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Structure/Function Studies of Hepatocyte Nuclear Factor-1α, a Diabetes-Associated Transcription Factor

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Mutations in the transcription factor hepatocyte nuclear factor-1α (HNF-1α) cause maturity-onset diabetes of the young type 3 (MODY3), a form of diabetes mellitus characterized by autosomal dominant inheritance, early onset, and pancreatic β-cell dysfunction. We have examined the effects of five diabetes-associated mutations (L12H, G191D, R263C, P379fsdelCT, and L584S585fsinsTC) on HNF-1α function including DNA binding ability, intracellular localization, and transactivation activity. L12H, P379fsdelCT, and L584S585fsinsTC mutations were found in patients with a clinical diagnosis of MODY, while G191D and R263C mutations were identified in patients diagnosed with type 2 diabetes. These mutations had diverse effects on the functional properties of HNF-1α. Comparison of the functional data with clinical information suggested that transactivation activity of mutant HNF-1α in β cells like MIN6 may be the primary determinants of the phenotypic differences observed among diabetic patients with HNF-1α mutations.

Key Words: **diabetes mellitus; MODY; HNF-1α.**

Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes mellitus characterized by an autosomal dominant inheritance, onset usually before 25 years of age and impaired insulin secretion (1). Recent studies have shown that heterozygous mutations in the gene encoding the liver-enriched transcription factor HNF-1α are the cause of the type 3 form of MODY (MODY3) (2). HNF-1α is a homeodomain containing transcription factor which is expressed at highest levels in liver, kidney, intestine and pancreas (3–7). It is composed of three functional domains: an N-terminal dimerization domain (amino acids 1–32), a DNA binding domain (amino acids 150–280) with POU-like and homeodomain motifs and a C-terminal transcription activation domain (amino acids 281–631) (8, 9). Formation of homodimers or heterodimers with the functionally-related transcription factor HNF-1β (10) are necessary for its specific recognition of the *cis*-acting elements.

Although mutations of HNF-1α gene were originally identified in patients with MODY (1, 11–14), further studies found mutations in small numbers of patients diagnosed with late-onset type 2 diabetes (15) as well as with type 1 diabetes (16). The reasons why mutations in HNF-1α are associated with differing clinical features including diverse severity and age-at-onset or diagnosis are unknown. One possibility is that different mutant proteins may have different functional properties. To address the question, we studied the functional properties of five HNF-1α mutants (L12H, G191D, R263C, P379fsdelCT and L584S585fsindTC) which were identified in Japanese subjects with diabetes mellitus.

MATERIALS AND METHODS

HNF-1α mutants. Five mutant proteins (L12H, G191D, R263C, P379fsdelCT and L584S585fsinsTC) (15) and wild-type (WT)-HNF-1α were studied. The mutation L12H (Leu to His at codon 12) is located in the dimerization domain, G191D (Gly to Asp at codon 191) and R263C (Arg to Cys at codon 263) are located in DNA binding domain, P379fsdelCT (a frameshift mutation caused by deletion of CT at codon 379) and L584S585fsinsTC (a frameshift mutation caused by insertion of TC at codon 584/585) are in the transcriptional activation domain. P379fsdelCT produces a protein of 416 amino acids and L584S585fsinsTC results in the synthesis of a mutant protein of 659 amino acids longer than the normal protein (631

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residues). The L12H, P379fsdelCT and L584S585fsinsTC mutations were found in patients with a clinical diagnosis of MODY each of whom was diagnosed with diabetes under 15 years of age (15–17). The G191D mutation was identified in a patient diagnosed with type 2 diabetes at 64 years of age and was also found in his brother who was diagnosed with type 2 diabetes at age 54 years (15). The R263C mutation was identified in a patient diagnosed with type 2 diabetes at 28 years of age without a family history of diabetes mellitus. Diabetic patients with G191D and R263C mutations are treated by diet therapy, while other patients with L12H, P379fsdelCT and L584S585fsinsTC mutations were treated by an oral hypoglycemic agent or insulin (15–17).

Plasmid constructs. The HNF-1α mutations (L12H, G191D, R263C, P379fsdelCT and L584S585fsinsTC) were generated using the Chameleon Double-Stranded Site-Directed Mutageneses Kit (Stratagene, La Jolla, CA). Wild type and mutant HNF-1α cDNAs were subcloned into pBluescript SK(+) or the mammalian expression vector pcDNA3.1/HisC (Invitrogen, Carlsbad, CA), which has an Xpress tag at upstream of the HNF-1α insert. A 251 bp fragment of the human transthyretin (TTR) gene promoter (-218 to $+33$ relative to the cap site) (18) was amplified by polymerase chain reaction (PCR) using primers TTRP1 (5′-ACCTAACTGGTCAAATGACCTA-3′) and TTRP2 (5′-GAAGCCATCCTGCCAAGAATGA-3′) and subcloned into the *Sma*I site of pGL3 basic luciferase reporter plasmid (Promega, Madison, WI). This fragment includes the HNF-1α binding site (18). The promoter region between positions -206 and -7 of the rat L-type pyruvate kinase (PKL) gene including the HNF-1α binding site was amplified by PCR using primers (*Hin*dIII-PKL7: 5′-ccaagcttGGGTCTGTGGGTCTGCTTTATA-3′ and *Xho*I-PKL206: 5′-cgctcgagCGGCTCTGCAGACAGGCCAAAG-3′) and subcloned to the *Hin*dIII/*Xho*I site of pGL3-basic vector. This region is sufficient to confer the hormonal and dietary regulation on the PKL promoter (19). The sequences of all of the constructs were verified by DNA sequencing.

Western blot analysis. HeLa cells (2×10^6) were transfected with 8 μg of WT or mutant HNF-1α-pcDNA3.1/HisC vector using LipofectAMINE PLUS (Life Technologies, Grand Island, NY). After 48 h, Western blot analysis was performed using anti-Xpress antibody (Invitrogen) (20).

DNA binding studies. WT and mutant HNF-1α proteins were synthesized using 1 μg of pBluescript SK(+) construct and TNT T7 Quick Coupled Transcription/Translation System (Promega). The size and quality of the *in vitro* translated proteins were verified by SDS–PAGE. Gel retardation experiment was carried out with ^{32}P-labeled oligonucleotide containing the HNF-1α binding site of the human TTR promoter (5′-TATGGGTTACTTATTCTCTCTTT-3′) (20). In the competition binding assay, 50-fold of unlabeled probes were used as the competitor. In order to determine the relative binding affinity of G191D-HNF-1α to the oligonucleotide, increasing amounts of unlabeled competitor (5, 10, 25 and 50-fold) was added at non-equilibrium conditions (5 minute incubation). The images were scanned and quantified using ScanningImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Transactivation assay. HeLa and MIN6 cells were maintained in D-MEM supplemented with 10% and 15% fetal bovine serum respectively. 1.5×10^5 HeLa cells or 3×10^5 MIN6 cells were transfected for 3 h with the indicated amounts of Xpress epitope tagged WT and mutant HNF-1α constructs in pcDNA3.1/HisC described above and 0.5 μg of TTR or PKL-pGL3-luciferase reporter gene together with 10 ng of pRL-SV40 internal control plasmid (Promega) using the LipofectAMINE PLUS Reagent (Life Technologies). After another 48 h incubation, the cells were lysed and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and Lumat LB9501 Measuring System for Bio- and Chemiluminescence (Berthold Japan, Osaka, Japan).

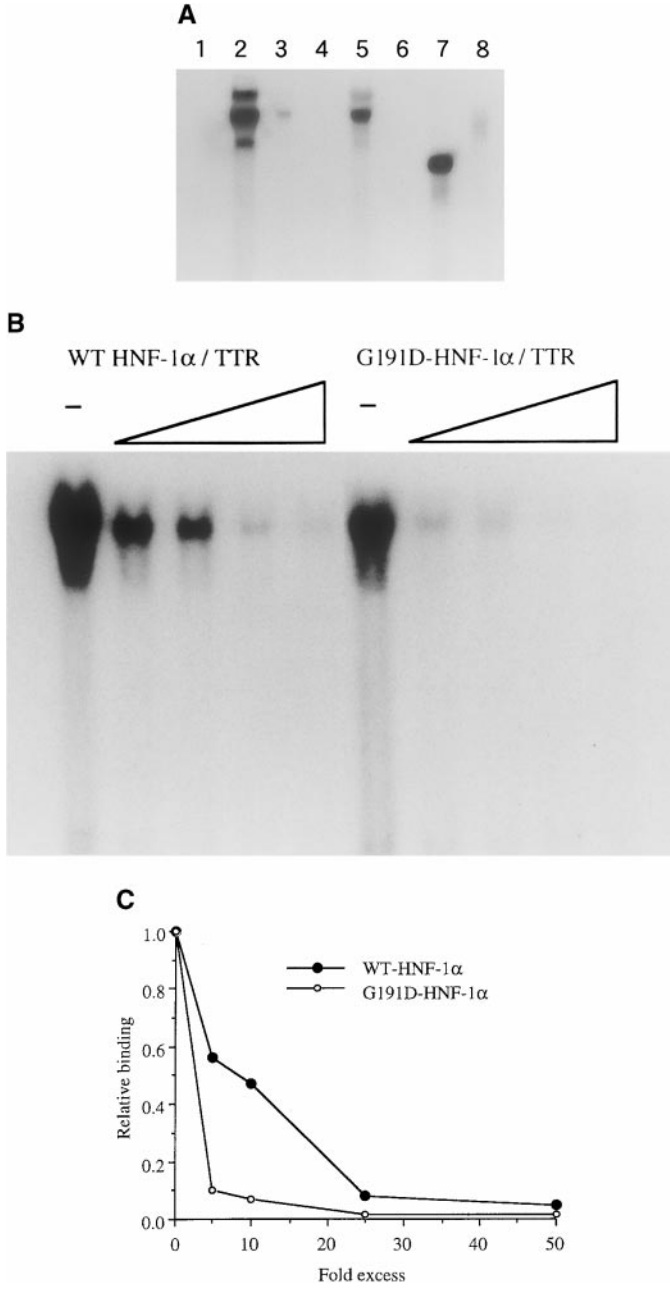

FIG. 1. *In vitro* transcription/translation and electrophoretic mobility shift assay of WT and mutant HNF-1α. (A) DNA binding ability of wild-type and mutant HNF-1α to the binding sequence in TTR promoter were assessed by EMSA. Lane 1, empty vector only; lane 2, WT-HNF-1α; lane 3, WT-HNF-1α and competitor; lane 4, L12H-HNF-1α; lane 5, G191D-HNF-1α; lane 6, R263C-HNF-1α; lane 7, P379fsdelCT-HNF-1α; lane 8, L584S585fsindTC. (B) Relative binding affinity of G191D-HNF-1α. WT and G191D-HNF-1α were bound to the labeled oligonucleotide in the presence of increasing amounts (5, 10, 25 and 50 molar excess) of unlabeled competitor in the non-equilibrium conditions. (C) A plot of relative binding of WT and G191D-HNF-1α to the target oligonucleotide. The data were obtained by densitometric scanning of the EMSA results indicated in B.

Immunolocalization of HNF-1α in transfected cells. MIN6 cells (1×10^5) grown on Lab-Tek chamber slides (Nalge Nunc, Naperville, IL) were transfected with Xpress epitope tagged WT and mutant

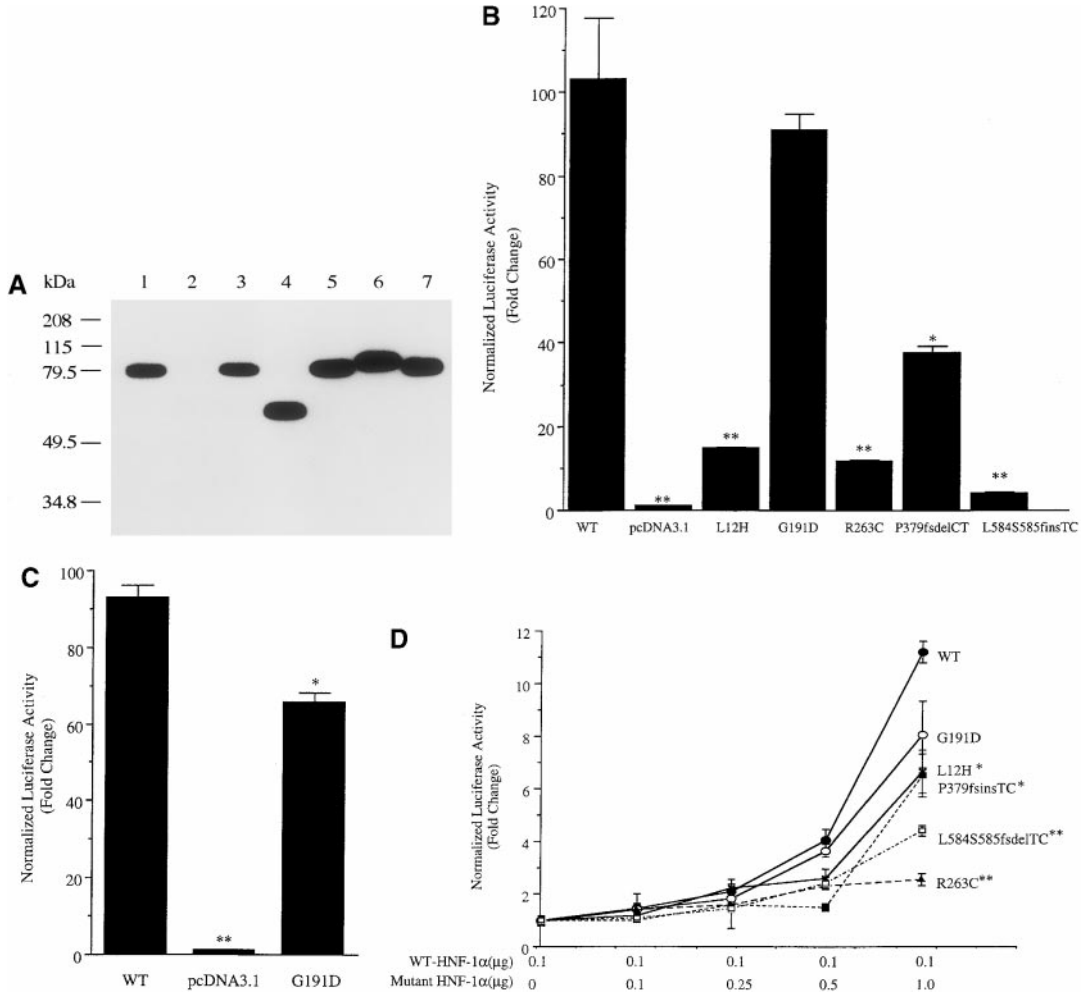

FIG. 2. Transactivation activities of mutant HNF-1α cells. (A) Expression of epitope tagged WT and mutant HNF-1α in HeLa cells. Lane 1, WT-HNF-1α; lane 2, empty pcDNA3.1/HisC vector only; lane 3, L12H-HNF-1α; lane 4, P379fsdelCT-HNF-1α; lane 5, G191D-HNF-1α; lane 6, L584S585fsinsTC-HNF-1α; lane 7, R263C-HNF-1α. (B) Transactivation activities of 1 μg of WT and mutant HNF-1α on TTR promoter in HeLa cells. Luciferase activities were normalized by the activity of internal control pRL-SV40. The results were from three independent experiments; mean $\pm$ SE, *$p < 0.05$, **$p < 0.01$. (C) Cells were transfected with 1 μg of WT and G191D-HNF-1α together with 0.5 μg of PKL reporter and pRL-SV40. The results were from three independent experiments; mean $\pm$ SE, *$p < 0.05$, **$p < 0.01$. (D) HeLa cells were transfected with increasing amounts of mutant HNF-1α expression vector together with constant amount of WT-HNF-1α DNA and TTR reporter. The results were from three independent experiments; mean $\pm$ SE, *$p < 0.05$, **$p < 0.01$.

HNF-1α constructs in pcDNA3.1/HisC described above. Immunohistochemical analysis was performed using anti-Xpress antibody. Biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) and Fluorescein Avidin D (Vector) were used for the detection of the signals. Nuclei were visualized by staining with propidium iodide (PI). The slides were examined using a Jena laser scanning microscope (LSM 510, Carl Zeiss Jena, Jena, Germany).

RESULTS

DNA binding studies. The ability of in vitro synthesized WT and mutant HNF-1α to bind the HNF-1α site in the human TTR promoter was assessed by EMSA. WT-HNF-1α bound to the ^{32}P-labeled oligonucleotide containing the TTR binding site (Fig. 1A). The binding was blocked by the addition of a 50-fold excess of unlabeled oligonucleotide (Fig. 1A, lane 3) and the DNA-protein complex was supershifted by the addition of anti-HNF-1α antiserum (data not shown). L12H-HNF-1α, a mutant in a dimerization domain, did not show any binding to the target sequence probably due to impairment of dimer formation. R263C-HNF-1α also did not bind to the target sequence. Arg 263 is located in the helix 3 of the homeodomain which is important for DNA binding (21). P379fsdelCT-HNF-1α which has a mutation in the transcriptional activation domain bound to the probe. However, L584S585fsinsTC-HNF-1α which have structurally abnormal transcriptional activation domain did not bind the probe. G191D-HNF-1α did bind the probe. However, the binding was decreased by 46% compared to that of WT. The decreased binding affinity of G191D-HNF-1α relative

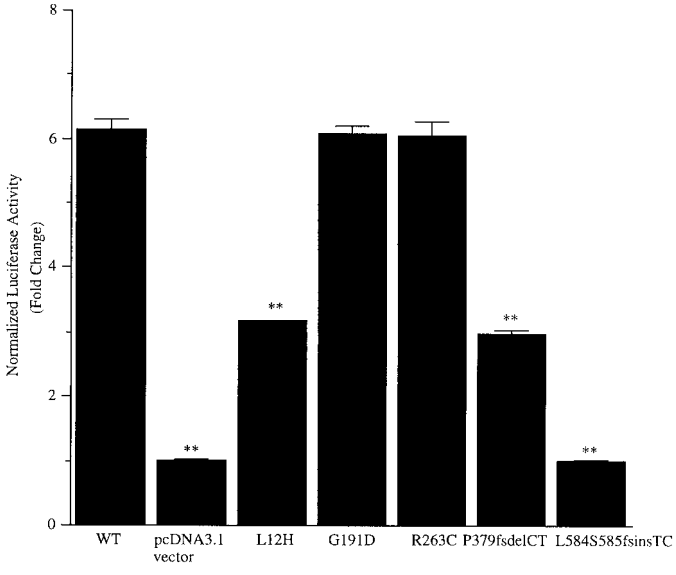

FIG. 3. Transactivation activities of mutant HNF-1α in MIN6 cells. MIN6 cells were transfected with 1 μg of expression vectors. Luciferase activities were normalized by the activity of internal control pRL-SV40. The results were from three independent experiments; mean ± SE, *$p < 0.05$, **$p < 0.01$.

to WT was also examined in non-equilibrium conditions (Figs. 1B and 1C). The slopes of the lines that represent the relative binding affinity (22) of WT and G191D-HNF-1α were 5.3 and 9.3 (Fig. 1C), respectively. The off-rate under nonequilibrium conditions of G191D-HNF-1α was approximately 75% faster than that of WT-HNF-1α. It is consistent with decreased affinity of G191D-HNF-1α compared to WT for the HNF-1α-binding site in the human TTR promoter.

Transactivation studies. The ability of Xpress-epitope tagged WT and mutant human HNF-1α to stimulate transcription of a human TTR promoter linked reporter gene was studied in HeLa cells which lack endogenous HNF-1α, and MIN6 cells which express the corresponding mouse protein. Expression of transfected epitope tagged HNF-1α protein was confirmed by Western blot analysis (Fig. 2A). The activities of L12H, R263C, P379fsdelCT and L584S585fsinsTC-HNF-1α on TTR promoter in HeLa cells were 14%, 11%, 36% and 3.7% of WT protein, respectively (Fig. 2B). The transactivation activity of G191D was reduced (88% of WT) in the system, but the difference was not significant. The expression of PKL, which is present in pancreatic islets and insulinoma cells, is regulated in part by HNF-1α (19, 20). PKL promoter-luciferase reporter was also used to test the transactivation activity of G191D-HNF-1α. The activity was significantly decreased by 29.4% ($p = 0.019$) compared with WT-HNF-1α (Fig. 2C). To determine whether any of these mutants acts in a dominant negative manner as observed for P291fsinsC-HNF-1α (20), increasing amounts of mutant HNF-1α plasmids were transfected in the presence of a constant amount of WT-HNF-1α. Increasing amounts of mutant HNF-1α did not inhibit the activity of WT-HNF-1α indicating that all of these mutants represent loss-of-function mutations (Fig. 2D).

The transactivation activities of the mutants were further investigated using MIN6 cells, an insulin secreting cell line which have endogenous HNF-1α. The transcription activity of L12H (51%), P379fsdelCT (48%) and L584S585fsinsTC (16%) was also impaired in MIN6 cells (Fig. 3). However, the activity of R263C-HNF-1α was normal in this system, suggesting that the WT endogenous protein may rescue the mutant. The transactivation activity of R263C-HNF-1α was also normal in HepG2 cells which have endogenous HNF-1α protein but was decreased in CHO cells which like HeLa cells lack HNF-1α (data not shown), consistent with this hypothesis. As in HeLa cells, the activity of G191D-HNF-1α was similar to WT-HNF-1α in MIN6 cells.

Subcellular localization of wild-type and mutant HNF-1α. The effect of mutation on subcellular localization of HNF-1α was studied in mouse insulinoma MIN6 cells. MIN6 cells were transfected with Xpress epitope tagged WT or mutant HNF-1α expression constructs and the localization of the epitope-tagged HNF-1α proteins was examined using the anti-Xpress antibody (Fig. 4). Although the number of transfected cells varies slightly from one experiment to another due to differences in transfection efficiency, the patterns of intracellular localization of the signals were consistent among experiments (Table 1). WT, L12H and G191D-HNF-1α were detected only in the nucleus, whereas R263C, P379fsdelCT and L584S585fsinsTC-HNF-1α were found in both the nucleus and cytoplasm of MIN6 cells. Thus, the various mutations can affect the localization or at least the rate of nuclear import.

DISCUSSION

Heterozygous mutations in the homeodomain-containing transcription factor HNF-1α are a common cause of MODY. They cause this form of diabetes because of their effect on the normal function of the insulin-secreting pancreatic β cells (23–25). Mutations in this transcription factor have also been found in patients thought to have type 1 diabetes indicating that some MODY patients may be diagnosed with type 1 diabetes. In addition, there have also been some patients with typical late-onset type 2 diabetes suggesting that these mutations may have a lower penetrance than those associated with MODY, the onset of which is normally before 25 years of age and often in childhood or adolescence.

HNF-1α is synthesized in the cytoplasm and then imported into the nucleus where it binds to a specific

TABLE 1

Intracellular Localization of Wild Type and Mutant HNF-1α in MIN6 Cells

HNF-1α	Signal positive cells/ counted cells (n)	Signals in nucleus (%)	Signals in cytoplasm (%)	Signals in nucleus and cytoplasm (%)
WT	11/281	100	0	0
L12H	10/813	100	0	0
G191D	15/560	100	0	0
R263C	19/839	26	53	21
P379fsdelCT	31/493	13	68	19
L584S585fsinsTC	8/244	50	50	0

cis-acting sequence in the promoter of the target genes and interacts with the basal transcription machinery and other factors to stimulate transcription. Thus, mutations in HNF-1α could affect multiple properties of this protein. Hence, we compared the functional properties of five diabetes-associated HNF-1α mutations: L12H (mutation in dimerization domain), G191D, R263C (mutation in DNA binding domain), P379fsdelCT and L584S585fsinsTC (mutation in transactivation domain). These mutations had diverse effects on the functional properties of HNF-1α (Table 2). The P379fsdelCT which disrupts the transactivation domain of HNF-1α affected transactivation activity and nuclear transport. The results are consistent with early reports describing that C-terminal half of HNF-1 is necessary and sufficient for transcriptional activity and residues 441–474 are associated with normal transportion to the nucleus (7, 26). On the other hand, our functional analysis on L584S585fsinsTC mutation provided a new aspect about the structure/function relationship of HNF-1α; DNA binding and intracellular localization were impaired in spite of the conserved DNA binding domain and the proper localization signal (amino acids 441–474). The impaired nuclear transport, DNA binding ability and transcriptional activity of L584S585fsinsTC suggest that the nonsense residues resulted from the frameshift mutation affect the function. The L12H mutation was impaired in DNA binding and transactivation but was efficiently imported into the nucleus. We also revealed that the transcriptional activation of the human insulin promoter by L12H-HNF-1α was impaired (27). However, Yamada *et al.* examined the transactivation activity of the L12H mutant using glucose transporter 2 (GLUT2) promoter and reported that the activity is even higher than that of WT when 48–96 ng of DNA was used for the transfection experiments (17). The reasons for the difference of the activities are unknown, but the difference of the reporter system (1.3 kbp GLUT2 promoter versus 251 bp TTR promoter) is a possible explanation. The R263C mutation was impaired in DNA binding and nuclear import. This mutation is quite interesting since it had reduced transactivation activity in HeLa cells that lack endogenous HNF-1α but normal activity in MIN6 cells that have endogenous HNF-1α. These data suggest that endogenous HNF-1α may rescue the function. The function in HeLa cells may reflect the true activity of the mutations. Since one allele of the MODY patients is normal, transactivation activity in MIN6 cells may reflect the *in vivo* function relevantly. Finally, the G191D mutation exhibited normal nuclear transport and transactivation activity in MIN6 cells, but a reduction in DNA binding and transactivation activity in HNF-1α-non-expressing cells. The results suggest that it is important to study multiple activities in order to understand the effect of mutations on HNF-1α function.

What do these studies tell us about HNF-1α mutations and diabetes? Mutations that are severely impaired in transactivation activities in MIN6 cells such as L12H, P379fsdelCT and L584S585fsinsTC are associated with MODY. Clinical features of the patients with the mutations are early-onset (diagnosis is under 15 years of age) and necessity of treatment by insulin or oral hypoglycemic agent. The effect on these activations may be the primary functional determinants leading to MODY. There is not a similar correlation between effect on DNA binding and nuclear localization and MODY. Mutations such as R263C and G191D which have apparently normal transactivation activity in MIN6 cells are associated with a mild-form of diabetes; the onset of the subjects with the mutations are delayed (the patients with G191D mutation were diagnosed at 64 and 54 years of age and one with R263C

TABLE 2

Effect of Mutations on HNF-1α Activity

Mutation	DNA binding	Transactivation	Nuclear import
L12H	↓	↓	N
G191D	↓	N ~ ↓	N
R263C	↓	↓ (HeLa), N (MIN6)	↓
P379fsdelCT	N	↓	↓
L584S585fsinsTC	↓	↓	↓

Note. N, normal; ↓, impaired.

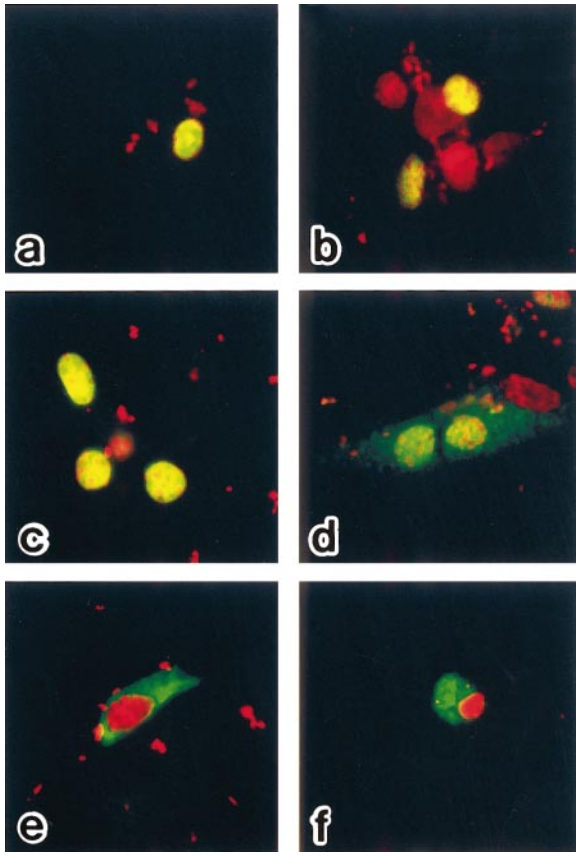

FIG. 4. Subcellular localization of wild-type and mutant HNF-1α in MIN6 cells. (A) Cells were transiently transfected with pcDNA3.1/HisC encoding wild-type and mutant HNF-1α, and the proteins were detected by anti-Xpress antibody. (a) WT-HNF-1α, (b) L12H, (c) G191D, (d) R263C, (e) P379fsdelCT, and (f) L584S585fsinsTC.

mutation was diagnosed at 28 years of age) and they can be treated by diet therapy. Our data suggest that transcriptional activity of mutant HNF-1α in β cells like MIN6 may be associated with the clinical severity of HNF-1α diabetes. Similar studies of other HNF-1α mutations may lead a better understanding of the correlation between the functional properties of the HNF-1α mutants and the clinical features of the patients.

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