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Article Genetics

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Genetic evidence that HNF-1α–dependent transcriptional control of HNF-4α is essential for human pancreatic β cell function

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Introduction

Maturity onset diabetes of the young (MODY) is a form of type 2 diabetes characterized by early onset (usually before 25 years of age) and autosomal dominant inheritance. Recent studies have shown that the disease is genetically heterogeneous. Mutations in the genes encoding hepatocyte nuclear factor 4α, (HNF-4α), glucokinase, HNF-1α, insulin promoter factor 1 (IPF-1), HNF-1β, and NeuroD1 are the cause of the six known forms of MODY (1–6).

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Jorge Ferrer directed the RNA expression and functional mutation studies described in this manuscript.
Sara K. Hansen and Marcelina Párrizas contributed equally to this work.

Conflict of interest: No conflict of interest has been declared.

Nonstandard abbreviations used: maturity-onset diabetes of the young (MODY); hepatocyte nuclear factor (HNF); insulin promoter factor (IPF); electromobility shift assay (EMSA).

Except for the *MODY2* gene, which encodes a liver- and β cell–specific glycolytic enzyme, the remaining *MODY* genes encode transcriptional regulators. HNF-1α and HNF-1β are atypical homeodomain proteins. *IPF-1* encodes a homeodomain-containing protein, while HNF-4α is a steroid nuclear receptor family member and NeuroD1 is a basic helix-loop-helix transcription factor (7). Because MODY is primarily associated with insulin secretory dysfunction (7), it is believed that these transcriptional regulators are critically involved in maintaining β cell function. However, the precise role of these proteins in adult pancreatic islets is only beginning to be unraveled.

Some of the transcriptional regulators encoded by MODY genes have been implicated in common regulatory circuits. HNF-4α has been shown to control the expression of the gene encoding HNF-1α (8–10). HNF-4α expression is in turn controlled by HNF-3α and HNF-3β (8). A precise operation of this circuit is crucial for normal hepatocyte differentiation and function (8, 10). On the other hand, we and others have very recently provided evidence for the existence of an HNF-1α–regulated circuit that is specifically active in pancreatic cells (11, 12).

Analysis of *hnf-1α*–null mutant mice has revealed that in pancreatic endocrine and exocrine cells, HNF-4α expression is dependent on HNF-1α (11), whereas

HNF-4α expression remains unaltered in the absence of HNF-1α in liver (11, 13). This differential regulation is exercised through an alternate tissue-specific *HNF-4α* promoter (P2) located 45.6 kb upstream of the known promoter (11, 14). A high-affinity HNF1 binding site in the P2 promoter was shown to bind HNF-1α in vivo in mouse islets (11), suggesting that HNF-1α regulates the expression of HNF-4α in a direct manner. HNF-1α also controls the expression of multiple other pancreatic transcriptional regulators, such as SHP, HNF-3γ, and HNF-4γ (11, 12). Interestingly, the HNF-1α dependence of many of these genes is initiated shortly after differentiated cells arise in the pancreas. This observation, together with the fact that HNF-4α and HNF-1α deficiency result in β cell dysfunction (15, 16), suggests that the tissue-specific regulatory circuit that is controlled by HNF-1α is likely to play a central role in maintaining differentiated pancreatic β cell function. However, because HNF-1α controls the expression of multiple transcriptional regulators and can also directly interact with its distal targets, the notion that HNF-4α is an essential downstream effector of HNF-1α in β cells has not been demonstrated. Moreover, evidence that this regulatory circuit operates in vivo in human cells is lacking.

Earlier studies have shown that a mutated binding site for the IPF-1 transcription factor in the P2 promoter of the *HNF-4α* gene cosegregates with diabetes (17). In this study we have identified a loss-of-function mutation in the HNF-1α binding site of the human *HNF-4α* P2 promoter that causes diabetes in a large family diagnosed with MODY. Our finding provides genetic evidence for the role of HNF-1α as a major regulator of HNF-4α expression in the human pancreas, and proves for the first time that the regulatory action of HNF-1α on the *HNF-4α* gene is crucial for the function of pancreatic cells.

Methods

MODY family. A Czech MODY family with autosomal dominant inherited diabetes and three family members with onset of diabetes before the age of 25 years were studied (see Figure 2). Using denaturing HPLC and direct sequencing as described previously (18–21), we excluded mutations in the known exons or promoter regions of the *HNF-4α*, *GCK, HNF-1α, IPF-1,* and *NeuroD1* genes. Subsequently, a linkage study was done using fluorescently labeled polymorphic microsatellite markers flanking genes previously shown to be associated with MODY. Markers were chosen from the ABI PRISM linkage mapping set, version 2 (Applied Biosystems, Torrance, California, USA), and from GeneMap99 (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/genemap99). Markers used were as follows. For the *HNF-4α* gene on chromosome 20: D20S96, ADA, D20S119, D20S17, and D20S197. For the *GCK* gene on chromosome 7: D7S667, GCK1, GCK2, D7S519, and D7S2506. For the *HNF-1α* gene on chromosome 12: D12S321, D12S807, and D12S342. For the *IPF-1* gene on chromosome 13: D13S221, D13S1254, and D13S289. For the *HNF-1β* gene on chromosome 17: D17S927, D17S1788, and D17S800. For the *NeuroD1* gene on chromosome 2: D2S335, D2S364, and D2S2188. The PCR products were loaded on an ABI 377 sequencer (Applied Biosystems) and analyzed using GeneScan 3.1 and Genotyper 2.0 software (Applied Biosystems). The multipoint linkage calculations were performed using the computer program LINKMAP in the FASTLINK package (ftp://fastlink.nih.gov/pub/fastlink/) (22). Four different liability classes were used for healthy carriers. Healthy carriers between 0 and 10 years of age have a 48% risk of being affected; between 10 and 25 years of age the risk is 25%; between 25–40 years of age there is a 10% risk of being affected; and healthy persons above 40 year of age have a 2% risk of being affected.

In the nondiabetic children (ID nos. 142, 145, 146, 149, and 150 in Figure 2), we performed an intravenous glucose tolerance test with injection of 50% glucose (0.3 g per kg body weight) after an overnight fast. Blood samples were drawn before glucose injection and at 2, 4, and 8 minutes after injection for analysis of serum insulin. Glucose-induced acute-phase serum insulin response was calculated as the area under the curve from 0 to 8 minutes as described (23). Serum insulin was determined by ELISA as described (24). Informed consent was obtained from all studied subjects prior to their participation in the study. The study was approved by the Ethical Committee of the Third Faculty of Medicine at Charles University and was carried out in accordance with the principles of the Declaration of Helsinki II.

Sequence analysis. Primers used to amplify the P2 promoter region and exon 1D were designed from human genomic clone AL117382.28 using Primer3 software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) (25). PCR amplification was carried out as described earlier (26). The PCR products were then sequenced using a BigDye Terminator cycle sequencing kit and analyzed on an ABI PRISM 377 automated sequencer (Applied Biosystems). After detection of the mutation in the proband, DNA from all family members was sequenced using primers 6F (5′-TGATGTCCCCATACACCTG-3′) and 6R (5′-GATTCTTC-TAATCACCCAAGG-3′).

RNA expression analysis. First-strand cDNA was prepared with Superscript II (Life Technologies Inc., Gaithersburg, Maryland, USA) using random hexamers (Promega Corp., Madison, Wisconsin, USA) on total RNA from human liver (Clontech Laboratories Inc., Palo Alto, California, USA) or gradient-enriched pancreatic islet and exocrine tissue (27). Primers were designed to amplify *HNF-4α* exons 1D to 2 (1DF–2Ra), 1A to 2 (1AF–2Rb), 8 to 10 (8F–10R), 8 (8F–8R), and β-actin (ACTF–ACTR) (Figure 1a). Oligonucleotide sequences were as follows. 1DF: 5′-GCGGGCCCCTGCTCCTC-CAT-3′; 2Ra: 5′-AGAAGCCCTTGCAGCCGTCACAG-3′; 1AF: 5′-ACATGGACATGGCCGACTAC-3′; 2Rb: 5′-CTCGAGG-CACCGTAGTGTTT-3′; 8F: 5′-AAGATCAAGCGGCTGC-GTTCC-3′; 10R: 5′-CTGGCGGTGAGGGCTGTGG-3′; 8R:

5′-ACTCCAACCCCGCCCCTCCTG-3′; ACTF: 5′-CAAGGCCAACCGCGAGAAGATG-3′; ACTR: 5′-CTGGCCAGCCAGGTCCAGA-3′. PCR was carried out under standard conditions with limited cycle numbers, and products were analyzed in ethidium bromide–stained nondenaturing acrylamide gels.

Electromobility shift assays. Electromobility shift assays (EMSAs) were carried out with synthetic HNF-1α prepared by in vitro transcription/translation, as described previously (11). Sequences of the double-stranded oligonucleotides are shown in Figure 3a. Quantification was performed with Molecular Imager FX (Bio-Rad Laboratories Inc., Hercules, California, USA) and Scion Image software (Scion Corp., Frederick, Maryland, USA). Competition curves were analyzed with GraphPad Prism (GraphPad Software, San Diego, California, USA).

Plasmids. P2 promoter fragments comprising positions –371 to –37 or –171 to –37 relative to the initiator codon were generated by PCR and inserted into pGL2-Basic (Promega Corp.) to generate P2.371 and P2.171. The mutations described above for EMSA studies were introduced into the P2.371 vector generating P2.371/HNF1G→A and P2.371/HNF1→SAC plasmids and were verified by sequencing.

Transient transfection assays. Human colon CaCo2 cells, mouse 10T/2 fibroblasts, and mouse MIN6 β cells were trypsinized 14 hours before transfection, distributed into 12-well plates (3×10^4 cells/well) and maintained in DMEM supplemented with 15% FBS. Cells were transfected with 400 ng P2 promoter plasmids using Effectene (Qiagen Inc., Valencia, California, USA). pCMV–β-galactosidase (1 ng) was added to control for transfection efficiency.

For stimulation experiments, 0, 0.05, 0.5, 5, or 10 ng pBJ5-HNF1α was added in the presence or absence of 50 ng pBJ5-DCoH. Fixed amounts of DNA were maintained with empty pBJ5 vector. After 48 hours of incubation, luciferase and β-galactosidase were measured with chemiluminescent assays (Roche Diagnostics Corp., Indianapolis, Indiana, USA). At least three independent experiments were performed, each of which tested two to three independent clones for each reporter plasmid construction in duplicate. For each well, luciferase/galactosidase activity was calculated and expressed as a percentage of average luciferase/galactosidase activity values measured with P2.371 alone or P2.371 plus empty pBJ5 plasmids in the same experiment. Differences between means were assessed by Student *t* test.

Results

Expression of HNF-4α transcripts in human pancreas. In order to elucidate *HNF-4α* promoter usage in human pancreatic tissue, primers were designed to selectively amplify exon 1D or exon 1A (corresponding to HNF-4α isoforms driven by the P2 and P1 promoters, respectively) (Figure 1a). Our results show that P2 is the predominant promoter driving HNF-4α expression in human islets and exocrine pancreatic cells, while in human liver cells, HNF-4α expression is primarily directed by the P1 promoter, and to a much lesser degree by P2 (Figure 1b). Further analysis revealed that all three known 3′ HNF-4α splice variations are expressed in human pancreatic islets (Figure 1, a and c). Because these HNF-4α transcripts contain exon 1D rather than 1A, we refer to these three isoforms as HNF-4α7, HNF-4α8, and HNF-4α9 (Figure 1a) (28). Taken together, the results indicate that

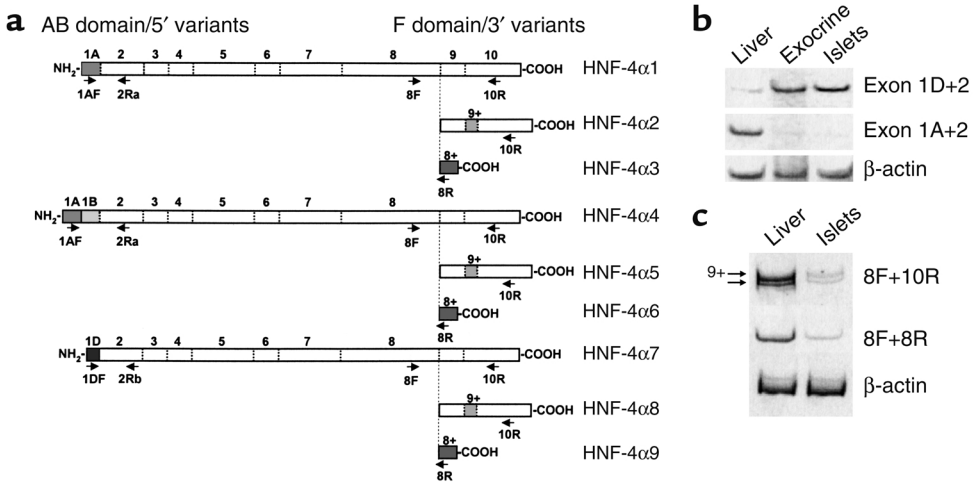

Figure 1
Expression of HNF-4α transcripts in human tissues. (**a**) Schematic representation of possible combinations of HNF-4α splice variations (adapted from ref. 27). Numbers indicate exons. Arrows indicate oligonucleotides used for RT-PCR. (**b**) RT-PCR analysis of HNF-4α exon 1A (transcribed from the P1 promoter) versus exon 1D (transcribed from the P2 promoter) in pancreatic tissues and liver. β-actin is used as internal control for the RT-PCR procedure. Only one band is amplified using primers designed to amplify *HNF-4α* exon 1A+2, indicating that transcripts originating in either tissue do not contain exon 1B. (**c**) RT-PCR analysis of HNF-4α 3′ end splice variations in human islets and liver. The 8F+10R primer set amplifies two fragments containing or lacking an extended exon 9 (9+) insertion. According to these results, liver contains predominantly HNF-4α1, -4α2, and -4α3 transcripts, whereas pancreatic cells contain HNF-4α7, -4α8, and -4α9 variants.

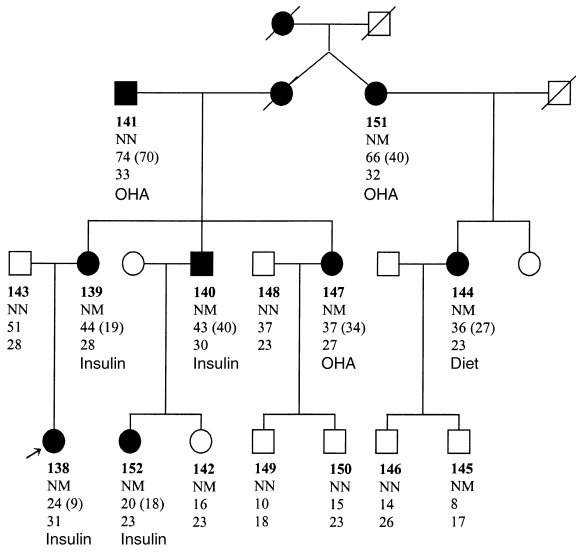

Figure 2
Pedigree of the MODY family with the *–181G→A* mutation in the P2 promoter of *HNF-4α*. Squares, male; circles, female; unfilled symbols are normal glucose tolerant; filled symbols are diabetic. Arrowhead indicates proband. The text below each individual represents the following: ID no., genotype (N, normal; M, mutant), age at diagnosis, body mass index (kg/m^2), treatment (OHA, oral hypoglycemic agents). Subject 144 was treated with insulin during her second pregnancy.

similar to mice, human pancreatic cells express diverse HNF-4α transcripts; the majority of these are regulated by the P2 promoter.

Screening for mutations in known MODY genes and linkage studies. Screening for mutations in the known exons and promoters of *HNF-4α, HNF-1α, GCK,* and *NeuroD1* genes in a large MODY pedigree did not reveal any mutations. Multipoint linkage studies did not reveal evidence of linkage in any of the six previously described MODY regions (HNF-4α/MODY1, GCK/MODY2, HNF-1α/MODY3, IPF-1/MODY4, HNF-1β/MODY5, NeuroD1/MODY6), but a suggestive lod score was observed on the MODY1 locus on chromosome 20 (z = 1.59). The GCK/MODY2 (z = –5.38), HNF-1β/MODY5 (z = –5.44), and NeuroD1/MODY6 (z = –5.38) regions were excluded. HNF-1α/MODY3 (z = –1.65) and IPF-1/MODY4 (z = –0.26) could not be excluded using a threshold for linkage exclusion of –2.0.

Identification of a mutation in the HNF-1α binding site of the P2 promoter. We sequenced 1 kb of the P2 promoter, exon 1D, and the exon/intron boundary in the proband from the MODY1 locus–linked family (Figure 2) without known mutations in the previously published sequences of the *HNF-4α* gene. The results revealed a G → A nucleotide substitution at position –181 from the translation start site, which is positioned in the previously described HNF-1α binding site (11, 14).

Genotyping of all family members revealed evidence of cosegregation of this mutation and diabetes in the family (Figure 2). Notice that subject 141 has diabetes but is a wild-type carrier. However, diabetes was diagnosed at the age of 70, and therefore it is plausible that he has a common form of late-onset type 2 diabetes and not MODY. Furthermore, as the deceased spouse of 141 is a monozygotic twin to 151, she is an obligate carrier of the mutation, and thus the mutation was inherited from this deceased spouse rather than from subject 141. Finally, the mutation was not found in 90 normal Czech chromosomes, or in more than 200 normal Danish chromosomes.

Examination of β cell function. Subjects 142 and 145 are heterozygous carriers, but are normal glucose tolerant. These subjects are 16 and 8 years old, respectively, and it is likely that they are too young to have developed a diabetic phenotype. We therefore examined their β cell function with an intravenous glucose tolerance test in order to test for a possible early β cell defect. Both mutation carriers have a relatively low insulin secretion capacity compared with the control population. Subjects 142 and 145 had a fasting serum insulin of 16 pmol/l and 11 pmol/l, respectively (reference interval obtained from data from 380 young Danes, median [10–90% percentiles]; 31 pmol/l, [17–66 pmol/l]). For comparison, subjects 146, 149, and 150 had fasting serum insulin concentrations of 49 pmol/l, 8 pmol/l, and 34 pmol/l. Estimation of the glucose-induced acute-phase serum insulin response for the first 8 minutes after an intravenous glucose load for subjects 142 and 145 was 1,154 min × pmol/l and 1,377 min × pmol/l, respectively; for subjects 146, 149, and 150, estimations were 5,043 min × pmol/l, 893 min × pmol/l, and 2,258 min × pmol/l, respectively (reference interval 2,252 min × pmol/l [1,078–4,457 min × pmol/l]). Furthermore, the majority of the diabetic subjects from the family are treated with insulin, indicating that, as expected, the mutation is associated with β cell insufficiency.

Functional analysis of the –181G→A mutation. Oligonucleotide competition assays were carried out to evaluate the impact of the *–181G→A* mutation on the affinity for HNF-1α. As shown in Figure 3, b and c, an amount of unlabeled HNF1G→A oligonucleotide sevenfold higher than the amount of wild-type oligonucleotide was required for half-maximal inhibition of binding of HNF-1α to its cognate site. For comparison, an artificially designed 4-bp substitution mutation (oligonucleotide HNF1→SAC) that modifies highly conserved nucleotides in the HNF1 site (29) completely disrupted sequence-specific binding.

The significance of these DNA-binding modifications on P2 promoter activity was assessed in 10T/2 fibroblasts, which do not express HNF-1α. Cells were transfected with increasing concentrations of pBJ5-HNF1α plus the luciferase reporter plasmid constructions shown in the schematic in Figure 4a. Figure 4b shows that HNF-1α stimulates the wild-type promoter (P2.371), but not a similar plasmid carrying the HNF1→SAC mutation. In keeping with the fact that the HNF1G→A mutation retains the ability to bind HNF-1α with reduced affinity, P2.371/ HNF1G→A can

be stimulated by HNF-1α, although higher concentrations of HNF-1α expression plasmid are required for half-maximal stimulation (ED_{50} for P2.371/HNF1G→A, 2 ± 0.4 vs. P2.371, 0.9 ± 0.4 ng), and maximal stimulation is significantly reduced (Figure 4b). Transactivation by HNF-1α of wild-type and P2.371/HNF1G→A promoters is specific, as it is potentiated by the dimerization cofactor of HNF-1α, DCoH, which does not elicit stimulation of the P2.371/HNF1→SAC–null mutant plasmid (Figure 4c) or of the P2.171 deletion plasmid lacking the HNF-1α binding site (not shown).

We then studied the intrinsic activity of P2 promoter plasmids in cells with endogenously expressed HNF-1α and HNF-4α exon 1D. In both MIN6 β cells and CaCo2 intestinal cells, P2.171, P2.371/HNF1G→A, and P2.371/HNF1→SAC plasmids had similarly reduced activity compared with the P2.371 wild-type plasmid (Figure 4d). These results suggest that under physiological activator concentrations, the reduction in the affinity for HNF-1α caused by the *HNF1G→A* mutation is sufficient to disrupt HNF-1α–dependent activation of the P2 promoter.

Discussion

Very recent studies have uncovered an alternate promoter of the mouse and human genes encoding HNF-4α (11, 14). This promoter, named P2, contains an HNF1 consensus element that is occupied in vivo by HNF-1α in mouse islets (11). Its transcriptional activity becomes dependent on HNF-1α as pancreatic endocrine and exocrine cells initiate terminal differentiation during mouse embryonic development (11). This suggests that HNF-4α may be a critical mediator of the function of HNF-1α in a genetic program destined to control differentiated pancreatic β cell function. However, the relative importance of HNF-4α as a downstream effector of HNF-1α cannot be concluded from those studies, as HNF-1α also controls the expression of multiple other transcriptional regulators (11, 12). Furthermore, previous studies did not establish whether HNF-1α acts on the P2 promoter through a single or multiple HNF1 sites, or whether intermediary HNF-1α–dependent transcriptional regulators are critically involved in the requirement for HNF-1α to control this promoter. In addition, formal evidence that the P2 promoter is the major HNF-4α transcription initiation site and that HNF-1α is necessary for HNF-4α expression in human pancreatic cells is lacking.

The present study provides evidence to address these issues. Using RT-PCR we demonstrate that the major HNF-4α isoforms in human exocrine and endocrine pancreas are transcribed from the P2 promoter. Although pure human β cells are not available to verify that the alternative HNF-4α isoform is present in this particular islet cell type in humans, previous studies using mouse MIN6 β cell lines indicate that HNF-4α exon 1D is indeed expressed in insulin-producing cells (30). Furthermore, we have identified a single nucleotide *–181G→A* mutation in the HNF-1α binding site of the P2 promoter of *HNF-4α* that cosegregates with diabetes in a large MODY pedigree. Several factors suggest that the identified mutation causes diabetes in the family. First, multipoint linkage studies revealed a suggestive linkage to the HNF-4α region on chromosome 20, whereas all other known MODY loci were excluded by linkage and/or direct mutation screening. Second, the mutation is not present in nearly 300 control chromosomes. Third, the low fasting insulin levels and the relative impaired insulin response to intravenous glucose in healthy mutation carriers indicate that the mutation causes an early β cell defect as also previously reported in MODY1 subjects (31).

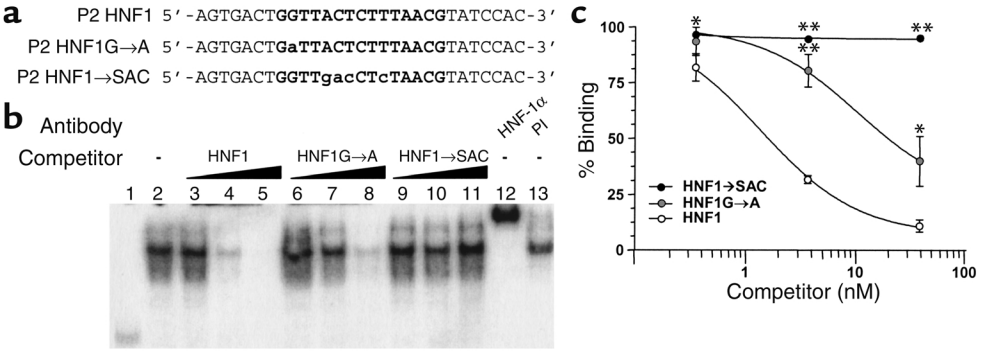

Figure 3

HNF-1α binding affinity in wild-type and mutant human P2 promoter oligonucleotides. (**a**) Schematic illustration of oligonucleotides containing the human HNF-4α P2 promoter HNF1 site (bold), and sites containing either the *–181G→A* mutation (P2 HNF1G→A) or an artificial mutation intended to completely disrupt HNF1 binding (P2 HNF1→SAC). Mutated bases are in lower case. (**b**) EMSA of radiolabeled wild-type HNF1 probe. Lanes 1 and 2: incubation with translation reactions using empty vector and pCMVTag-HNF1α, respectively. Lane 2 represents the maximal binding obtained in the absence of any cold competitor. Lanes 3–11: same as 2, except for preincubation with the indicated unlabeled probes at 1×, 10×, and 100× excess relative to the labeled probe. Lanes 12 and 13: same as 2, except for preincubation with either anti–HNF-1α or preimmune antisera, respectively. Similar results were obtained with mouse pancreatic nuclear extracts (not shown). (**c**) Results from two experiments such as the one shown in **b** were used to calculate oligonucleotide concentrations required for half-maximal displacement (HNF1G→A, 9.67 ± 1.45 nM; HNF1, 1.36 ± 0.22 nM). *$P < 0.05$; **$P < 0.01$.

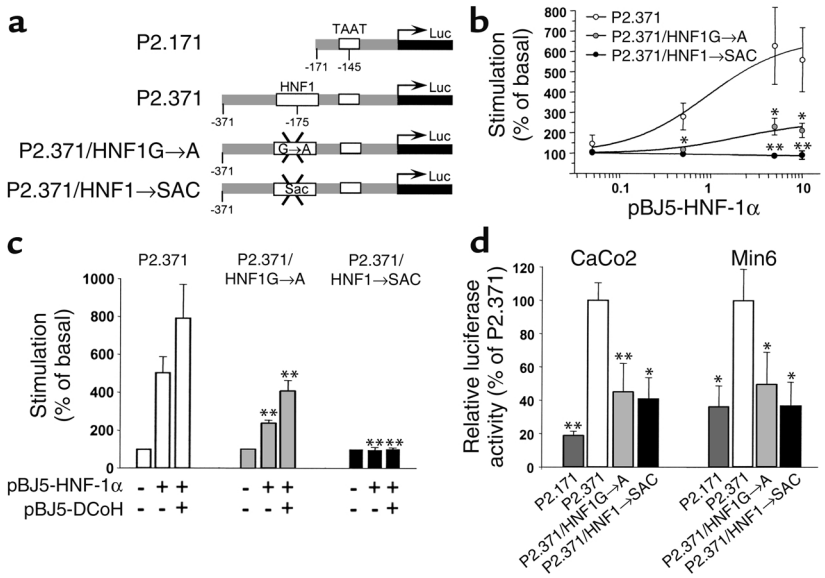

Figure 4
Effect of P2 promoter mutations on HNF-1α–dependent activation. (**a**) Schematic representation of the P2 promoter plasmids used in the transfection experiments described. Positions of the HNF1 site and the previously reported TAAT box are depicted. (**b**) Cotransfection of fibroblasts with indicated reporter constructs plus increasing amounts (0.05–10 ng) of pBJ5-HNF1α. Data are expressed as percentages of transfections performed with empty pBJ5. (**c**) Effect of DCoH on HNF-1α–dependent activation of indicated constructs. (**d**) Effect of HNF1G→A and HNF1→SAC mutations in cell lines expressing endogenous HNF-1α. Data are expressed as percentages of results obtained with P2.371. $*P < 0.05$; $**P < 0.01$.

Also, we performed an oral glucose tolerance test in the diabetic subject cz147 that revealed low serum insulin levels at all time points during the test compared with subjects having type 2 diabetes (data not shown). These observations point to a progressive β cell defect. Obviously however, prospective data are needed to illuminate the natural fate of the β cell function in mutation carriers. Fourth, the mutation results in reduced in vitro binding of HNF-1α to its cognate site and impairs HNF-1α–dependent transcriptional activation. The results indicate that the diabetic phenotype of *–181G→A* mutation carriers is caused by the decreased expression of HNF-4α as a consequence of a disruption of HNF-1α–dependent activation of the P2 promoter. While it is also possible that the mutation disrupts regulation by HNF-1β, which in vitro possesses a DNA-binding sequence specificity almost identical to that of HNF-1α, there is currently insufficient evidence that HNF-1β is important for P2 promoter activity in adult pancreas, as HNF-4α expression is severely impaired in *hnf-1α*–null mice in the presence of normal levels of HNF-1β (11, 12). Thus, these results represent the first in vivo indication that HNF-1α is required for HNF-4α expression in humans.

The observation that the disruption of a single *cis* element is linked and associated with diabetes provides important novel information concerning the mechanism whereby HNF-1α controls HNF-4α expression. It shows that the essential role of HNF-1α in the P2 promoter can be narrowed down to a direct interaction with a single *cis* element. Furthermore, while defective expression of multiple transcriptional regulators has been documented in hnf-1α–deficient pancreatic cells (11, 12), the *–181G→A* mutation shows that HNF-1α deficiency per se (rather than the loss of intermediary factors) underlies the HNF-1α dependence of the P2 promoter.

It is currently not established whether decreased expression of HNF-4α in HNF-1α deficiency is relevant to the mechanism of β cell dysfunction. For example, β cell dysfunction resulting from primary HNF-4α deficiency (e.g., in MODY1) could theoretically be exclusively mediated through the resulting HNF-1α deficiency, while the phenotype in *hnf-1α*–null mutant mice could primarily result from the direct effects of HNF-1α on its distal targets, as well as from defective expression of other intermediary transcriptional regulators. The *–181G→A* mutation provides for the first time proof that HNF-1α control of the *HNF-4α* P2 promoter is on its own essential for β cells to function normally. Thus, despite the apparent complexity of the transcriptional regulatory circuit in which this interaction is immersed (7, 11, 12), this mutation reveals a discrete regulatory site that is indispensable to its function. It is hypothesized that modulators of the *HNF-4α* P2 promoter, or more specifically modulators of HNF-1α action on this promoter, may be of importance to control the differentiation or function of human pancreatic β cells.

The genetic and RT-PCR findings described here also confirm the essential role of the P2 promoter in the expression of HNF-4α in human pancreatic cells. This is consistent with a recent report by Thomas et al.

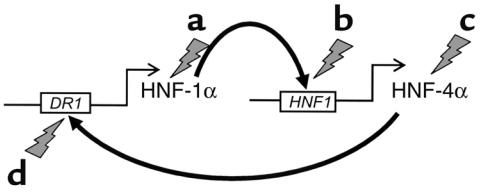

Figure 5
Summary of human genetic findings supporting positive cross regulation between HNF-1α and HNF-4α in human pancreatic cells. MODY has been found in subjects with loss-of-function mutations in the coding region of HNF-1α (**a**), HNF-1α binding site in the P2 promoter of the *HNF-4α* gene (**b**), HNF-4α coding region (**c**), and the HNF-4α binding site (DR1) in the promoter of the *HNF-1α* gene (**d**). References are provided in the text.

describing a loss-of-function mutation located in a TAAT element in the *HNF-4α* P2 promoter that binds recombinant PDX-1 (14). This mutation cosegregates with diabetes in a large British MODY family. Obviously, these findings should stimulate further studies of MODY families of unexplained genetic background to estimate the prevalence of MODY due to variability in exon 1D and the P2 promoter. Furthermore, they warrant an assessment of the possible role of genetic variability in this promoter region in the pathogenesis of type 2 diabetes, as several studies have demonstrated linkage to chromosome 20q12-q13.1 (32).

In conclusion, the current study indicates that HNF-1α is a major regulator of HNF-4α expression in the human pancreas, acting directly through a distinct essential *cis* element in the *HNF-4α* P2 promoter. Together with previous human and rodent studies indicating that HNF-4α controls the expression of HNF-1α, this finding provides independent evidence for the existence of a positive cross-regulatory loop between HNF-1α and HNF-4α in human pancreatic cells (9, 11, 12, 33, 34) (Figure 5). Furthermore, it proves that despite the complexity of the pancreatic HNF-1α–dependent regulatory network (11, 12), the interaction between HNF-1α and the *HNF-4α* P2 promoter is indispensable for human pancreatic β cell function.

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