Functional Characterization of the MODY1 Gene Mutations HNF4(R127W), HNF4(V255M), and HNF4(E276Q)

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Genetic studies have shown that mutations in the gene encoding hepatocyte nuclear factor (HNF)-4α, a member of the steroid/thyroid hormone receptor superfamily, give rise to early-onset type 2 diabetes (MODY1). The functional properties of mutant HNF-4α proteins and the molecular mechanisms by which they impair insulin secretion are largely unknown. In the present study, we have investigated transcriptional activation, DNA binding properties, and protein dimerization activity of three HNF-4α missense mutations—HNF4(R127W), HNF4(V255M), and HNF4(E276Q)—that have been associated with type 2 diabetes. We demonstrate that HNF4(E276Q) has lost its ability to bind to HNF-4 consensus binding sites and activate transcription. HNF4(E276Q) had no effect on the functional activity of wild-type HNF-4α in the pancreatic β-cell line HIT-T15, but it exhibited weak dominant-negative activity in other cell types. Analysis of HNF4(E276Q) protein showed that it exists in two forms: a full length 54-kDa protein and a 40-kDa COOH-terminal protein lacking the NH_2-terminal transactivation domain and the DNA binding domain. Immunoprecipitation experiments indicate that this truncated protein can bind to wild-type HNF-4α and may be responsible for the weak dominant-negative effects seen in these cells. In addition, we show that the transcriptional transactivation of HNF4(R127W) and HNF4(V255M) is indistinguishable from that of wild-type HNF-4α, suggesting that they are sequence polymorphisms. Our results demonstrate that HNF4(E276Q) is a loss-of-function mutation and that it identifies glutamic acid 276 in α-helix 8 of the ligand-binding domain of HNF-4α protein as a critical residue for DNA binding, transcriptional activation, and protein stability in vivo. ***Diabetes*** **48:1459–1465, 1999**

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CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FLAG, pharmaceutical name for product; HNF, hepatocyte nuclear factor; MODY, maturity-onset diabetes of the young; PCR, polymerase chain reaction.

Maturity-onset diabetes of the young (MODY) is a distinct and heterogenous form of diabetes characterized by an early onset (generally before age 25 years), autosomal dominant inheritance, and impaired insulin secretion with minimal or no defects in insulin action (1). Mutations in five genes are associated with different forms of MODY. The MODY2 gene encodes the glycolytic enzyme glucokinase, and MODY genes 1, 3, 4, and 5 encode transcription factors hepatocyte nuclear factor (HNF)-4α, HNF-1α, and HNF-1β, respectively (2–6).

The gene responsible for MODY1 encodes HNF-4α, a transcription factor that belongs to the steroid/thyroid hormone receptor superfamily. It was first identified by its interaction with cis-regulatory sequences of liver-specific gene promoters (7). HNF-4α contains a zinc finger DNA binding domain and binds DNA as a homodimer. Like other members of the steroid hormone receptor superfamily, HNF-4α contains two transactivation domains: AF1, an NH_2-terminal (acidic) amphipathic α-helical domain spanning the first 24 amino acids, and AF2, a complex transactivator domain spanning amino acids 128–366, which also includes the dimerization interface and the ligand binding domains (8) (Fig. 1). Functionally, HNF-4α plays a critical role in development, cell differentiation, and metabolism and is essential for the normal functioning of visceral endoderm, liver, intestine, kidney, and pancreatic β-cells (9–11). HNF-4α also regulates the expression of transcription factor HNF-1α, thereby defining a transcriptional hierarchy responsible for two phenotypically indistinguishable forms of early-onset type 2 diabetes (12–14).

The first mutation associated with diabetes that was identified in the gene encoding HNF-4α was found to be a nonsense mutation in codon 268, Q268X (3). This mutation generates a truncated protein containing an intact DNA binding domain but lacking part of the AF2 region. Functional studies of this mutation have shown that the cause of diabetes is due to a loss-of-function, rather than a dominant-negative, mechanism (10).

Seven additional HNF-4α mutations have been associated with type 2 diabetes: F75fsdelT, K99fsdelAA, R154X, R127W, V255M, E276Q, and V393I (15–21). Deletion mutants F75fsdelT and K99fsdelAA lead to frame shifts and truncated proteins. Nonsense mutation R154X produces a truncated protein containing only the DNA binding domain and the AF1 transactivation domain. These proteins can be predicted to lack

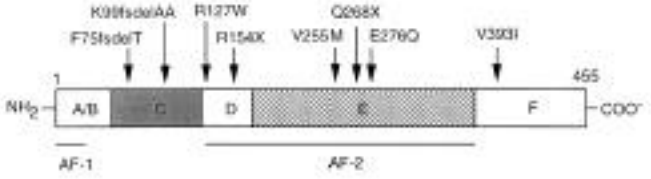

FIG. 1. Schematic representation of the functional domains of HNF-4α. The most NH_2-terminal part of region *A/B* contains the transactivation domain AF1. The DNA binding domain is located in region *C* and the transactivation region AF2 is spanning regions *D* and *E*. Region *E* also contains the dimerization domain and the putative ligand domain. Arrows indicate the locations of mutations found in HNF-4α.

transcriptional transactivation activity (8). The missense mutation V393I located in the AF2 domain has been shown to lead to a modest decrease in transactivation potential (21). The biological activities of three point mutations, R127W, V255M, and E276Q, which were identified in Japanese, Danish, and U.K. families, respectively, have not been determined (18–20). HNF4(R127W) is located between the DNA binding and the AF2 domain, and HNF4(V255M) and HNF4(E276Q) are positioned within the AF2 region. The present study was undertaken to study the functional properties of mutations HNF4(R127W), (V255M), and (E276Q). We demonstrate that HNF4(E276Q) is a loss-of-function mutation and that HNF4(R127W) and HNF4(V255M) are functionally normal and most likely represent rare polymorphisms.

RESEARCH DESIGN AND METHODS

Plasmid constructs. The mutant HNF-4α plasmids were generated by polymerase chain reaction (PCR)-mediated site-directed mutagenesis, using appropriate primers. Oligonucleotides HNF4-127F (5′-cgggattggatcagcacgcg-3′) and HNF4-840R (5′-tcgaggatgcgaatggacaca-3′), as well as HNF4-127R (5′-cgcgtgctgatc caatcccg-3′) and universal primer T3, were used to amplify two fragments from wild-type HNF4 (plasmid pf7) containing the R127W mutation at their 5′ and 3′ ends, respectively. PCR products were purified and used as templates to generate an 870-bp fragment using oligonucleotides T3 and HNF4-840R. The PCR fragment was digested with *Not*I and *Nhe*I and cloned into pf7 in which the corresponding wild-type fragment had been deleted (7).

HNF4(E276Q) was produced using oligonucleotides HNF4-276F (5′-atcgat gataatcaatacgcctg-3′) and HNF4-1640R (5′-ctcctcaaggcaaagccctg-3′) to generate a 768-bp fragment and HNF4-276R (5′-caggcgtattgattatcatcgat-3′) and HNF4-766F (5′-aggcaatgactacatcgtcc-3′) to amplify a 129-bp fragment containing the E276Q mutation at their 5′ and 3′ ends, respectively. PCR products were then used as templates to generate an 897-bp fragment using oligos HNF4-766F and HNF4-1640R. This PCR product was digested with *Nhe*I and *Nsi*I and cloned into the respective sites of plasmid pf7.

HNF4(V255M) was generated using oligonucleotide HNF4-255F (5′ccagagc tagcagagatgagccgta tgtccattc-3′) containing the valine-to-methionine mutation and oligo HNF-1640R to generate an 843-bp fragment. This DNA fragment was digested with *Nhe*I and *Nsi*I and cloned into the respective sites of plasmid pf7.

Both constructs were sequenced completely through the regions amplified by PCR to verify the mutation and exclude polymerase errors. Mutant HNF-4α cDNA sequences were also subcloned into expression vector pCMVβ for transient transfection experiments (22).

In vitro translation of proteins and immunoblot analysis. cDNAs for wild-type and mutant rat HNF-4α were transcribed using T3 RNA polymerase, and transcripts were translated using the rabbit reticulocyte lysate TNT system (Promega, Madison, WI) as described (23). The relative amounts of in vitro–translated proteins were determined by SDS-PAGE. For immunoprecipitation, equal amounts of wild-type pharmaceutical name for product (FLAG)-tagged HNF-4α and [^{35}S]methionine-labeled proteins were incubated in the presence of anti-FLAG M2 affinity gel (Kodak, New Haven, CT) for 16 h at 4°C in a buffer containing 15 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.55 mmol/l EDTA, 0.55 mmol/l dithiothreitol (DTT), 0.1% NP40 Complete protease inhibitor (Boehringer, Mannheim, Germany), and 0.5% nonfat dry milk. The pellet was washed five times, and immunoprecipitates were run on SDS-PAGE. Western blot analysis was carried out using a polyclonal anti-HNF4 antiserum, anti-445, directed against amino acids 455–465 of the rat HNF-4α protein or a monoclonal anti-FLAG M2 commercial antibody (Kodak).

Electrophoretic mobility shift assay. In vitro–translated proteins or nuclear cell extracts were incubated with ^{32}P-labeled oligonucleotides containing a high-affinity HNF4 binding site (LF-A1) 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, 0.5mmol/l DTT, and 4% Ficoll and 2 µg poly(dIdC) at 25°C for 20 min. Supershift analysis was performed by incubating the antibody with the protein or 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 (0.023 M Tris-borate, 0.5 mmol/l EDTA) and run at 4°C. Nuclear extracts were prepared as described (24).

Tissue culture, transient transfections, and luciferase assay. cos7 and HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% and 15% fetal calf serum, respectively. HIT cells were grown in DMEM supplemented with 5% fetal calf serum and 10% horse serum. A modified calcium phosphate precipitation procedure was used for transient transfections as described (10); 0.5 ml of the precipitate containing 1 µg of

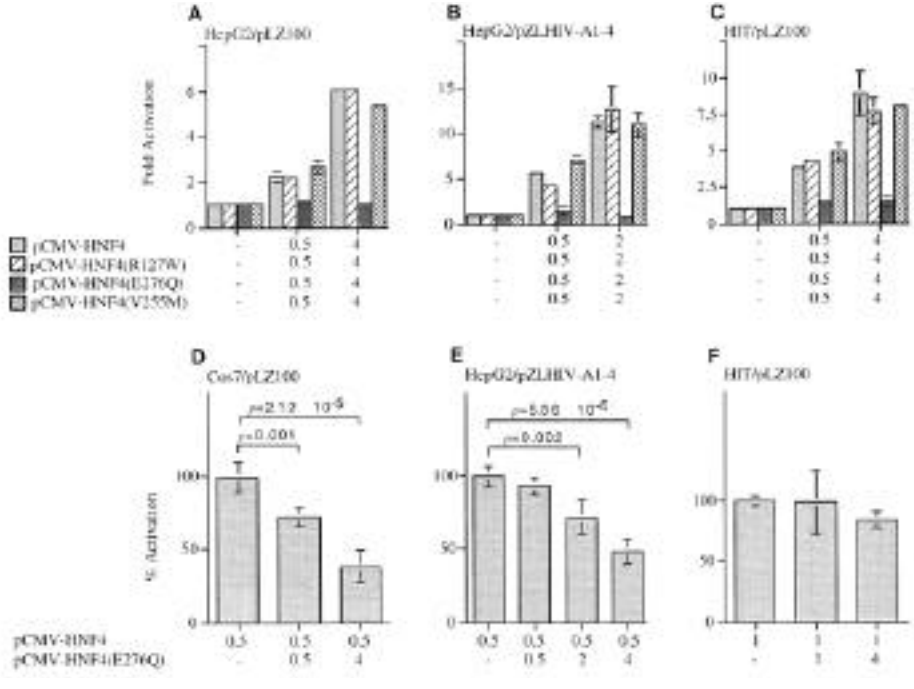

FIG. 2. Transcriptional activity of mutants HNF4(R127W), HNF4(V255M), and HNF4 (E276Q). cos7, HepG2, and HIT cells were cotransfected with the reporter constructs pLZ100 or pZLHIV-A1-4, CMV-LacZ, and the indicated wild-type or mutant expression vectors. Cells were harvested 40 h after transfection and assayed for luciferase and β-galactosidase activities. The amount (in micrograms) of expression vector used is indicated. All experiments were performed in duplicates and repeated twice; experiments *D–F* were repeated five times. The average fold inductions were normalized to β-galactosidase activity. Error bars indicate SE. Student's *t* test was used for statistical analysis.

cytomegalovirus (CMV)-LacZ, 1 µg of luciferase reporter construct, the indicated amount of expression vectors, and carrier DNA (up to 10 µg) were added per 60 mm dish. Luciferase was normalized for transfection efficiency by the corresponding β-galactosidase activity (22). The pLZ100 reporter contains the apolipoprotein CIII minimal promoter upstream of the luciferase gene. The pZLHIV-A1-4 contains four HNF4 binding sites and has been described previously (10).

RESULTS

Transcriptional activation activity of HNF4(R127W), V255M, and E276Q. To study the ability of HNF4(R127W), HNF4(V255M), and HNF4(E276Q) to stimulate transcription, mutant and wild-type cDNAs were cloned into the mammalian expression vector CMV-β (22). Mutant and wild-type HNF-4α constructs were cotransfected into cos7, HepG2, and HIT cells along with either reporter plasmid pZLHIV-A1-4, which contains four HNF4 binding sites of the apolipoprotein A1 promoter upstream of an HIV-LTR basal promoter, or pLZ100, in which transcriptional activation is driven by the minimal apolipoprotein CIII promoter containing one HNF4 binding site.

Figure 2*A–C* shows that wild-type HNF-4α activated the transcription of both reporter genes in all cell lines 5- to 15-fold. Mutations R127W and V255M had no effect on transactivation potential and activated the expression of the reporters to the same extent as the wild-type protein. In contrast, HNF4(E276Q) failed to transactivate gene transcription from pZLHIV-A1-4 and pLZ100 promoter constructs in cos7, HepG2, and HIT cell lines (Fig. 2*A–C*). To examine whether HNF4(E276Q) can inhibit the activity of wild-type HNF-4α, we coexpressed mutant proteins with wild-type HNF-4α. HNF4(E276Q) exhibited a mild but significant dominant-negative effect on wild-type HNF4 in cos7 and HepG2 cells (Fig. 2*D* and *E*). However, this effect was not present in HIT cells (Fig. 2*F*).

In vitro–translated HNF4(E276Q) protein is stable, dimerizes, and binds to DNA. Since HNF4(R127W) and HNF4(V255M) were functionally indistinguishable from wild type, no further detailed analysis of these proteins was performed. However, HNF4(E276Q) completely lacked any transcriptional activation potential. Amino acid 276 is located within the AF2 region, which contains the transactivation, dimerization, and putative ligand domain. To study whether the loss of transcriptional activity was caused by an inability of HNF4(E276Q) to bind to DNA, we compared the ability of wild-type and mutant HNF-4α proteins to interact with a high-affinity HNF4 binding site (LF-A1) by electrophoretic mobility shift assay (EMSA) analysis (Fig. 3). Wild-type and mutant proteins were produced in vitro using the Promega TNT transcription/translation system (23). A 54-kDa band could be identified by PAGE analysis for wild-type and mutant proteins. The relative amount of these in vitro synthesized proteins is shown in Fig. 3*A*. DNA binding of these proteins was then studied in EMSA analysis. Figure 3*C* shows that wild type and HNF4(E276Q) both bind strongly to DNA. To show that HNF4(E276Q) can interact with wild-type HNF-4α, we performed coimmunoprecipitation experiments. [^{35}S]methionine-labeled HNF4(E276Q) was able to bind to FLAG-tagged wild-type HNF-4α, suggesting that in vitro–translated mutant protein can heterodimerize with wild-type HNF-4α (Fig. 3*B*).

HNF4(E276Q) is unstable in cells. Since the in vitro expression and DNA binding studies did not reveal an explanation for the lack of transcriptional activity of HNF4 (E276Q), we expressed this mutant, as well as wild-type and HNF4(R127W) controls, in cos7 and HIT cells. Immunoblot analysis was then performed using either a polyclonal antibody that recognizes the 10 COOH-terminal amino acids of HNF-4α or an anti-FLAG monoclonal antibody. Figure 4 shows that, depending on the cell type, 30–90% of mutant HNF4(E276Q) protein migrated as an ~40-kDa protein, suggesting that an ~14-kDa aminoterminal portion of HNF4(E276Q) was degraded. A faint 40-kDa band was also

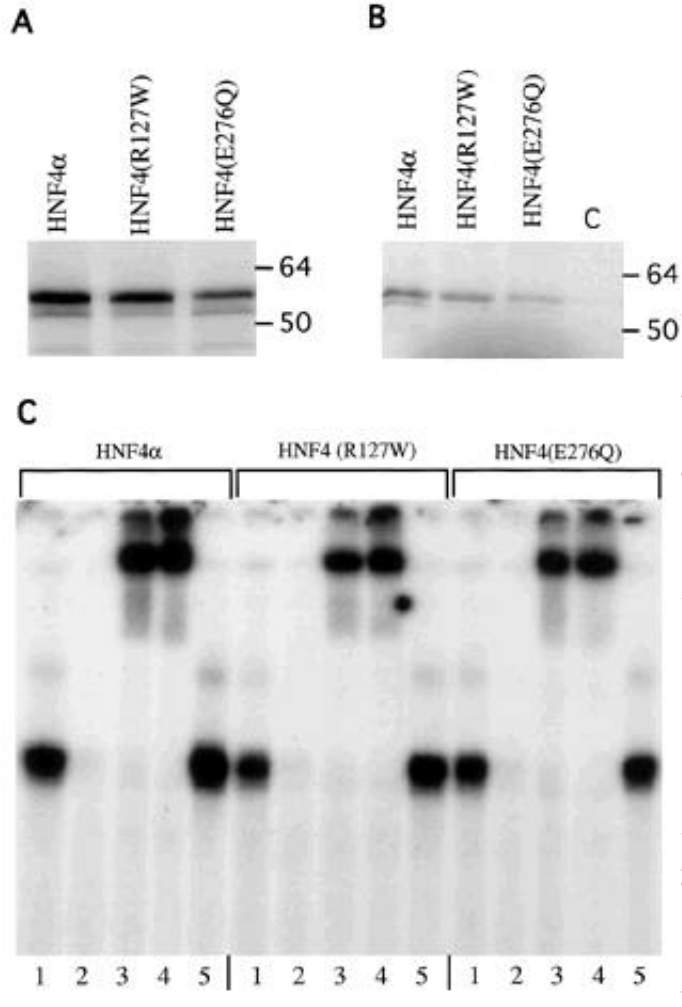

FIG. 3. Functional characterization of in vitro–translated wild-type HNF-4α protein and mutants HNF4(R127W) and HNF4(E276Q). *A*: SDS-PAGE analysis of in vitro–translated and [^{35}S]-labeled wild-type HNF4α and mutants HNF4(R127W) and HNF4(E276Q) followed by autoradiography. *B*: In vitro–translated mutant HNF4(R127W) and HNF4(276Q) proteins dimerize with wild-type HNF-4α. FLAG-tagged wild-type HNF-4α protein bound to an anti-FLAG (M2; Kodak) monoclonal antibody attached to agarose was incubated with 10 µl of [^{35}S]methionine-labeled wild-type HNF-4α and mutants HNF4(R127W) and HNF4(E276Q). Immunoprecipitates were run on 15% SDS-PAGE, and bound HNF-4α proteins were detected by autoradiography. HNF-4α-FLAG, [^{35}S]methionine-labeled FLAG-tagged HNF-4α; C, [^{35}S]methionine-labeled HNF-4α containing no FLAG-tagged HNF4 protein in the immunoprecipitation reaction. *C*: EMSA analysis of DNA binding activity of 2 µl of in vitro–translated wild-type and mutant HNF4(R127W) and HNF4(E276Q) proteins. The probe used for this experiment consisted of the ^{32}P-labeled double–stranded oligonucleotide LF-A1. Supershift analysis was carried out with 5 ng of polyclonal anti-HNF4 antiserum (anti-445) or 1 ng of an anti-STAT-5 control antiserum. *Lanes 1*, band shift; *2*, competition using 50 times cold probe; *3*, supershift using 1 ng of anti-HNF-4; *4*, supershift using 5 ng of anti-HNF4; *5*, supershift using anti-STAT-5 control antiserum.

occasionally detected in immunoblots of cells transfected with wild-type HNF-4α or HNF4(R127W), suggesting that it is a natural proteolytic product and that protein degradation is accelerated in mutant HNF4(E276Q) (Fig. 4*A* and *B*). The ratio of 40- over 54-kDa forms varied in different cell types and was highest in cos7 cells (0.6–0.9) (Fig. 4*A*) and lowest in HIT cells (0.3) (Fig. 4*B*). No significant differences in expression ratios were noted when whole cell extracts instead of nuclear extracts were studied in immunoblot analysis (results not shown), indicating that a defect in nuclear translocation of HNF4(E276Q) is unlikely to account for these differences. Overall expression levels of HNF4(E276Q) tended to be lower than that of wild-type protein, but equal expression of full-length HNF4(E276Q) protein could be achieved when transfected at a sixfold excess (Fig. 4*B*). The above data suggest that HNF4(E276Q) protein is degraded in cells, which results in the formation of a stable NH_2-terminal truncated ~40-kDa protein.

In vivo–translated HNF4(E276Q) does not bind to DNA. To investigate the DNA binding properties of the mutant HNF4(E276Q) proteins, we used the above nuclear extracts from transfected cos7 and HIT cells for EMSA analysis (Fig. 4*A* and *B*). Figures 5 and 6 show that HNF4(E276Q) completely lost its ability to bind to DNA. The lack of binding activity was shown in cos7 (in which 90% of the protein is present as a truncated 40-kDa form) and HIT cells (in which the majority of the HNF4[E276Q] exists as a full-length protein). Lack of DNA/heterodimer complexes was demonstrated using nuclear extracts of cos7 cells that were cotransfected with wild-type and mutant HNF-4α expression vectors (data not shown). Incubation of in vitro–translated wild-type HNF-4α protein and nuclear extracts of FLAG-tagged HNF4(E276Q)–transfected cos7 cells did not supershift with anti-FLAG antibodies, indicating that no heterodimers bound to DNA. Furthermore, we also did not detect a band shift with an abnormal electrophoretic mobility pattern in HIT cells, which might have indicated heterodimer formation of endogenous HNF-4α and the truncated version of HNF4(E276Q). The presence of HNF-4α proteins in the DNA binding complexes was confirmed by supershift analysis using a monospecific anti-HNF4 antiserum (anti-445) or a monoclonal anti-FLAG antibody (Kodak). The data demonstrate that in vivo–expressed full–length HNF4(E276Q) protein, as well as the NH_2-terminal truncated 40-kDa form, lack DNA binding activity.

The 40-kDa degradation product of HNF4(E276Q) can bind to wild-type HNF4α in vivo. HNF4(E276Q) inhibits the transactivation potential of wild-type HNF4α in cos7 or HepG2 cells. In these cells, the majority of the mutant protein exists a truncated 40-kDa form. To study whether the dominant-negative effect could be explained by the formation of heterodimers of the HNF4(E276Q) degradation product and wild-type HNF-4α protein, thus limiting the amount of wild-type homodimers that can bind to DNA and transactivate transcription, we cotransfected FLAG-tagged wild-type HNF-4α and HNF(E276Q) into cos7 cells. Proteins from nuclear extracts were then immunoprecipitated with anti-FLAG agarose beads and analyzed by Western blotting. Figure 7 shows that wild-type HNF-4α binds to the 40-kDa degradation product of HNF4(E276Q). This suggests that the dominant-negative effect of HNF4(E276Q) is in part the result of heterodimerization of mutant and wild-type HNF-4α proteins. However, EMSA analysis from nuclear extracts of cos7 cells that were cotransfected with wild-type HNF-4α and increasing amounts of HNF4(E276Q) did not lead to a significant decrease in band shift activity, suggesting that heterodimer formation may not be the only mechanism responsible for the dominant-negative effect in cos7 and HepG2 cells.

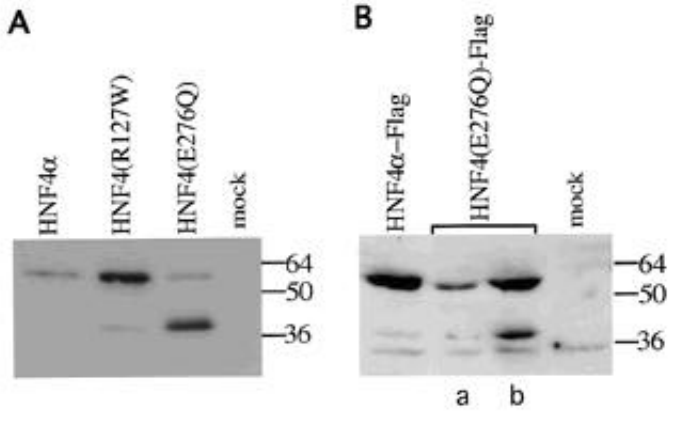

FIG. 4. Western blot analysis of nuclear extracts from cells transfected with wild-type and HNF4α variants. *A*: Nuclear extracts of cos7 transfected cells; 60 mm dishes of confluent cells were transfected with 10 µg of expression vectors. Nuclear extracts were prepared as described previously (10). Nuclear extracts were subjected to 15% SDS-PAGE, followed by Western blotting analysis using the polyclonal anti-HNF-4 antiserum (anti-445). Mock, nuclear extract from cos7 transfected with 10 µg of carrier (salmon sperm) DNA. *B*: nuclear extracts of HIT-T15 transfected cells. Cells were transfected with 5 µg of wild-type HNF-4-FLAG (*lane 1*), or 5 µg (*a*) and 30 µg (*b*) of HNF4(E276Q) expression plasmid DNA (*lanes 2* and *3*, respectively). Western blotting analysis was performed using anti-FLAG M2 antibodies.

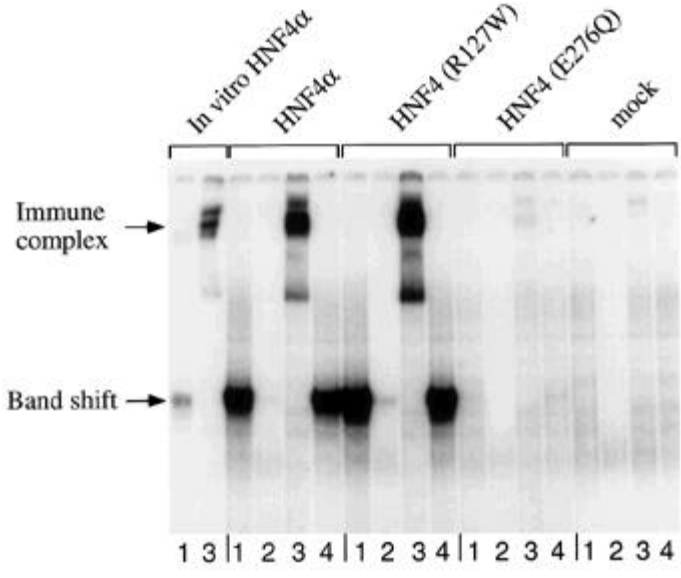

FIG. 5. HNF4(E276Q) does not bind DNA when expressed in cos7 cells. EMSA analysis of DNA binding activity of 3 µl of nuclear extracts of cos7 cells transfected either with expression vector for mutants HNF4(R127W) and HNF4(E276Q) or wild-type HNF-4α. Mock, nuclear extract of cos7 transfected with 10 µg of carrier DNA. Probe: ^{32}P-labeled LF-A1 oligonucleotide (7). *Lanes 1*, band shift; *2*, competition using 50 times of cold probe; *3*, supershift using 1 ng polyclonal anti-HNF-4 (anti-445) antiserum; *4*, 1 ng of anti-STAT-5 control antiserum.

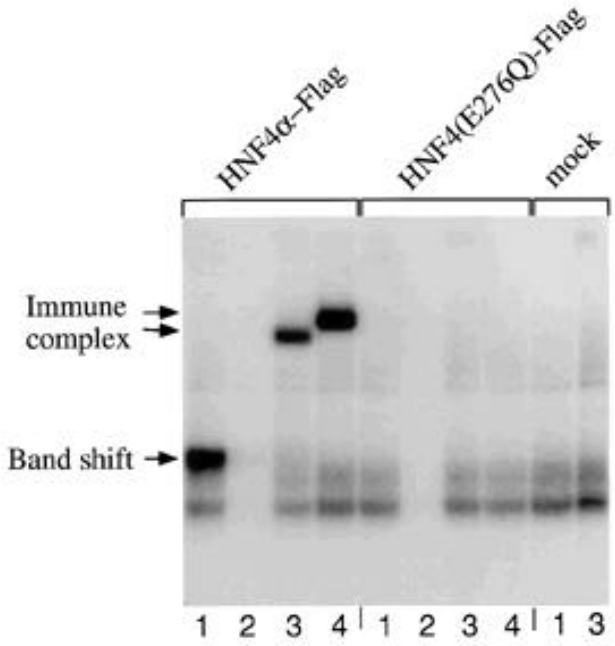

FIG. 6. Nuclear extracts of HNF4(E276Q)-transfected pancreatic β-cells lack DNA binding activity. EMSA analysis of DNA binding activity of nuclear extracts from HIT-T15 cells transfected with expression vector for either wild-type HNF-4α or HNF4(E276Q). Mock, nuclear extract of HIT-T15 cells transfected with 10 μg of carrier DNA. *Lanes 1,* band shift using ^{32}P-labeled LF-A1 probe; *2*, competition using 50 times of cold probe; *3*, supershift using 1 ng polyclonal anti-HNF-4 (anti-445) antiserum; *4*, 1 ng of anti-FLAG M2 antiserum.

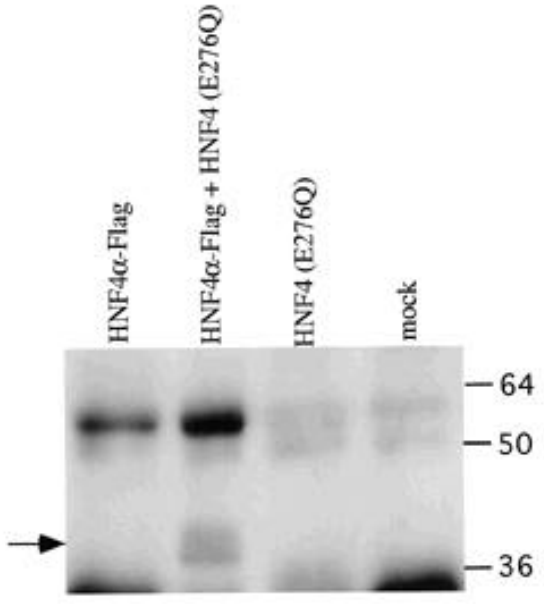

FIG. 7. Degradation product of HNF4(E276Q) coimmunoprecipitates with wild-type HNF-4α. cos7 cells were transfected with 5 μg of FLAG-tagged wild-type HNF-4α or mutant HNF4(E276Q) or were cotransfected with 5 μg of both plasmids. Nuclear extracts from transfected cells were immunoprecipitated with anti-FLAG agarose beads, and immunoprecipitates were subjected to Western blotting analysis using the polyclonal anti-HNF-4α antiserum (anti-445). The arrow indicates the truncated form of mutant HNF4(E276Q) that coimmunoprecipitates with wild-type HNF-4α.

DISCUSSION

Genetic studies have shown that mutations in HNF-4α result in an autosomal dominant form of type 2 diabetes characterized by defects in glucose-stimulated insulin secretion. HNF-4α is a transcription factor of the steroid hormone receptor superfamily and has an important role as a key regulator of hepatic and pancreatic islet gene expression. HNF-4 ligands have not been identified, although in one report, fatty acyl CoA thioesters have been shown to moderately affect HNF-4α activity (25). HNF-4α binds to its promoter binding sites as a homodimer and can be coactivated by CREB binding protein, steroid receptor coactivator-1, and glucocorticoid receptor interacting protein-1 (26,27). The molecular mechanisms by which mutations in HNF-4α may affect its transcriptional activity include changes in DNA binding, dimerization, putative ligand binding affinities, or disruption of receptor-interaction domains of transcriptional coactivators.

A number of mutations associated with early- and late-onset type 2 diabetes have been reported (3,15–21). However, statistically significant proof that a certain genetic variation is responsible for the diabetic phenotype has only been provided in two families (3,21). Since most families are not large enough to independently establish linkage with a given mutation, it is important to functionally characterize the mutant gene products and compare them with wild-type proteins.

In the present study, we have characterized three missense mutations in HNF-4α that have previously been linked to the development of type 2 diabetes. HNF4(R127W) was previously found in three of five diabetic members of a Japanese MODY family characterized by severe microvascular complications (18). HNF4(V255M) was found in 0.8% of Danish type 2 diabetic patients but was absent in 217 control patients (19). Our results indicate that the ability of these variant HNF-4α proteins to activate transcription is indistinguishable from that of wild-type HNF-4α. We conclude that HNF4(R127W) and HNF4(V255M) are rare polymorphisms and that the genetic susceptibility in those families/individuals has yet to be identified or that a functional defect cannot be revealed in in vitro transcriptional activation assays.

HNF4(E276Q) was originally identified in a U.K. pedigree with early-onset type 2 diabetes. Five members diagnosed as diabetic and two unaffected subjects, 17 and 34 years old, were shown to exhibit the mutation (20). The logarithm of odds score for the mutation in this pedigree (using a disease frequency of 0.001) was calculated to be 1.46 at $\theta = 0$. The HNF4(E276Q) mutations leads to a Glu to Gln substitution at position 276 in helix 8 of the ligand binding domain of the HNF-4α protein (8). Although helix 8 has not been shown to directly participate in the dimerization surface, DNA, or ligand binding, a glutamic acid at amino acid residue 276 is highly conserved throughout the steroid receptor superfamily, including the progesterone, estrogen, and retinoic acid receptors (28,29). Our biochemical analysis shows that the E276Q substitution abolishes HNF-4α transcriptional activity. HNF4(E276Q) protein is stable and binds to high-affinity DNA binding sites when synthesized in vitro. In vivo, however, HNF4(E276Q) is unstable and has lost the ability to bind to DNA.

Nuclear extracts of cells expressing HNF4(E276Q) revealed the presence of a truncated ~40-kDa protein that can be detected by two independent antibodies directed against the carboxyterminal domain of HNF-4α. The mutation may

introduce structural changes that make the protein more susceptible to degradation through proteolytic cleavage by cellular proteases. Based on the size of the truncated protein, we estimate that the NH_2-terminus lacks ~14 kDa that includes the AF1 transactivation domain, as well as part of the DNA binding domain. Because the truncated degradation product is stable and retains its ligand binding and dimerization domain, we tested whether it can heterodimerize in solution with wild-type HNF-4α protein. The immunoprecipitation demonstrates that the mutant degradation product can bind to HNF-4α. We also detected a clear correlation between dominant-negative activity and the expression levels of the 40-kDa truncated protein, which leads us to believe that this form is responsible for the weak but significant inhibition of wild-type HNF-4α in cos7 and HepG2 cells. Since we did not detect DNA/heterodimer complexes in EMSA analysis, it is unlikely that heterodimers bind to DNA and block access of functional homodimers on promoters of HNF-4α target genes.

The discrepancy of DNA binding activities between in vitro– and in vivo–translated proteins remains unclear and cannot simply be accounted for by a partial degradation of the mutant protein. One possible explanation for the inability of full-length in vivo–translated HNF4(E276Q) protein to bind to DNA is a posttranslational modification that only takes place in vivo. Alternatively, the mutant protein may have acquired affinity to proteins inhibiting DNA binding that are not present or active in reticulocyte extracts.

The dominant-negative effect may in part result from sequestering wild-type HNF-4α protein, hereby decreasing its availability to bind DNA as homodimers. In addition, the truncated form could also bind to coactivators of HNF-4α dependent transcription, general transcription factors, or HNF-4α ligands that are limiting, thereby inhibiting transcriptional activation (26,27). The latter explanation is the mechanism more likely to explain the dominant-negative activity in cos7 and HepG2 cells because EMSA analysis did not show significant reduction in DNA binding of wild-type HNF-4α when coexpressed with HNF4(E276Q). Since no dominant-negative activity could be demonstrated for HNF4(E276Q) in the pancreatic β-cell line HIT-T15, we believe that a loss-of-function mutation is the most likely molecular mechanism for this defect. However, it cannot be ruled out that dominant-negative activity contributes to a hepatic defect that may manifest as abnormalities of impaired gene expression of HNF4 liver-specific target genes such as the lipoproteins, transferrin, or α1-antitrypsin. Future genotype/phenotype relationship studies will be able to assess whether impairment of hepatic function, insulin secretion, and disease progression is more severe in patients with mutation HNF4(E276Q) compared with patients with pure loss-of-function mutations.

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