HNF-1α to bind and activate a subset of target genes.

MATERIALS AND METHODS

Patients and Mutation Screening

The diabetic patients involved in this study were referred to our laboratory for a molecular diagnosis of monogenic diabetes. The probands of families P36 and P56 were referred to us by author MG, family P20 by author J-JG, family P30 by author SA, family P37 by author MD and family P52 by author V-MA. The clinical diagnosis was made using classical crite-

ria (1). All families were of Spanish Caucasian descent except family P56, who was Hispanic. Informed consent was obtained from the subjects or their parents. The studies were performed according to the Declaration of Helsinki as revised in 2000 and approved by the corresponding ethical committees.

To screen for HNF-1α mutations, the exons, their flanking intron regions and the minimal promoter of the *HNF1A* gene were polymerase chain reaction (PCR) amplified, and their products were sequenced. The upstream and downstream primers used were as follows: exon 1, 5'-gcttggcctagtggggttttg-3' and 5'-cctctaggctctcctgggag-3'; exon 2, 5'-tgggctccataactgctttc-3' and 5'-ccagg gaagatgcggggtag-3'; exon 3, 5'-agaatcaagg gcaaggtcag-3' and 5'-gaccaaaccagcatgtttc-3'; exon 4, 5'-tcagaaccctccccttcatg-3' and 5'-cacgtgtcccttgtccccac-3'; exon 5, 5'-caatg gagtttgaagtgctg-3' and 5'-gccaaggaaa gatgaggttg-3'; exon 6, 5'-agggagattctggag cagtc-3' and 5'-cacccaggtgcccacccatc-3'; exon 7, 5'-cagctgattccctccccttc-3' and 5'-atccattgacagccaacctc-3'; exon 8, 5'-gaggcctgggactagggctg-3' and 5'-tctgt cactggccgagggag-3'; exon 9, 5'-cctgtgacag agcccctcac-3' and 5'-ggacagcaacagaag gggtg-3'; exon 10, 5'-gtaccctagggaca ggcag-3' and 5'-cccccaagcaggcagtacag-3'; and promoter, 5'-agcactgttcttggcacatg and 5'-ggcagacacaaaccaaactc-3'. Screening for the presence of novel mutations on a panel of 54 healthy control individuals was done by single-strand conformation polymorphism of PCR fragments under two conditions (14).

Site-Directed Mutagenesis and Construction of HNF-1α Mutants

A construct encoding the mouse HNF-1α cDNA (pcDNA3-mHNF1A) was prepared by cloning a 2.2-Kb *Eco*RI-*Eco*RV fragment from pBJ5mHNF (15) into pcDNA3 (Invitrogen, Carlsbad, CA, USA).

To generate plasmid pGEX5.2-HNF1A(1–281), expressing GST-HNF-1α(1–281) fusion protein, a DNA fragment containing amino acids 1–281 of HNF-1α was amplified from pcDNA3-mHNF1A using oligonucleotides GST1AF:

5'-gggatccccatggtttctaagctg-3' and GST1AR: 5'-gggatcctcacaacttgtgccggaag-3' and inserted into the *Bam*HI site of the polylinker of pGEX-5X-2 (GE Healthcare, Barcelona, Spain).

Mutations were introduced into the pcDNA3-mHNF1A or into the pGEX5.2-HNF1A(1–281) constructs by PCR using a kit (QuikChange II Site Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA). To generate missense mutations p.Val133Met, p.Thr196Ala, p.Glu235Gly, p.Arg271Trp and p.Pro379Arg (note that residue Pro379 corresponds to residue Pro378 in the mouse protein), the following oligonucleotides were used, respectively: 5'-ccagcgggag atggtggacaccacgggtc-3', 5'-gagcccacaggcgat gagctcccagctaagaagg-3', 5'-gaagagcgag agaccttggtggaggggtgtaatag-3', 5'-ctacaactgg tttgccaattggcgcaaggagg-3' and 5'-gtccc ctgccgcgggtcagcaccctg-3'. Constructs carrying mutations were checked by sequencing and digestion with *Sac*I, *Mfe*I and *Kps*I in case of mutants mHNF1A(T196A), mHNF1A(R271W) and mHNF1A(P378R), respectively.

Plasmids pGL3-1AP and pGL3-1APm (which contain the proximal promoter and, wild-type or mutant, human HNF1A 5'UTR, driving the luciferase gene) were generated by amplifying a DNA fragment containing bases from –504 to +9 of *HNF1A* (positions 211–723 of GenBank® accession number U73499) from genomic DNA of patient P37 with primers 1AP2F: 5'-tctcctgtctcagcatatg-3' and 1A1-1R: 5'-cttctccagccaggtag-3' and cloned into pGEMTeasy (Promega, Madison, WI, USA). After sequencing, the inserts were cut off with *Sma*I and *Sac*I and introduced into pGL3basic (Promega).

Tissue Culture, Transient Transfections, Immunoblot and Luciferase Assays

Cos7 and Min6 cells were grown as described elsewhere (16,17). Transient transfections were performed with lipofectAMINE Reagent (Invitrogen), in 80% confluent 12-well culture dishes, including the corresponding luciferase reporter construct, HNF1A expression vectors and pCMVβ (Clontech) to control for efficiency of transfection. The amounts of

each plasmid are indicated in the figures. Luciferase activity was measured after 24 h in cell lysates using the Luciferase Assay System (Promega) and normalized for transfection efficiency by the β-galactosidase activity of pCMVβ. Transfection efficiency, measured by β-galactosidase staining with X-gal was in a range of 16–24% from experiment to experiment. Western blot analysis was carried out using the polyclonal antibody HNF-1α C19 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Reporter plasmid pGL3-HNF4AP2 contains the human HNF-4α P2 promoter, which includes one HNF-1α binding site, cloned into the *Sma*I site of pGL3 basic (Promega). The HNF-4α P2 promoter was cloned from human genomic DNA with primers HNF4AP2F: 5'-gacacacccatagtttggag-3' and HNF4AP2R: 5'-tcacgctgaccatggccaag-3' amplifying bases –418 to +13 of the P2 promoter (bases 8036943 to 8037373 of the Homo sapien chromosome 20 genomic contig, reference assembly, GeneBank® accession number NT011362.9). Reporter plasmid pGL3-GLUT2 contains base pairs –1308 to +46 of the mouse GLUT2 promoter (18). Reporter plasmid β28-Luc contains three copies of the β-fibrinogen HNF1 binding site (15). Reporter plasmid pGL3-RIP, containing base pairs –872 to –169 of the rat insulin 2 gene promoter, was provided by Dr. A. Barberá (Institut d'investigations Biomediques August Pi i Sunyer [IDIBAPS], Barcelona, Spain).

Production of Recombinant Wild-Type and Mutant GST-HNF-1α(1–281) Fusion Proteins

The *Escherichia coli* BL21 strain was transformed with the indicated pGEX5.2-HNF1A(1–281) plasmids and grown at 37°C to mid-log phase. Expression of fusion proteins was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.2 mmol/L, and cultures were incubated with orbital shaking for 16 h at 22°C. GST-HNF-1α(1–281) fusion proteins were purified from crude *E. coli* extracts by single-step affinity chro-

matography using glutathione-agarose beads (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany), essentially as described by Galan *et al.* (19). Fusion proteins were stored at a concentration of about 1 mg/mL at –80°C with 30% glycerol. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany). GST-HNF-1α(1–281) purification resulted in a single band of approximately 56 kDa on Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

DNA Binding Studies

Purified GST-fusion proteins or nuclear cell extracts were incubated with double-strand ^{32}P-labeled oligonucleotides (*1A*: 5'-tcgacttggttaataattcaccagag-3', based on the 21-mer primer described by Chi *et al.* [20], and *4AP2*: 5'-tcgaagtgactggttactctt-taacgtatccac-3', based on the oligonucleotide P2 described by Boj *et al.* [5]) containing high-affinity HNF-1α binding sites in a 15-μL reaction mixture containing 20 mmol/L HEPES buffer, pH 7.9, 40 mmol/L KCl, 1 mmol/L MgCl$_2$, 0.1 mmol/L EGTA, 0.5 mmol/L DTT, 4% Ficoll and 1 μg poly(dIdC) at 25°C for 20 min. Supershift analysis was performed by incubating 0.2 μg of the polyclonal antibody HNF-1α C-19 with the nuclear extracts for 5 min on ice before adding the probe. The reaction mixture was loaded on a 6% nondenaturating polyacrylamide gel containing 0.25 × TBE buffer (23 mmol/L Tris-borate, 0.5 mmol/L EDTA) and run at 4°C. Nuclear extracts were prepared as described by Navas *et al.* (16) and the relative amounts of HNF-1α expressed proteins were determined by Western blotting. To determine relative binding affinity (K_a) and maximal binding (B_{max}), mobility shift analysis was performed with increasing amounts of ^{32}P-labeled oligonucleotides and set levels of HNF-1α proteins as indicated. To quantify the bound and free oligonucleotides, radioactive bands were analyzed with a Phosphorimager (Bio-Rad Laboratories GmbH). Specific counts were determined after sub-

tracting the background, and the data were analyzed by the Scatchard method (21). Statistical significance was analyzed by the two-tailed Student *t* test.

RESULTS

Identification of *HNF1A* Mutations in Patients with Young-Onset Familial Diabetes

Probands from 28 independent diabetic families were screened for MODY3 mutations. Six of them carried *HNF1A* changes. The previously described p.Val133Met, p.Thr196Ala, p.Arg271Trp and p.Pro379Arg and the novel mutations p.Glu235Gly and c-57-64delCACGCGGT;c-55G>C were identified by DNA sequencing (Figure 1A and Table 1). The novel mutations cosegregated with diabetes in the available subjects in their respective families (Table 1) and were not found in 55 unrelated healthy control individuals, who showed normal conformers of single-strand conformation polymorphism compared with the probands (not shown).

The available clinical and anthropometric data from the index patients carrying *HNF1A* changes are given in Table 1. Most of the patients studied as probands for genetic diagnosis had a clinical history of diabetes from at least two consecutive generations. Hyperglycemia values vary from one patient to another as well as hemoglobin (Hb)A_{1c} and treatment at the moment of genetic study.

Effect of Mutation c-57-64delCACGCGGT;c-55G>C on the 5′ UTR of HNF1A

Mutation c-57-64delCACGCGGT;c-55G>C is located within the 5′UTR of *HNF1A* (Figure 1A). To investigate whether this mutation had an effect on *HNF1A* expression, we prepared plasmids containing the luciferase reporter gene under the control of the minimal promoter, and wild-type or mutant, 5′UTR of *HNF1A* (bases c.–504 to +9) and transfected them into insulinoma Min6 cells. We found

Table 1. Clinical characteristics of probands and *HNF1A* mutations.

Family	Age at diagnosis/ actual age (years)	Patient sex	Body mass index (kg/m²)	Fasting plasma glucose (mmol/L)	OGTT[a] (mmol/L)	HbA$_{1c}$ (%)	Basal C-peptide (ng/mL)	Exon	Nucleotide change	Protein change	Affected family members	Treatment[b]
P37	20/22	Female	21	6.9	6.9	5.9	NA	1	c–57 to –64 delCACGCGGT;c-55G>C[c]	None[c]	S, F, 2 pU, pGM	Diet
P20	17/65	Male	24	10.2	NA	7.4	NA	2	c.397G>A	p.Val133Met	F, S, D	Glimepiride + metformin
P30	24/26	Female	20	7.9	17.5	9.6	1.9	2	c.586A>G	p.Thr196Ala	S[d]	Repaglinide
P36	14/23	Female	22	14.2	NA	10.6	1.0	3	c.704A>G[c]	p.Glu235Gly[c]	M, S, mGM	Metformin + insulin
P52	17/20	Female	22	4.7	14.7	5.8	0.8	4	c.811C>T	p.Arg271Trp	M	Repaglinide
P56	34/35	Male	25	7.0	5.3	5.8	0.76	6	c.1136C>G	p.Pro379Arg	M, 4 mU, mGM	Diet

[a]OGTT, plasma glucose at 120 min after a 75-g oral glucose tolerance test. Nucleotide numbering uses +1 as the A of the ATG initiation codon, on the basis of the GenBank sequence number NM_000543. F, father; M, mother; Sn, son; D, daughter; S, sister; pU, paternal uncle; mU, maternal uncle; pGM, paternal grandmother; mGM, maternal grandmother; NA, not analyzed. Underlined are those affected family members where mutation was checked by genotyping.
[b]Proband treatment at the time of genetic diagnosis.
[c]Novel mutation.
[d]Diabetic family member without mutation.

that this mutation results in an ~50% decrease in luciferase activity (Figure 1B), thus indicating that transcription of the *HNF1A* gene is impaired for this mutant allele.

Effect of HNF-1α Missense Mutations on Transcriptional Activation

To characterize the functional consequences of missense mutations p.Val133Met, p.Thr196Ala, p.Glu235Gly, p.Arg271Trp and p.Pro379Arg, direct mutagenesis of the mouse cDNA was performed to prepare the mutants mHNF1A(V133M), mHNF1A(T196A), mHNF1A(E235G), mHNF1A(R271W) and mHNF1A(P378R), respectively. HNF-1α protein is highly conserved between the human and mouse species. The amino acid sequence homology is 94.6% for the entire protein and 99.5% for the DNA binding domain. With the exception of mutation Pro379Arg, which is located in the transactivation domain, all the other missense mutations are located in the DNA binding domain and involve amino acid residues strictly conserved between mouse and human. Pro379 is also conserved in the mouse protein, but its position is 378 because of the insertion of a His residue after position 359 in the human protein.

HNF-1α is an important regulator of gene expression in the β cell as well as in other tissues involved in glucose homeostasis such as the liver. To evaluate the effects of HNF-1α missense mutations, we compared the ability of the mutant proteins to activate transcription from a selected group of tissue-specific target promoters activated in liver (β-fibrinogen), in β -cells (HNF-4α P2, insulin) or in both (GLUT2). Additionally, we tested the effects of these mutations in two different cellular contexts: Min6, an insulinoma-derived cell line that expresses endogenous HNF-1α as well as some of the target genes whose promoters are used, and Cos7, which does not. Although Min6 cells are more comparable to the pancreatic β cells, some of the effects of the exogenous mutant proteins could be masked by the en-

dogenous wild-type. This limitation does not apply to Cos7; however, in this case, the lack of some specific coactivators may also be limiting. The comparison of the results with both cell lines is useful to understand the effect of the HNF-1α mutations on transcriptional activation, and similar approaches have been used previously in the analysis of other MODY3 mutations (22).

Mutant and wild-type HNF-1α constructs were cotransfected in increasing amounts along with a set amount of reporter plasmids pGL3-GLUT2, pGL3-RIP, pGL3-HNF4AP2 or β28-Luc into Cos7 and Min6 cells. Stimulation of transcription triggered by activation of the respective promoters was measured by luciferase reporter gene activity. Figure 2A–G shows that wild-type HNF-1α activates the transcription of all reporter genes in both cell lines in a dose-dependent manner, from 2- to 20-fold. In all assays, we found that the activation of the reporter no longer increases with increasing amount of the wild-type expression vector DNA above the maximal value showed in the figure (not shown). Mutation p.Thr196Ala has no significant effect on the transactivation function of HNF-1α except a slight decrease, if any, on the GLUT2 promoter in Min6 cells (Figure 2E). Similar results were obtained with mutation p.Pro378Arg, except for the activation of the β28-luc reporter, which was reduced to about 50% in Cos7 cells (Figure 2B). In contrast, transcriptional activation by the mutant mHNF1A(R271W) was strongly impaired in the four reporters. Interestingly, mutations p.Val133Met and p.Glu235Gly had heterogeneous effects. Both mutant proteins behaved as the wild-type with respect to transactivation of the *HNF4A* P2 promoter (Figure 2C, G). However, they were defective in the transcriptional activation of the other promoters tested. The behavior of mHNF1A(V133M) was more variable. At low DNA concentrations, this mutant activated β-fibrinogen and insulin promoters at significant lower levels than the wild-type protein. However, when

higher amounts of the expression vector were used in Cos7 cells, β28-luc and pGL3-RIP reporters were activated at the wild-type level (Figure 2B, D). On the other hand, when equal amounts of wild-type and mutant expression vectors were cotransfected into Cos7 cells, together with the reporter plasmid pGL3-GLUT2, no significant reduction of the wild-type activity was observed (Figure 2H). It is known that mutants of HNF-1α that are unable to bind DNA may have a dominant-negative effect through the formation of nonproductive heterodimers with the wild-type protein (8). We did not observe this effect, which is not unexpected, since these mutants still maintain some capacity to bind DNA. These results are consistent with a previous study showing that one of these mutations, p.Arg271Trp, does not show any dominant-negative effect on the rat albumin promoter in HeLa cells (23).

Effect of Missense Mutations on HNF-1α DNA Binding

Because most of the missense mutations analyzed in this study are localized to the DNA binding domain (Figure 1A), we studied the ability of the mutant proteins to bind DNA. We purified GST-fusion proteins to the first 281 amino acids of HNF-1α [GST-1α(1–281)] containing the dimerization and DNA binding domains (Figure 3A). Using electrophoretic mobility shift assay (EMSA), we measured the DNA binding affinity of wild-type and mutant GST-fusion proteins. Binding site titration experiments were performed using probe 1A, which contains a consensus HNF-1 high-affinity binding site, to calculate the association constant (K_a) and maximal binding (B_{max}) (Figure 3B). We found a clear correlation between the effect of these mutations on HNF-1α transactivation function and DNA binding. Mutation p.Arg271Trp produced the strongest effect on DNA binding and decreased both affinity and maximal binding by 3.6- and 2.5-fold, respectively. In contrast, the K_a and B_{max} values of the fusion protein carrying mu-
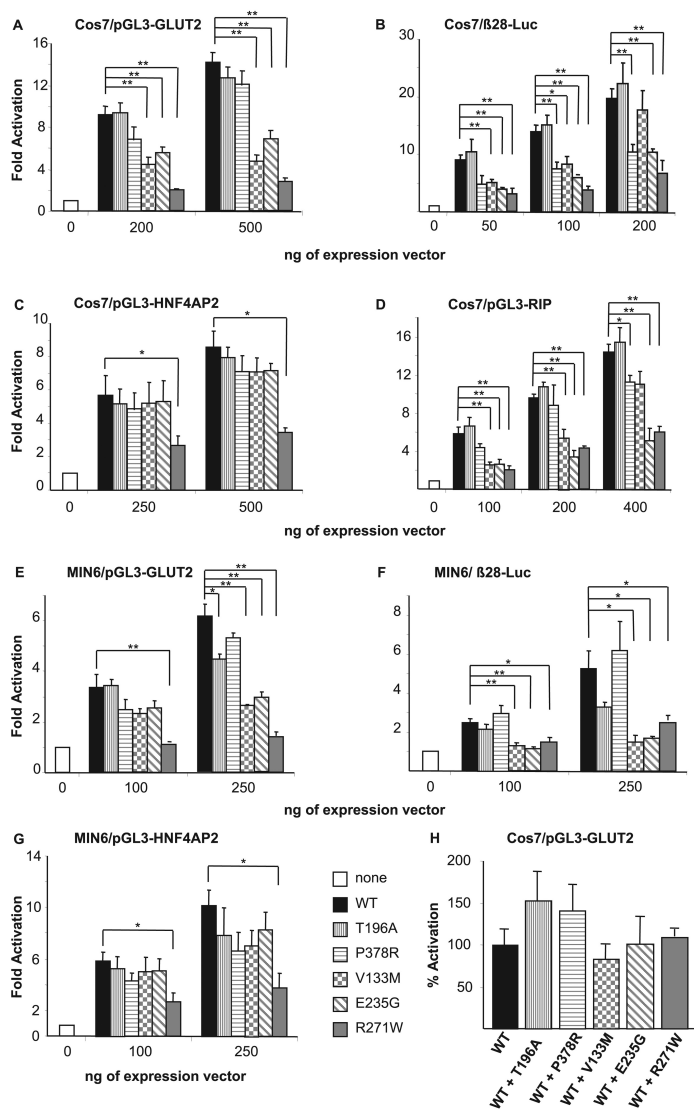
Figure 2. Transcriptional activity of mutants mHNF1(V133M), mHNF1(T196A), mHNF1(E235E), mHNF1(R271W) and mHNF1(P378R). Cos7 (A–D) and Min6 (E–G) cells were cotransfected with 250 of the reporter constructs pGL3-GLUT2, β28-Luc, pGL3-HNF4AP2 or pGL3-RIP; 200 ng pCMVβ; and the indicated amounts of wild-type or mutant HNF1A expression vectors, which were defined to obtain a sufficiently strong response with the wild-type (at least a two-fold activation) but avoid saturation of the system. Cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized by the β-galactosidase activity of the internal transfection efficiency control pCMVβ. For a typical experiment, β-galactosidase activity was 1.8 ± 0.1 (mean ± SEM) optical density (OD)$_{420}$ units/(min • mg total protein). Normalized luciferase values obtained from cells cotransfected with HNF1A expression vectors are shown as fold activation relative to the controls lacking any HNF1A expression vector, which were set as 1. (H) Cos7 cells were cotransfected with 200 ng pGL3-GLUT2, 200 ng pCMVβ and 100 ng wild-type pcDNA3-mHNF1A expression vector alone or together with 100 ng of each mutant expression vector, as indicated. Normalized luciferase values obtained from cells transfected with wild-type HNF-1α alone are set as 100%. All experiments were performed in duplicate and repeated at least 3× with different DNA preparations. The Student *t* test was used to compare the mean relative fold activation values between groups. Error bars indicate SEM. *$P \leq$ 0.05; **$P \leq$ 0.005. Note that human residue Pro379 corresponds to mouse Pro378.

tation p.Thr196Ala did not differ significantly to that of the wild-type fusion protein. Finally, although mutations p.Val133Met and p.Glu235Gly did not affect B_{max}, K_a was 3.9- and 2.1-fold higher, respectively.

To confirm the results obtained with the GST fusion to the HNF-1α DNA binding domain, we carried out EMSA experiments with the full-length HNF-1α and mutant derivatives expressed in Cos7 cells. Additionally, and because these mutations appeared to have a differential effect on the transcriptional activation of pGL3-HNF4AP2 with respect to the other reporters tested, we used two high-affinity HNF-1 binding sites: the previously used 1A probe and the probe 4AP2, which contains the HNF-1α binding site found in the pancreatic P2 promoter of HNF-4α. We then compared the ability of wild-type and mutant HNF-1α proteins to interact with these two binding sites. Steady-state levels of wild-type and mutant proteins were similar, as shown by Western blot of nuclear extracts of Cos7 cells transfected with equal amounts of expression vectors (Figure 4A). In agreement with the transactivation assays, mutation p.Arg271Trp impaired HNF-1α binding to both DNA probes, whereas mutants mHNF1A(T196A) and mHNF1A(P378R) bound DNA as well as the wild-type protein (Figure 4B). Interestingly, DNA binding of the mutants mHNF1A(V133M) and mHNF1A(E235G) differed depending on the HNF-1 binding site used as probe. As expected from the GST-fusion experiments, binding to the 1A probe was impaired by both mutations. In contrast, binding of the wild-type and mutant proteins to the 4AP2 probe was not markedly different (Figure 4B). These results were confirmed by carrying out binding site titration experiments with full-length HNF-1α and mutant derivatives, from nuclear extracts. K_a values for the 1A probe did not differ much from that found using the GST-fusion proteins, thus confirming the effects produced by mutations p.Val133M, p.Glu235Gly and p.Arg271Trp (Table 2). Additionally, mutation p.Glu235Gly did not produce any effect
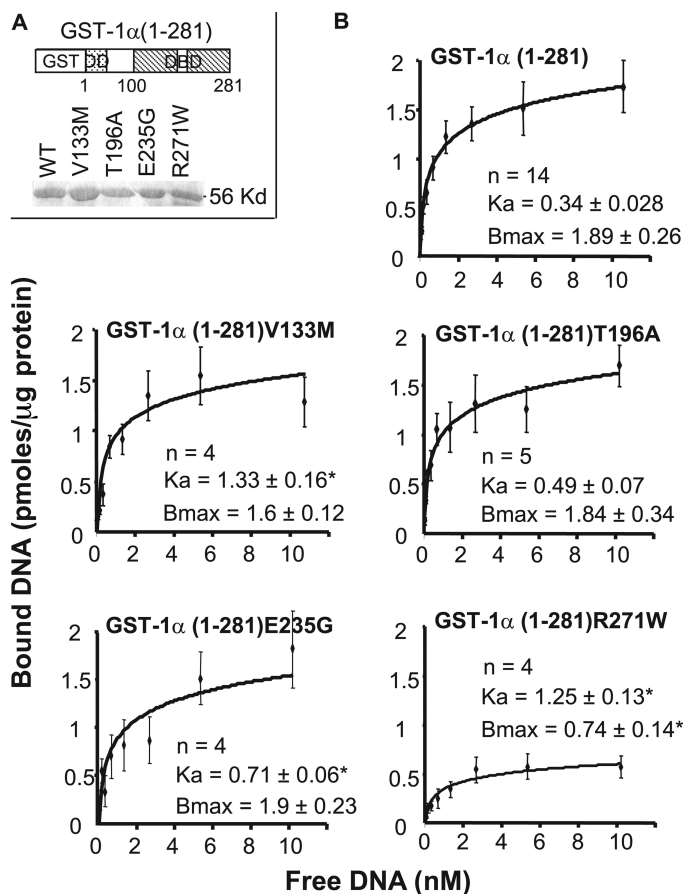
Figure 3. (A) Schematic representation of the GST-fusion proteins containing mouse HNF-1α amino acids 1–281. DD, dimerization domain. DBD, DNA binding domain. Numbers indicate amino acid positions of the HNF-1α protein. (B) Binding site titration experiments using GST-fusion proteins GST-1α(1–281) and radiolabeled probe 1A. A total of 2 ng purified GST-1α(1–281) proteins were incubated with a varying amount of radiolabeled oligonucleotide (0.042, 0.084, 0.168, 0.336, 0.420, 0.672, 1.34, 2.68, 5.36 and 10.2 nmol/L). GST-1α(1–281)/DNA complexes were assayed by EMSA and quantified in a phosphorimager. Relative binding affinities (K_a) and maximal binding (B_{max}) for the mutant and wild-type GST-1α(1–281) proteins were calculated by using the Scatchard plot. Data indicate mean ± SEM. K_a is given in nmol/L; B_{max} is given in pmol DNA bound μg protein. n, Number of independent experiments. *$P ≤ 0.05$.

on 4AP2 binding affinity, whereas mutant mHNF1A(V133M) produced only a slight effect. As expected, the greatest effect was observed for mutation p.Arg271Trp, which caused a 2.5-fold increase in the K_a value.

DISCUSSION

Mutations in *HNF1A* are a common cause of monogenic diabetes characterized by a progressive deficiency in glucose-stimulated insulin secretion. Functional analysis of MODY3 mutations to identify the corresponding molecular defect might contribute to uncover the mechanisms of β-cell impairment in these diabetic patients. In this work, we characterized six different *HNF1A* variants found in patients diagnosed with familial, young-onset diabetes.

Mutation c-57-64delCACGCGGT;c-55G>C is located within the 5′UTR of *HNF1A* and was found in a family with a mild clinical phenotype. This mutation was previously reported as a rare polymorphism identified in a patient homozygous for the p.Arg54X mutation (8). However, this mutation cosegregates with diabetes in our patient family and was absent in 55 non-diabetic control subjects. The 5′UTR region of *HNF1A*, starting at position c-225, could be involved in transcription/translation processes or mRNA stability, as has been shown for other genes (24–26). Other MODY3 mutations have been previously identified in this region. In particular, the substitution c-62C>G, which is contained in the deleted region of our family, has been proposed to modify a Myc/Max binding site (8,27). Interestingly, the c-41-58 sequence is fully conserved among human, mouse and rat species, and the mutation found in family P37 modify three bases within that region. Our results suggest that this mutation affects the transcription of *HNF1A* in the β cell, although we cannot exclude other additional effects on the posttranscriptional regulation of this gene, nor define its importance with respect to the different mRNA isoforms (13).

The effects of missense mutations on HNF-1α transcriptional activity are heterogeneous and appear to depend on the reporter under study. Mutation p.Pro379Arg is located in a region of the transactivation domain of HNF-1α, which has been proposed to contribute to the transcriptional activity of HNF-1α in a gene- and cell type–dependent manner (28,29). Our results show that mutant mHNF1A(P378R) fails to fully transactivate in a promoter- and cell-specific manner, since it shows a specific reduction of the β-fibrinogen promoter transactivation when tested into Cos7 cells. Consistent with the localization of the mutated residue in the transactivation domain of HNF-1α, the mutant protein binds DNA as well as the wild-type. Thus, our results could be explained by an impairment of the interaction of the mutant protein with a specific coactivator in Cos7 cells. The lack of effect in Min6 cells could be due to the presence of endogenous HNF-1α, as previously proposed for other MODY3 mutations (22).

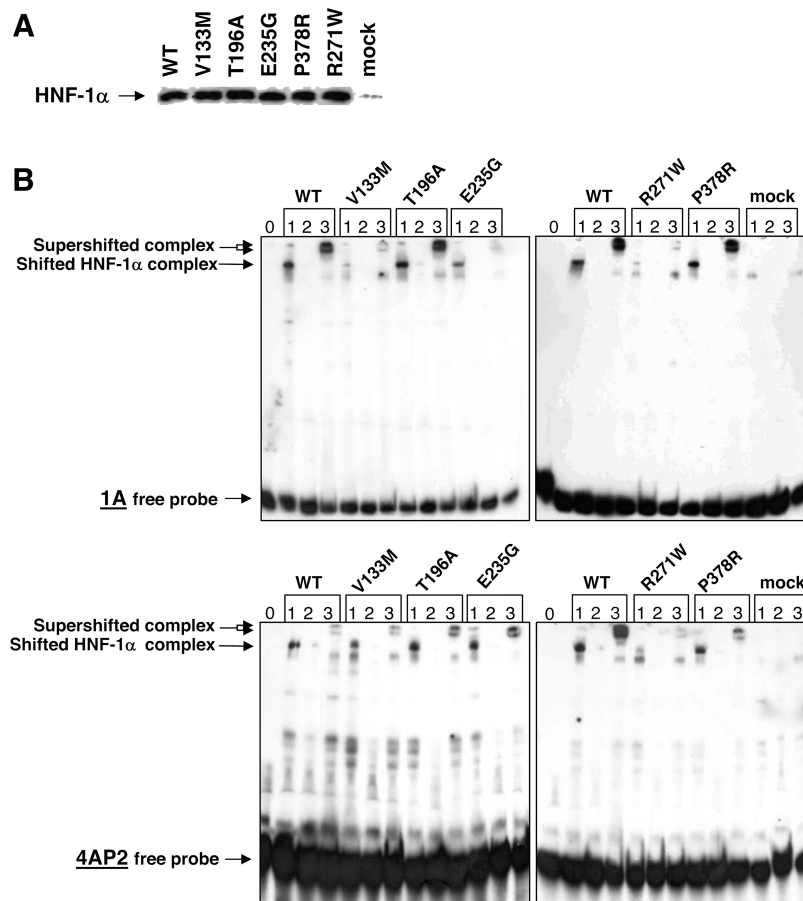Mutations pVal133Met, p.Thr196Ala, p.Glu235Gly and p.Arg271Trp are all

Figure 4. A Western blot analysis of nuclear extracts from Cos7 cells transfected with wild-type and HNF-1α variants. The 60-mm dishes of confluent cells were transfected with 10 μg of expression vectors using a modified calcium phosphate precipitation procedure (16). The 10 μL of nuclear extracts were subjected to 12% SDS-PAGE, followed by Western blotting analysis using the polyclonal anti–HNF-1α antiserum (C-19; Santa Cruz). mock, Cos7 cells transfected with no expression vector. (B) EMSA analysis of HNF-1α DNA binding activity. The 1 μL of nuclear extracts from Cos7 cells transfected with the expression vectors shown in (A) were incubated with 0.42 nmol/L ^{32}P-labeled 1A or 4AP2 probes, as indicated. *Lane 0*, free probe; *lane 1*, band shift; *lane 2*, competition using a 25-fold excess of cold probe; *lane 3*, supershift using the polyclonal anti–HNF-1α antibody C-19. A representative experiment is shown.

located in the DNA binding domain. The DNA binding domain of HNF-1α is a bipartite motif containing a Pit 1, Oct 1, and Oct 2, unc-86 (POU)-homeodomain (POU$_H$), involved in DNA recognition, and a POU-specific domain (POU$_S$) that cooperates with the POU$_H$ to enhance the binding affinity and specificity (Figure 1A) (20). Residue Arg271 is located in the third α-helix of the POU$_H$ domain and is highly conserved in many other homeodomains (30). This residue is required for the conformational stability

of the homeodomain and for optimal DNA binding (30). Accordingly, mutation p.Arg271Trp decreased both affinity and maximal DNA binding and, consequently, impaired transactivation in all conditions tested here as well as previously (23). In contrast, mutation p.Thr196Ala does not appear to affect HNF-1α activity in our experimental conditions. The Thr196 residue is conserved in human, mouse, rat and chicken HNF-1α and is localized in the linker between POU$_S$ and POU$_H$ do-

mains. The mHNF-1A(T196A) mutant shows normal DNA binding and transactivation capacities, such as other missense mutations directed to this flexible interdomain (20). The apparent lack of effect of this mutation is not unexpected, since there is not a clear genotype/phenotype correlation in the P30 family. In particular, the sister of the patient, who was diagnosed at age 31 years, does not bear the p.Thr196Ala change (Table 1). However, this rare polymorphism, which does not appear to be a MODY3 mutation, might be a risk factor for developing diabetes, since it was not detected in 54 healthy subjects studied in our laboratory and has been reported in two other diabetic patients, including one where this change coexists with type 1 diabetes and overweight (9,31).

Notably, we found that the effect of mutations *p.Val133Met* and *p.Glu235Gly* on HNF-1α transactivity depends on the promoter tested. These residues are located in the third α-helix within the POU$_S$ and in the second α-helix within the POU$_H$, respectively. Val133 is highly conserved among other POU$_S$ and its implication in protein stability has been suggested (20). An impaired protein stability of this mutant could explain the normal level of activation obtained with the β-fibrinogen and insulin promoters when higher amounts of DNA were transfected into Cos7 cells. However, this compensatory effect was not observed with the GLUT2 promoter or when MIN6 cells were used. Additionally, wild-type and mutant HNF-1α levels were similar in nuclear extracts of transfected Cos7 cells. In contrast, our results suggest that these two mutations affect DNA binding of HNF-1α. Although residue Val133 as well as Glu235 are not predicted to directly contact the DNA (20), DNA binding affinity of both mutants for the consensus 1A probe was clearly decreased, suggesting that this defect may be responsible for the reduced HNF-1α–dependent transactivation of most of our reporters. Strikingly, mutations p.Val133Met and p.Glu235Gly did not produce any significant defect in

Table 2. Affinity of HNF-1α wild-type and mutant proteins expressed in Cos7 for 1A and 4AP2 probes.[a]

mHNF-1α mutation	K_a (nmol/L) 1A probe (n)	K_a (nmol/L) 4AP2 probe (n)
None	0.32 ± 0.042 (12)	0.4 ± 0.04 (4)
V133M	1.4 ± 0.26[b] (4)	0.67 ± 0.05[b] (3)
T196A	0.27 ± 0.04 (4)	ND
E235G	0.73 ± 0.09[b] (4)	0.45 ± 0.04 (3)
R271W	1.33 ± 0.16[c] (4)	1.0 ± 0.07[b] (4)
P378R	0.26 ± 0.036 (3)	ND

[a]Relative amounts of protein were determined using Western blot. Results are shown as mean ± SEM of *n* independent experiments. For each experiment, equal amounts of wild-type and mutant proteins were used. EMSA was performed using constant amounts of proteins and increasing amounts of ^{32}P-labeled probes (0.042, 0.084, 0.168, 0.336, 0.420, 0.672, 1.34, 2.68, 5.36 and 10.2 nmol/L). K_a was calculated as described in "Materials and Methods." ND, not determined.
[b]$P < 0.05$.
[c]$P < 0.01$.

the transactivation of the P2 promoter of HNF-4α, which is the main functional promoter for this gene in the β cell (6). In agreement with these findings, DNA binding affinity of mHNF1A(E235G) to the 4AP2 probe was similar to that of the wild-type protein. Affinity of mHNF1A(V133M) for this probe was slightly decreased, but this effect was smaller than that observed with the consensus 1A binding site. The difference between the binding site sequence of 4AP2 (GTTACTCTTTAAC) and 1A (GTTAATAATTCAC) probes could be responsible for the differential effect of these two mutations on DNA binding affinity. Alternatively, adjacent sequences to the binding site might also contribute to this differential sensibility, as previously shown for the steroidogenic factor-1 (32).

Despite the common progressive β-cell function defect, which characterized the MODY3 phenotype, there is certain variability in the severity of its clinical expression in patients with respect to age of diagnosis, treatment requirements and development of complications. In particular, age of diagnosis has been shown to depend on the type of mutation and HNF-1α isoform affected as well as the presence of additional genetic modifiers (9,12,13). Besides these factors, the individual lifestyle is another important factor that may influence the evolution of

the disease. In this regard, for example, it is worth noting the strict control on diet and pharmacological treatment exerted by patient P52 opposed to that exerted by patient P36. The results of our molecular study suggest that the differential effects on the regulation of specific target genes caused by the HNF-1α mutations could also contribute to the differences in the development of the clinical phenotype. HNF-1α is an important transcriptional regulator in liver and in pancreatic islets controlling tissue-specific transcriptional programs (33). In particular, HNF-1α is a major regulator of HNF-4α in pancreas, and there is evidence of a cross-regulatory feedback circuit between HNF-1α and HNF-4α in the β cell (34). Mutations in HNF4A are responsible for another form of monogenic diabetes, MODY1, which shares many clinical features with MODY3. Strikingly, some of the MODY3 missense mutations found in our diabetic patients do not appear to affect HNF4A expression. These results suggest that regulation of HNF4A expression by HNF-1α may not be the simplest explanation for the HNF-1α–deficient phenotype and that regulation of target genes other than HNF4A may also play an important role. Indeed, both HNF-1α and HNF-4α regulate synergistically common target genes involved in β-cell function (35). Considering the key role of HNF-4α in the β-cell

function, we would expect that HNF1A mutations resulting in a decrease of HNF-4α expression will have a stronger impact on the regulation of common target genes, thus contributing in a major way to reduced insulin secretion and the progress of diabetes.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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