The Diabetic Phenotype in *HNF4A* Mutation Carriers Is Moderated By the Expression of *HNF4A* Isoforms From the P1 Promoter During Fetal Development

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OBJECTIVE—Mutations in the alternatively spliced *HNF4A* gene cause maturity-onset diabetes of the young (MODY). We characterized the spatial and developmental expression patterns of HNF4A transcripts in human tissues and investigated their role as potential moderators of the MODY phenotype.

RESEARCH DESIGN AND METHODS—We measured the expression of *HNF4A* isoforms in human adult tissues and gestationally staged fetal pancreas by isoform-specific real-time PCR. The correlation between mutation position and age of diagnosis or age-related penetrance was assessed in a cohort of 190 patients with *HNF4A* mutations.

RESULTS—HNF4A was expressed exclusively from the P2 promoter in adult pancreas, but from 9 weeks until at least 26 weeks after conception, up to 23% of expression in fetal pancreas was of P1 origin. HNF4A4–6 transcripts were not detected in any tissue. In whole pancreas, HNF4A9 expression was greater than in islets isolated from the endocrine pancreas (relative level 22 vs. 7%). Patients with mutations in exons 9 and 10 (absent from *HNF4A3*, *HNF4A6*, and *HNF4A9* isoforms) developed diabetes later than those with mutations in exons 2–8, where all isoforms were affected (40 vs. 24 years; $P = 0.029$). Exon 9/10 mutations were also associated with a reduced age-related penetrance (53 vs. 10% without diabetes at age 55 years; $P < 0.00001$).

CONCLUSIONS—We conclude that isoforms derived from the HNF4A P1 promoter are expressed in human fetal, but not adult, pancreas, and that their presence during pancreatic development may moderate the diabetic phenotype in individuals with mutations in the *HNF4A* gene. ***Diabetes*** **57:1745–1752, 2008**

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AF, activation function; β2M, β-2-microglobulin; Ct, crossing point; HNF, hepatocyte nuclear factor; MODY, maturity-onset diabetes of the young.

See accompanying commentary, p. 1461.

The *HNF4A* gene codes for hepatocyte nuclear factor (HNF)-4α, which has an important role in pancreatic development and maintenance of β-cell function (1). P2 promoter region variants are associated with susceptibility to type 2 diabetes in some populations (2–6). Rare mutations in the *HNF4A* gene or the P2 promoter cause young-onset autosomal-dominant diabetes known as maturity-onset diabetes of the young (MODY) (7,8). These mutations also cause increased birth weight and macrosomia with hyperinsulinemic hypoglycemia in neonates (9). This phenotypic variation is paradoxical, since *HNF4A*-MODY is characterized by a failure of β-cells to appropriately increase insulin secretion in response to hyperglycemia (10), while the macrosomia and hypoglycemia reflect increased insulin secretion in utero and during the neonatal period, respectively. Increased insulin secretion has also been noted in utero and in early life in the β-cell–specific *Hnf4a* knockout mouse (9,11). These findings may indicate different roles for HNF-4α in fetal and adult β-cells.

The *HNF4A* gene encodes a 465–amino acid protein with five functional domains: A/B to F (Fig. 1). HNF-4α plays a key role in the development and differentiation of β-cells. In the mouse, the *Hnf4a* gene is first expressed in primary endoderm at E4.5 but is restricted to the visceral endoderm from E5.5 to 8.5 days (1,12). Thereafter, *Hnf4a* expression is evident in the liver diverticulum, the hindgut, the mesonephric tubules of the kidney, and the developing stomach, intestine, and pancreas (12–15). The requirement for HNF-4α in early development is also evident in animal models where the gene has been inactivated. In knockout studies, $Hnf4a^{-/-}$ mice display defective gastrulation and die around day E9 (16). The presence of HNF-4α appears to be critical for the processes that occur from day E6.5 onwards, as replacement of $Hnf4a^{-/-}$ endoderm with the wild-type counterpart before that point restores embryonic viability (14). The rescued embryos fail to express the correct profile of liver-specific genes, indicating an additional role for HNF-4α in the terminal differentiation of hepatocytes (17). In the adult mouse, *Hnf4a* expression has been reported in liver, kidney, intestine, stomach, and pancreas (12,13), where HNF-4α is responsible for the regulation of genes involved in processes such as insulin secretion (11), gluconeogenesis (18), bile acid synthesis (19), and lipid metabolism in adult humans (20).

In the adult β-cell, the expression of HNF-4α is regulated by the pancreatic transcription factor network (21–23), positively by a number of proteins including the *HNF1A*, *PDX1*, and *HNF1B* gene products (22,24,25) and negatively by itself (21). The key regulatory relationship

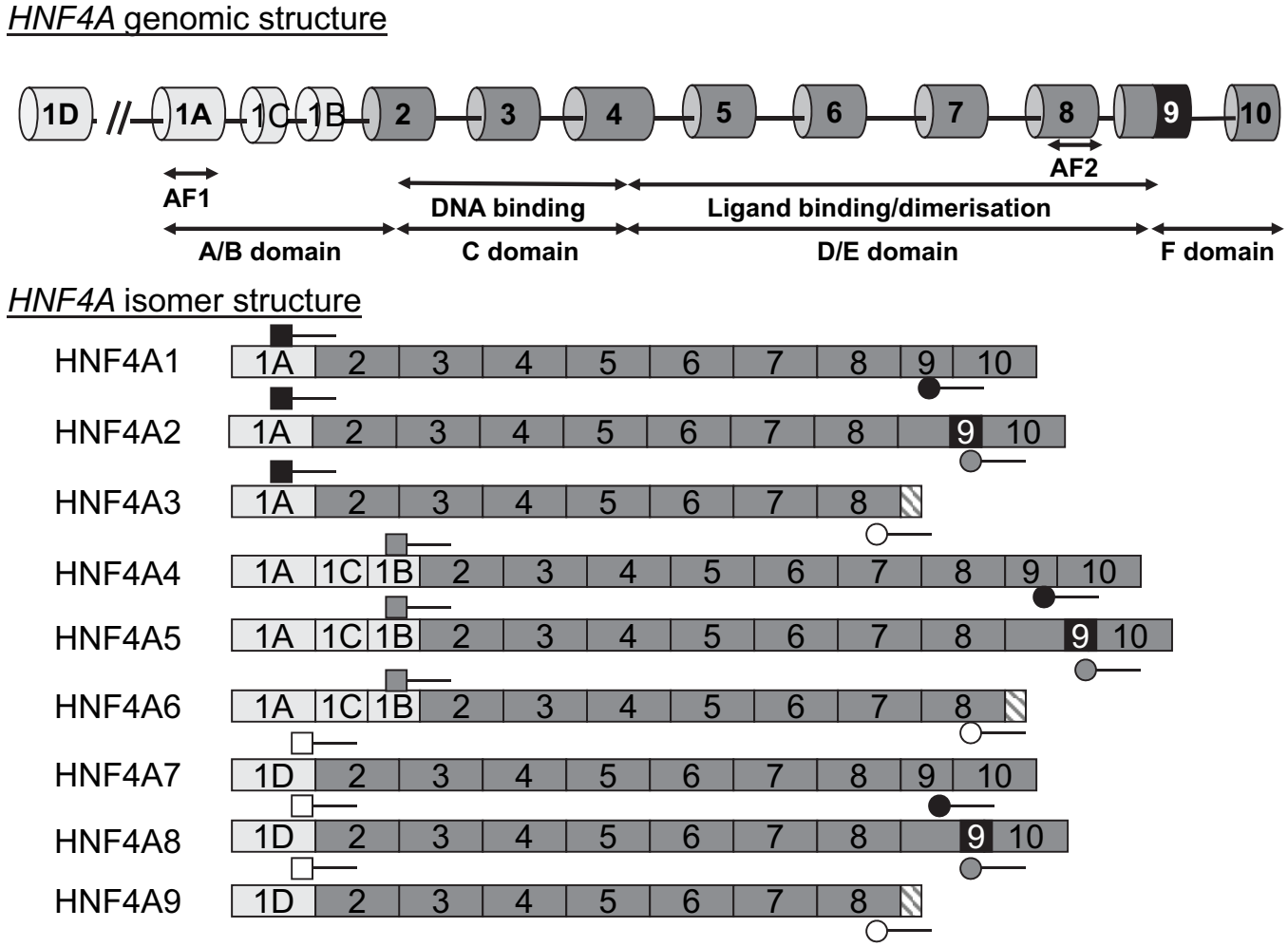

FIG. 1. Isoforms of the *HNF4A* gene. The genomic and isoform structure of the *HNF4A* gene are given. Alternate exons are coded in gray; the 10–amino acid insertion present in exon 9 in isoforms *HNF4A2*, *HNF4A5*, and *HNF4A8* is marked in black. Intronic sequences included in isoforms *HNF4A3*, *HNF4A6*, and *HNF4A9* are indicated by hatched boxes. AF-1 and AF-2 refer to activation function motifs. The domain structure of the HNF-4α protein and the positions of the isoform-specific probes used are given; probe set 1 is marked by black, gray, and white squares, and probe set 2 is marked by black, gray, and white circles.

appears to be that with HNF-1α (26). In hepatocytes, the expression of *HNF4A* is not dependent on the presence of HNF-1α, although other components of the pathway remain unchanged (21,22). Mutations in the *HNF1A* and *HNF4A* genes were thought to result in similar phenotypes as a consequence of an autoregulatory relationship between the genes (21,22,27). However, the recent association of neonatal hyperinsulinemia and macrosomia in *HNF4A* but not *HNF1A* mutation carriers (9) suggests that the network may be configured differently in adult and fetal β-cells.

Alteration of the configuration of the pancreatic transcription factor network could be mediated by changes in the abundance or nature of its components. One important mechanism that may underlie these processes is alternate mRNA processing. Many of the genes involved in this pathway code for multiple gene products (28). The related *HNF1A* gene produces three variant isoforms, *HNF1A(A)*, *HNF1A(B)*, and *HNF1A*(C), which are subject to both spatial and temporal variation in expression pattern. The predominant *HNF1A* isoform in human adult pancreas is *HNF1A(B)*, whereas in fetal pancreas it is *HNF1A(A)* (29). We previously showed that mutations that do not affect *HNF1A(B)* present at a median of 7.5 years later than mutations affecting all three isoforms (29).

The *HNF4A* gene produces a total of nine potential isoforms (*HNF4A1–HNF4A9*) by a combination of differential promoter usage, variation in polyadenylation site, and alternate splicing (Fig. 1) (27,30–32). Isomers *HNF4A1* to *HNF4A6* are coded from the P1 (hepatic) promoter. Isoforms *HNF4A7* to *HNF4A9* are transcribed from the P2 (pancreatic) promoter. The 3′ termini are also distinct. Isoforms *HNF4A2*, *HNF4A5*, and *HNF4A8* include a 10–amino acid insertion in exon 9 relative to *HNF4A1*, *HNF4A4*, and *HNF4A7*. *HNF4A3*, *HNF4A6*, and *HNF4A9* are truncated in intron 8 (1,28). There are conflicting reports of the spatial distribution of *HNF4A* isoforms. In hepatic and renal tissues, P1-derived isoforms are most abundant (27,33,34). The situation is less clear in the β-cell. Although two studies report the absence of P1 transcripts (27,32), one report suggests the expression of transcripts derived from the P1 promoter (33). However, it is noteworthy that the only study to examine isoform-specific *HNF4A* expression at the protein level detected only P2-derived isoforms in adult pancreas (35).

The aim of our study was to determine whether alternate mRNA processing of the *HNF4A* gene could contribute to differences in the composition and function of the pancreatic transcription factor network in fetal and mature pancreatic tissues. To achieve this, we developed a

complete spatial and temporal profile of *HNF4A* isoforms in adult liver, kidney, pancreas, isolated islet, and gestationally staged human fetal pancreas. We subsequently examined the role of specific isoforms in the development and maintenance of mature function by correlating mutation phenotype with mutation position relative to the known structure of the isoforms. We report that the *HNF4A* gene, like the *HNF1A* gene, undergoes alternate mRNA processing and that the isoforms demonstrate differences in spatial and temporal expression. These expression profiles may explain the observed correlation of the mutation position with the age at diagnosis of diabetes in *HNF4A* mutation carriers.

RESEARCH DESIGN AND METHODS

RNA samples used for study. RNA samples were obtained from kidney (pooled from six Caucasian women, 28–52 years old) (Clontech, Oxford, U.K.), liver (51-year-old Caucasian man), total pancreas (pooled from five Caucasian men and women, 24–77 years), fetal pancreas (pooled at 19/27 weeks' gestation), colon (pooled from five Caucasian men and women, 20–55 years), small intestine (pooled from five Caucasian men and women, 20–61 years), stomach (50-year-old Caucasian man), and isolated human islets (two independent samples) (National Disease Resource Interchange [NDRI], Philadelphia, PA). The collection of early human fetal material and its ethical approval have been described previously (36,37). Material from eight human fetuses assessed for gestational age according to foot length was studied. Mouse islet RNA was a gift of Dr. Anna Gloyn. All RNA samples were DNAse treated before reverse transcription using the TURBO DNAse kit (Ambion, Huntingdon, U.K.).

Assay design. Reference sequences corresponding to the six known 5′and 3′ HNF4A variations were identified from the NCBI Database (http://www.ncbi.nlm.nih.gov). The ubiquitously expressed β-2-microglobulin (*β2M*) gene was selected as a control on the basis of its constant expression levels across the tissues and developmental stages studied. An assay for this gene (assay Hs00187842) was purchased by Assays-on-Demand from Applied Biosystems (Foster City, CA). Custom real-time PCR assays were obtained from the Assays-by-Design service available from Applied Biosystems. Probe and primer sequences are given in supplementary Table 1 (available in an online appendix at http://dx.doi.org/10.2337/db07–1742). Where possible, probes were chosen to span introns to ensure amplification of cDNA rather than genomic DNA. Assays were validated by standard curve analysis of serial 1:2 dilutions of pooled liver, kidney, and pancreas cDNA. The efficiency of each assay was assessed by reference to the slope of the corresponding standard curve. The correlation between cDNA concentration and crossing point (r^2 value) was assessed from the fit of the crossing point corresponding to each dilution point to the curve.

Reverse transcription and real-time PCR amplification. Complementary DNA (cDNA) was synthesized from 3 μg of the total RNA using the Thermoscript RT-PCR system (Life Technologies, Paisley, U.K.) using 50°C as the incubation temperature. Real-time PCR reactions to determine *β2M* and *HNF4A* expression levels were carried out using the ABI Prism 7000 platform (Applied Biosystems). Each sample was amplified in triplicate to ensure accuracy of quantification. Where multiple samples per tissue were tested, each sample was from a separate mRNA extraction and reverse transcription. PCRs contained 10 μl TaqMan Universal Mastermix (no AMPerase) (Applied Biosystems), 0.9 μmol/l each primer, 0.25 μmol/l probe, and 2 μl cDNA reverse transcribed as above in a total volume of 20 μl. PCR conditions were a single cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Human fetal pancreas RNA was subject to a pre-amplification step before quantification of targets using the Applied Biosystems TaqMan PreAmp Master Mix kit (Applied Biosystems), which allows the study of multiple targets from small amounts of tissue.

Expression profiling. Expression levels of *β2M* and *HNF4A* isoforms were calculated by reference to the average crossing point of the triplicate samples. For each RNA sample, the difference between the average crossing point (Ct) obtained from *β2M* amplification ($Ct^{\beta 2M}$) and the average crossing point obtained from the test probe set (Ct^{test}) was termed the ΔCt value. ΔCt values were calculated for each isoform. The level of each transcript relative to *β2M* levels could then be calculated from the equation $2^{-\Delta\Delta Ct}$ (38), where ΔΔCt is the ΔCt value of the test transcript (ΔCt^{test}) normalized to a reference transcript, which was taken to be the levels of *HNF4A* for the probe set in question found in human liver (ΔCt^{ref}). Transcripts were identified as originating from the P1 promoter or the P2 promoter using probe set 1, which identified *a*) *HNF4A1–4A3* (P1), *b*) *HNF4A4–4A6* (P1), and *c*) *HNF4A7–4A9* (P2) transcripts. The occurrence of each 3′ variant was then determined using probe set 2, which identified *a*) *HNF4A1/4A4/4A7*, *b*) *HNF4A2/4A5/4A8*, and *c*) *HNF4A3/4A6/4A9* transcripts. Where expression was exclusively from one promoter, the identity of each isoform could be determined in this manner by combining data.

HNF4A subject. Data were gathered from families that had been referred for testing in our laboratories in the period between 1996 and 2006 ($n = 84$) and from families reported in the literature for which age of diagnosis data were available ($n = 106$) (8). We studied the possible effect of alternate mRNA processing on phenotype in a total of 45 different mutations found in 190 subjects from 58 families, shown in online supplementary Fig. 1. Of these, 50 (26%) patients had mutations in the P2 promoter or exon 1D, 125 (66%) grouped in exons 2–8, and 15 (8%) had mutations in exons 9 and 10.

Correlation of mutation phenotype with the position of the mutation and HNF4A protein domain structure. We correlated the age at diagnosis with mutation position relative to the known structure of the *HNF4A* isoforms. To confirm that any effect noted was due to the effect of mutation on different *HNF4A* isomers and was not related to differences in mutational mechanism, we also correlated age at diagnosis with mutation type (missense or premature termination codon forming) and with the functional domain structure of the HNF-4α protein by Mann-Whitney *U* analysis. To account for relatedness between family members, we also carried out additional analysis using generalized estimating equation (GEE) analysis. Age of diagnosis was used as the dependent variable, and isomer structure was added to the model, coded as a dummy variable, with mutations affecting all isoforms treated as baseline. To determine whether there was any difference in age-related penetrance between the three cohorts, we examined the number of individuals carrying mutations but with no diabetes at the age of 55 years using Kaplan-Meier survival curves and χ^2 analysis. All statistics were carried out using the SSPS software package (SSPS, Chicago, IL).

RESULTS

Validation of real-time PCR for the detection and quantification of alternatively spliced transcripts. Real-time PCR assays specific to *HNF4A* proved quantitative across a dynamic range of more than seven 1:2 serial dilutions. The efficiencies of detection were −3.4, −3.7, −3.7, −3.5, −3.7, and −3.3 for probe sets 1 (*a*, *b*, and *c*) and 2 (*a*, *b*, and *c*), respectively, where a gradient of −3.3 represents an optimally efficient PCR. This is a reflection of the fact that where amplification efficiencies are near 100%, there should be 3.3 cycles between each dilution point on the standard curve. The correlations between the crossing point and input template (r^2 value) were also good ($r^2 = 0.99, 0.91, 0.98, 0.99, 0.98$, and 0.95 for probe set 1 [*a*, *b*, and *c*] and 2 [*a*, *b*, and *c*], respectively).

HNF4A isoforms demonstrate spatial variation in expression profile. In adult human liver and kidney, *HNF4A* expression was derived exclusively from the P1 promoter and comprised *HNF4A1*, *HNF4A2*, and *HNF4A3* at 37, 46, and 17% (liver) and 42, 42, and 16% (kidney) of total *HNF4A* expression, respectively. In human adult pancreas and isolated islet samples, however, *HNF4A* expression was driven only from the P2 promoter with isoforms *HNF4A7*, *HNF4A8*, and *HNF4A9* being present at 36, 42, and 22% (total pancreas) and 45, 48, and 7% (islet) (Fig. 2*A* and *B*). We also examined adult mouse islets, where only P2 isoforms *HNF4A7*, *HNF4A8*, and *HNF4A9*, which vary at the 3′ terminus of the gene, were expressed at 49, 47, and 4% of total *HNF4A* expression. A noteworthy species difference in the levels of *HNF4A* transcripts in liver and islets is also apparent. In our human study, levels of *HNF4A* mRNA are similar in liver and islets, whereas in the mouse, *Hnf4a* expression is much greater in the liver (39). This is in agreement with our own experiments in mouse liver and islets, where islet-specific expression of *HNF4A* is only ~10% of that in the liver. In human stomach, small intestine, and colon, transcripts from both

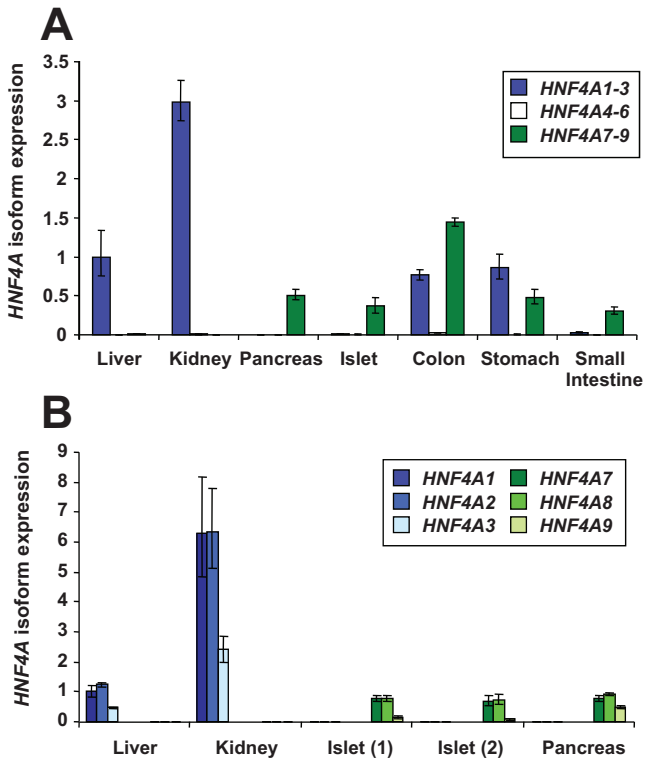

FIG. 2. Spatial expression profiles of *HNF4A* isoforms. The profiles given here refer to the tissue-specific expression patterns of *HNF4A* isoforms in human tissues. Tissues studied are shown on the *x*-axis, and the expression level of each isoform relative to the endogenous control gene β-2-microglobulin (*β2M*) are given on the *y*-axis. *A*: The expression profile of P1 and P2 *HNF4A* isoforms relative to adult liver as determined by probe set 1. *B*: The specific identities of *HNF4A* isoforms relative to adult human liver as determined using probe set 2. A color key to the identity of the isoforms is given within each section.

the P1 and P2 promoters were identified, although tissue-specific variation in the P1:P2 balance was noted (Fig. 2*A*). In adult stomach, the majority of *HNF4A* expression derived from the P1 promoter was present at 64% of *HNF4A* expression, whereas these isoforms were present only at 9% in the small intestine. Isoforms *HNF4A4*, *HNF4A5*, and *HNF4A6* were not identified at significant levels in any tissue (Fig. 2*A* and *B*).

HNF4A isoforms show differences in pancreatic developmental expression. The expression levels of *HNF4A* transcripts differed between human adult and fetal total pancreas mRNA (Fig. 3*A*–*C*). In mature pancreas, only P2 transcripts were identified. However, analysis of human fetal pancreas (staged with respect to gestational age according to foot length) revealed the presence of P1 isoforms at ~9 weeks post-conception (14% of total *HNF4A* expression; Fig. 3*C*). Expression was detected until the most advanced fetal pancreas was analyzed, a sample pooled from 19 and 26 weeks post-conception (Fig. 3*C*). The mixed derivation of fetal *HNF4A* isoforms made it impossible to determine the specific identity of each isoform, but the distribution of *HNF4A1/HNF4A7*, *HNF4A2/HNF4A8*, and *HNF4A3/HNF4A9* isoforms, characterized by variation at the 3′ end of the *HNF4A* gene, showed the presence of an apparent switch in *HNF4A* expression between adult and fetal human pancreas (Fig. 3*B* and *C*). In the adult pancreas, *HNF4A7* is present at 32% of total expression, whereas in fetal pancreas, only 8% of *HNF4A* expression is identified by the *HNF4A1/HNF4A7* probe set (with the shorter exon 9). In the adult pancreas, *HNF4A8* and *HNF4A9* are present at 42 and 22%, respectively. In human fetal pancreas at 19/26 weeks post-conception, however, 77 and 15% of total *HNF4A* expression is accounted for by *HNF4A2/HNF4A8* (with the longer exon 9) and *HNF4A3/HNF4A9* (terminating in IVS8), respectively.

HNF4A mutation position influences age of diagnosis of MODY and age-related penetrance. The median age at diagnosis for individuals with mutations in exons 2–8, which affect all isoforms, was 24 years (Fig. 4). In contrast, patients with mutations in exon 9 or 10 that do not affect *HNF4A3*, *HNF4A6*, or *HNF4A9* were diagnosed at 40 years ($P = 0.029$). Mutations in the P2 promoter or exon 1D affect the P2 isoforms *HNF4A7*, *HNF4A8*, and *HNF4A9*, and the median age at diagnosis of diabetes was 31 years ($P = 0.001$). Because factors such as age at diagnosis may also be influenced by other factors within the families, we also carried out general estimating equation analysis to account for family relatedness. After this correction, mutations affecting only P2 isoforms caused diabetes 8.1 years later than mutations affecting all isoforms (95% CI 2.7–13.6; $P = 0.010$), and mutations not affecting isoforms *HNF4A3*, *HNF4A6*, and *HNF4A9* caused diabetes 13.1 years later than baseline (95% CI 3.15–23.1) ($P = 0.004$). Kaplan-Meier analysis showed similar differences in the age-related penetrance of *HNF4A* mutations affecting different sets of isoforms (Fig. 5). By the age of 55 years, 90% of individuals with exon 2–8 mutations had diabetes, compared with 78% of those with P2/exon 1D mutations and only 47% of those with mutations in exons 9 or 10 ($P < 0.00001$).

We also correlated age of diagnosis and the position of the mutation with respect to the functional domain structure (A/B to F) of the *HNF4A* gene (Fig. 1). Although individuals with mutations in the F domain did present later than mutations in other regions (40 vs. 26 years; $P = 0.029$), this domain is also encoded entirely by exons 9 and 10. It is therefore unclear whether the effects are related to the position of the mutation within the F domain or by compensation from the unaffected P1 isoforms. Clarification of the relative contribution of these factors requires consideration of the molecular mechanisms by which the F domain exerts its effects.

The F domain interacts physically with areas of the HNF-4α protein in the D and E domains (amino acids 128–366) that are essential for its transactivational activity (31,40–44). These interactions induce a conformational change that blocks the access of cofactors such as p300 to the COOH-terminal activation function (AF)-2 motif (44,45). If the effect on age of onset is due to the position of the exon 9 and 10 mutations within the F domain, a similar effect should be noted for those regions that interact with the F domain (i.e., the D and E domains; amino acids 128–366). However, patients with mutations in amino acids 128–366 were not diagnosed later than those with mutations in other regions of the gene (24 vs. 27 years, respectively; $P = 0.121$). No effect of mutation type on age of diagnosis was seen (24 years for missense [$n = 78$] and 20 years for prematurely terminating mutations [$n = 38$], respectively; $P = 0.139$). This suggests that the observed delay in age of diagnosis of diabetes is probably attributable to compensation from unaffected isoforms rather than the position of these mutations within the F domain.

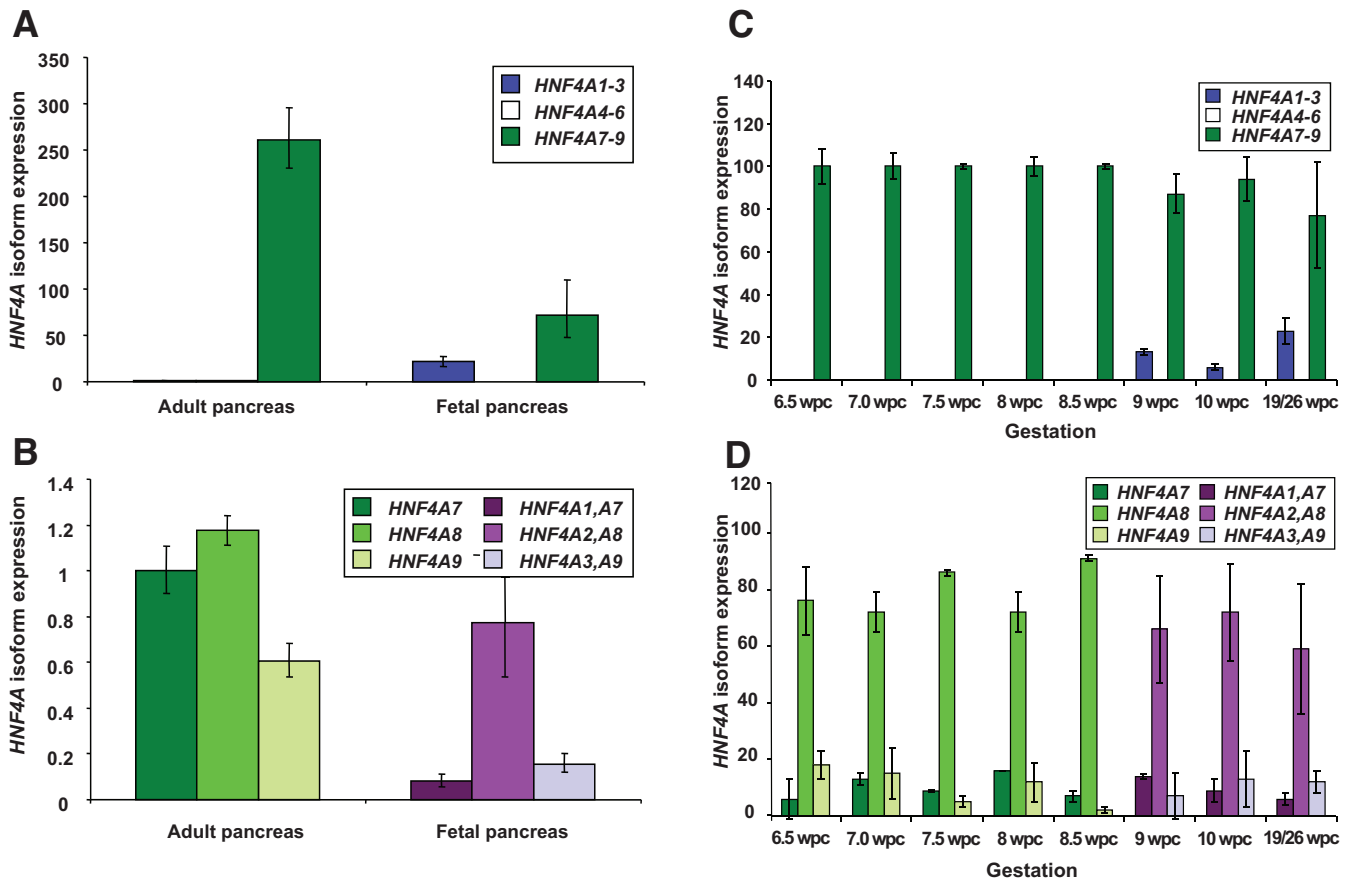

FIG. 3. Developmental expression profiles of *HNF4A* isoforms. The profiles given here refer to the developmental expression patterns of *HNF4A* isoforms in adult and fetal human pancreas. Tissues studied are shown on the *x*-axis, and the expression level of each isoform relative to the endogenous control gene β-2-microglobulin (*β2M*) are given on the *y*-axis. *A*: This graph refers to the expression profile of P1 and P2 *HNF4A* isoforms in adult and fetal tissues, as determined by probe set 1, relative to adult human pancreas. Transcripts *HNF4A4–HNF4A6* were not evident in any sample. *B*: The diagram shows the expression of *HNF4A* isoforms in adult and fetal pancreas as determined using probe set 2. In fetal pancreas, it was not possible to identify specific isoforms, since expression was noted from both P1 and P2 promoters. *C*: The expression profile of P1 and P2 *HNF4A* isoforms in a series of gestationally staged fetal pancreas as determined by probe set 1, expressed as percentages. Transcripts *HNF4A4–HNF4A6* were not evident in any sample. *D*: The expression of *HNF4A* isoforms in a series of gestationally staged fetal pancreas as determined using probe set 2, expressed as percentages. Where signal was obtained from both P1 and P2 isoforms, it was not possible to identify specific isoforms, since expression was noted from both P1 and P2 promoters. A color key to the identity of the isoforms is given within each section. wpc, weeks post-conception.

DISCUSSION

We have shown that *HNF4A* isoforms exhibit both temporal and spatial variation in expression, as previously reported for the *HNF1A* gene (29). Only the P2-derived *HNF4A* isoforms (*HNF4A7–HNF4A9*) were found in adult total pancreas RNA; the relative expression levels were 36, 42, and 22% respectively. However, in islets, *HNF4A9* represented only 7% of the transcripts, suggesting that the expression of this isoform may be greater in exocrine rather than endocrine pancreas. We found no evidence for the presence of *HNF4A* P1 isoforms *HNF4A4–HNF4A6* in any tissue. Their ubiquitous absence may indicate that they have a very restricted expression pattern or may not influence normal development or function.

HNF4A isoforms also demonstrate a developmental switch in relative expression. Transcripts derived from the P1 promoter were not observed in the adult pancreas but were present in fetal pancreas from 9 weeks until at least 19–26 weeks post-conception, comprising up to 23% of total *HNF4A* expression. We were unable to characterize the precise point at which P1 expression ceases, since human fetal tissues at later stages of development were not available. A developmental switch in the relative isoform expression was previously found for *HNF1A*, where the predominant fetal isoform is *HNF1A(A)*, but *HNF1A(B)* is more abundant in the adult pancreas (29). The difference in the relative expression of the *HNF1A* and *HNF4A* isoforms implies that the pancreatic transcription factor network may be subject to isoform-specific differences in regulation.

Alternate mRNA processing of the *HNF4A* gene appears to be acting as a moderator of *HNF4A*-MODY phenotype in our study. The age at which diabetes is diagnosed and the age-related penetrance are both influenced by the position of the mutation with respect to isoform structure. The location of the mutation will determine the number and derivation of isoforms that are affected. Mutations in exons 9 or 10 are predicted to affect all isoforms except *HNF4A3* and *HNF4A6*, which are expressed from the P1 promoter, and *HNF4A9*, which is expressed from the P2 promoter. Mutations in the P2 promoter or those in exon 1D are predicted to only affect P2-derived isoforms, leaving the isoforms under the control of the P1 promoter unaffected.

Our statistical analysis suggests that where some of the P1-derived isoforms escape mutation, the age of onset of diabetes may be delayed by up to 16 years. The effect is strongest for variants located in the terminal 2 exons of

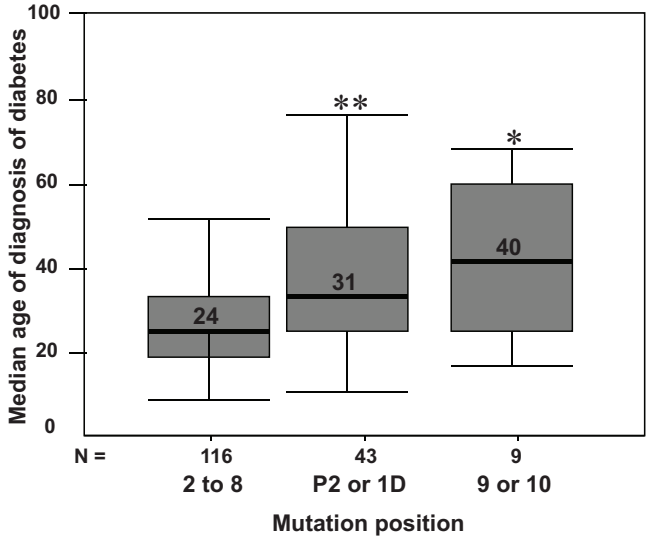

FIG. 4. The effect of mutation position relative to isoform structure on age of diagnosis of diabetes. The position of the mutation relative to the exon structure of the *HNF4A* gene is given on the *x*-axis. *N* refers to the number of subject in each category. The age of diagnosis of diabetes is given on the *y*-axis. Data are presented as median age of onset. Stars indicate statistically significant differences between categories as determined by the Mann-Whitney *U* test.

the *HNF4A* gene (median age at diagnosis 40 vs. 24 years for mutations in exons 2–8, which affect both P1 and P2 isoforms; age-related penetrance at 55 years, 43 vs. 90%), but is also apparent for mutations located in exon 1D and in the P2 promoter (median age at diagnosis 31 vs. 24 years for mutations in exons 2–8, which affect both P1 and P2 isoforms; age-related penetrance at 55 years, 78 vs. 90%). Given the small sample numbers, we cannot definitively rule out that other factors may be acting to influence age at diagnosis within our cohort, but our data still represent the largest *HNF4A*-MODY cohort available for study and represent a unique resource.

The reduction in effect size noted for mutations affecting the 5′ end of the *HNF4A* gene relative to variants affecting the 3′ terminus of the gene may reflect different modes of action for mutations located in these regions. The majority of the mutations affecting the 5′ end of the gene lie within the P2 promoter itself and are regulatory rather than coding. This raises the possibility that they may be hypomorphic and pathologically less severe. However, most of the mutations in this region lie within well-characterized binding sites for important transcription factors such as IPF-1 and HNF-1α and have been shown in in vitro transient transactivation studies and by band shift assays to profoundly affect both the binding of transcription factors and resultant *HNF4A* activity (27,32). The results relating to variants at the 5′ end of the gene may also be subject to some bias, since around half (27–50) of the P2 cohort are from seven families with the same C>G substitution at position −192 within the P2 promoter (5,46). The evidence for the pathogenicity of this mutation is equivocal. Although the −192 C>G mutation had only a minimal effect on *HNF4A* activity in in vitro studies, it did appear to disrupt the binding of an unidentified islet-specific protein to a putative regulatory motif (46). The median age at diagnosis in patients with this mutation was 45 years (5,46), which is later than for other mutations in the P2 promoter. This may be because −192 C>G, unlike the other mutations in the P2 promoter (−181 G>A and −146 T >C), does not lie within any known binding sites for pancreatic transcription factors, although it is located just outside the HNF-1α binding site.

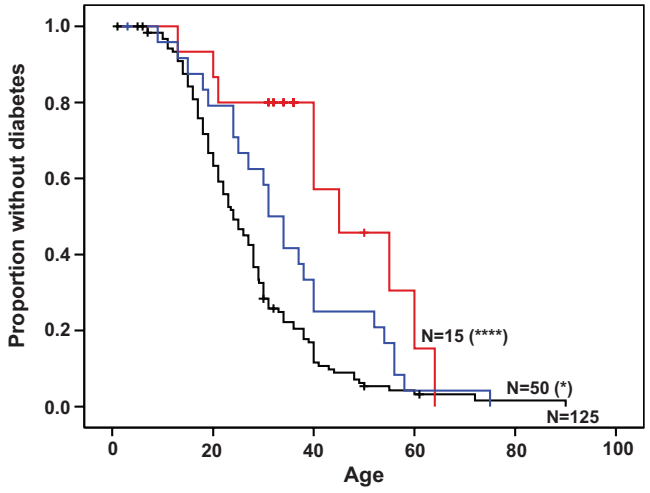

FIG. 5. Age-related penetrance of *HNF4A* mutations. Kaplan-Meier curve relating age at diagnosis on the *x*-axis to cumulative occurrence of diabetes on the *y*-axis. The black line refers to mutations in exons 2–8, the blue line for mutations in the P2 promoter or exon 1D, and the red line to mutations in exons 9 or 10. Each step down on the curve refers to a diagnosis of diabetes within the cohort. Crosses refer to the present age of a patient with no diabetes. In each case, the number of individuals in each category is given by *N*. Stars indicate statistically significant differences between categories as determined by χ^2analysis.

We have demonstrated that P1-derived transcripts are present in human fetal pancreas and that mutations that do not affect these isoforms are associated with a later median age of diagnosis and also with reduced age-related penetrance. These results indicate that the differences in the expression patterns of the *HNF4A* isoforms between mature and fetal pancreas may play a role in moderating the activity of the pancreatic transcription factor network during β-cell development and mature function. Although further functional studies to delineate the specific roles of *HNF4A* isoforms are required, our analysis suggests that the *HNF4A3*, *HNF4A6*, or *HNF4A9* isoforms may moderate the *HNF4A* diabetic phenotype. Because *HNF4A6* is not expressed in either adult or fetal pancreas, and we found predominantly exocrine expression of *HNF4A9* (Fig. 2*B*), we suggest that this effect may be attributable to the P1-derived isoform *HNF4A3* expressed during development. It is possible that mutations in exons 9 and 10 could in theory disturb the expression profile of *HNF4A* isoforms and lead to the upregulation of *HNF4A9*. However, there is no evidence to support this possibility, and in the absence of islet samples from patients with such mutations, the possibility of such an occurrence is difficult to determine.

We therefore conclude that the expression in human fetal pancreas of *HNF4A* isoforms derived from the P1 promoter may be important in the development of the fetal β-cell and may also influence the diabetic phenotype.

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REFERENCES

1. Ryffel GU: Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. *J Mol Endocrinol* 27:11–29, 2001
2. Weedon MN, Owen KR, Shields B, Hitman G, Walker M, McCarthy MI, Love-Gregory LD, Permutt MA, Hattersley AT, Frayling TM: Common variants of the hepatocyte nuclear factor-4alpha P2 promoter are associated with type 2 diabetes in the U.K. population. *Diabetes* 53:3002–3006, 2004
3. Bonnycastle LL, Willer CJ, Conneely KN, Jackson AU, Burrill CP, Watanabe RM, Chines PS, Narisu N, Scott LJ, Enloe ST, Swift AJ, Duren WL, Stringham HM, Erdos MR, Riebow NL, Buchanan TA, Valle TT, Tuomilehto J, Bergman R, Mohlke KL, Boehnke M, Collins FS: Common variants in maturity-onset diabetes of the young genes contribute to risk of type 2 diabetes in Finns. *Diabetes* 55:2534–2540, 2006
4. Silander K, Mohlke KL, Scott L, Peck EC, Hollstein P, Skol A, Jackson A, Deloukas P, Hunt S, Stavrides G, Chines PS, Erdos MR, Narisu N, Conneely KN, Li C, Fingerlin TE, Dhanjal SK, Valle TT, Bergman R, Tuomilehto J, Watanabe R, Boehnke M, Collins F: Genetic variation near the hepatocyte nuclear factor-4 alpha gene predicts susceptibility to type 2 diabetes. *Diabetes* 53:1141–1149, 2004
5. Raeder H, Bjørkhaug L, Johansson S, Mangseth K, Sagen J, Hunting A, Følling I, Johansen O, Bjorgaas M, Paus PN, Sovik O, Molven A, Njolstad PR: A hepatocyte nuclear factor-4 α gene (HNF4A) P2 promoter haplotype linked with late-onset diabetes: studies of HNF4A variants in the Norwegian MODY Registry. *Diabetes* 55:1899–1903, 2006
6. Valle T, Tuomilehto J, Bergman RN, Ghosh S, Hauser ER, Eriksson J, Nylund SJ, Kohtamaki K, Toivanen L, Vidgren G, Tuomilehto-Wolf E, Ehnholm C, Blaschak J, Langefeld CD, Watanabe RM, Magnuson V, Ally DS, Hagopian WA, Ross E, Buchanan TA, Collins F, Boehnke M: Mapping genes for NIDDM: design of the Finland-United States Investigation of NIDDM Genetics (FUSION) Study. *Diabetes Care* 21:949–958, 1998
7. Owen K, Hattersley AT: Maturity-onset diabetes of the young: from clinical description to molecular genetic characterization. *Best Pract Res Clin Endocrinol Metab* 15:309–323, 2001
8. Ellard S, Colclough K: Mutations in the genes encoding the transcription factors hepatocyte nuclear factor 1 alpha (HNF-1a) and 4 alpha (HNF-4a) in maturity-onset diabetes of the young. *Hum Mutat* 27:854–869, 2006
9. Pearson ER, Boj SF, Steele AM, Barrett T, Stals K, Shield JP, Ellard S, Ferrer J, Hattersley AT: Macrosomia and hyperinsulinaemic hypoglycaemia in patients with heterozygous mutations in the HNF4A gene. *PLoS Med* 4:e118, 2007
10. Byrne MM, Sturis J, Clement K, Vionnet N, Pueyo ME, Stoffel M, Takeda J, Passa P, Cohen D, Bell GI, et al.: Insulin secretory abnormalities in subjects with hyperglycemia due to glucokinase mutations. *J Clin Invest* 93:1120–1130, 1994
11. Gupta R, MZ Vatamaniuk, Lee C, Flaschen R, Fulmer J, Matschinsky FM, Duncan S, Kaestner K: The MODY1 gene HNF-4alpha regulates selected genes involved in insulin secretion. *J Clin Invest* 115:1006–1015, 2005
12. Cereghini S: Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J* 10:267–282, 1996
13. Sladek FM, Zhong W, Lai E, Darnell JE Jr: Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev* 4:2353–2365, 1990
14. Duncan SA, Manova K, Chen WS, Hoodless P, Weinstein DC, Bachvarova RF, Darnell JE Jr: Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proc Natl Acad Sci U S A* 91:7598–7602, 1994
15. Tavaviras S, Monaghan AP, Schultz G, Kelsey G: Characterization of the mouse HNF-4 gene and its expression during mouse embryogenesis. *Mechanisms of Development* 48:67–79, 1994
16. Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE Jr: Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev* 8:2466–2477, 1994
17. Li J, Ning G, Duncan S: Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes Dev* 14:464–474, 2000
18. Rhee J, Inoue Y, Yoon Y, Puigserver P, Fan M, Gonzales FJ, Spiegelman B: Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. *Proc Natl Acad Sci U S A* 100:4012–4017, 2003
19. Inoue Y, Yu AM, Yim SH, Ma X, Krausz KW, Inoue J, Xiang CC, Brownstein MJ, Eggertsen G, Bjorkhem I, Gonzales FJ: Regulation of bile acid biosynthesis by hepatocyte nuclear factor 4alpha. *J Lipid Res* 47:215–227, 2006
20. Chen W, Chiang JY: Regulation of human sterol 27-hydroxylase gene (CYP27A1) by bile acids and hepatocyte nuclear factor 4alpha (HNF4alpha). *Gene* 313:71–82, 2003
21. Boj SF, Parrizas M, Maestro MA, Ferrer J: A transcription factor regulatory circuit in differentiated pancreatic cells. *Proc Natl Acad Sci U S A* 98:14481–14486, 2001
22. Ferrer J: A genetic switch in pancreatic beta-cells: implications for differentiation and haploinsufficiency. *Diabetes* 51:2355–2362, 2002
23. Gupta RK, Kaestner K: HNF-4a: from MODY to late-onset type 2 diabetes. *Trends Mol Med* 10:521–524, 2004
24. Odom DT, Dowell R, Jacobson E, Nekludova L, Rolfe P, Danford T, Gifford D, Fraenkel E, Bell G, Young R: Core transcriptional regulatory circuitry in human hepatocytes. *Mol Syst Biol* 2:E1–E5, 2006
25. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M: Regulation of a transcription factor network required for differentiation and metabolism. *Science* 281:692–695, 1998
26. Hansen L, Urioste S, Petersen HV, Jensen JN, Eiberg H, Barbetti F, Serup P, Hansen T, Pedersen O: Missense mutations in the human insulin promoter factor-1 gene and their relation to maturity-onset diabetes of the young and late-onset type 2 diabetes mellitus in Caucasians. *J Clin Endocrinol Metab* 85:1323–1326, 2000
27. Thomas H, Jaschkowitz K, Bulman M, Frayling TM, Mitchell SMS, Roosen S, Lingott-Frieg A, Tack CJ, Ellard S, Ryffel GU, Hattersley AT: A distant upstream promoter of the HNF-4alpha gene connects the transcription factors involved in maturity-onset diabetes of the young. *Hum Mol Genet* 10:2089–2097, 2001
28. Harries LW: Alternate mRNA processing of the hepatocyte nuclear factor genes and its role in monogenic diabetes. *Expert Review of Endocrinolgy and Metabolism* 1:715–726, 2006
29. Harries L, Ellard S, Stride A, Morgan N, Hattersley A: Isomers of the TCF1 gene encoding hepatocyte nuclear factor-1 alpha show differential expression in the pancreas and define the relationship between mutation position and clinical phenotype in monogenic diabetes. *Hum Mol Genet* 15:2216–2224, 2006
30. Drewes T, Senkel S, Holewa B, Ryffel GU: Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. *Mol Cell Biol* 16:925–931, 1996
31. Sladek FM, Ruse MD Jr, Nepomuceno L, Huany SM, Stallcup MR: Modulation of transcriptional activation and coactivator interaction by a splicing variation n the F domain of nuclea receptor hepatocyte nuclear factor 4a1. *Mol Cell Biol* 19:6509–6522, 1999
32. Hansen SK, Parrizas M, Jensen ML, Pruhova S, Ek J, Boj SF, Johansen A, Maestro MA, Rivera F, Eiberg H, Andel M, Lebl J, Pedersen O, Ferrer J, Hansen T: Genetic evidence that HNF-1alpha-dependent transcriptional control of HNF-4alpha is essential for human pancreatic beta cell function. *J Clin Invest* 110:827–833, 2002
33. Eeckhoute J, Moerman E, Bouckenooghe T, Lukoviak B, Pattou F, Formstecher P, Kerr-Conte J, Vandewalle B, Laine B: Hepatocyte nuclear factor 4 alpha isoforms originated from the P1 promoter are expressed in human pancreatic beta-cells and exhibit stronger transcriptional potentials than P2 promoter-driven isoforms. *Endocrinology* 144:1686–1694, 2003
34. Hata S, Tsukamoto T, Osumi T: A novel isoform of rat hepatocyte nuclear factor 4 (HNF-4). *Biochim Biophys Acta* 1131:211–213, 1992
35. Tanaka T, Jiang S, Hotta H, Takano K, Iwanari H, Sumi K, Daigo K, Ohashi R, Sugai M, Ikegame C, Umezu H, Hirayama Y, Midorikawa Y, Hippo Y, Watanabe A, Uchiyama Y, Hasegawa G, Reid P, Aburatani H, Hamakubo T, Sakai J, Naito M, Kodama T: Dysregulated expression of P1 and P2 promoter-driven hepatocyte nuclear factor-4alpha in the pathogenesis of human cancer. *J Pathol* 208:662–672, 2006
36. Ostrer H, Wilson DI, Hanley NA: Human embryo and early fetus research. *Clin Genet* 70:98–107, 2006
37. Piper K, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, Hanley NA: Beta cell differentiation during early human pancreas development. *J Endocrinol* 181:11–23, 2004
38. Applied-Biosystems: Relative quantitation of gene expression. *User bulletin #2*:11–15, 2001
39. Ihara A, Yamagata K, Nammo T, Miura N, Yuan M, Tanaka T, Sladek FM,

Matsuzawa Y, Miyagawa J, Shimomura I: Functional characterization of the HNF4alpha isoform (HNF4alpha8) expressed in pancreatic beta-cells. *Biochem Biophys Res Commun* 329:984–990, 2005
40. Wang JC, Stafford JM, Granner DK: SRC-1 and GRIP1 coactivate transcription with hepatocyte nuclear factor 4. *J Biol Chem* 273:30847–30850, 1998
41. Iyemere VP, Davies NH, Brownlee GG: The activation function 2 domain of hepatic nuclear factor 4 is regulated by a short C-terminal proline-rich repressor domain. *Nucleic Acids Res* 26:2098–2104, 1998
42. Ruse MD Jr, Privalsky ML, Sladek FM: Competitive cofactor recruitment by orphan receptor hepatocyte nuclear factor 4alpha1: modulation by the F domain. *Mol Cell Biol* 22:1626–1638, 2002
43. Chou WC, Prokova V, Shiraishi K, Valcourt U, Moustakas A, Hadzopoulou-Cladaras M, Zannis VI, Kardassis D: Mechanism of a transcriptional cross talk between transforming growth factor-beta-regulated Smad3 and Smad4 proteins and orphan nuclear receptor hepatocyte nuclear factor-4. *Mol Biol Cell* 14:1279–1294, 2003
44. Eeckhoute J, Formstecher P, Laine B: Maturity-onset diabetes of the young type 1 (MODY1) associated mutations R154X and E276Q in hepatocyte nuclear factor 4 alpha (HNF4A) gene impair recruitment of p300, a key trancriptional activator. *Mol Endocrinol* 15:1200–1210, 2006
45. Hadzopoulou-Cladaras M, Kistanova E, Evagelopoulou C, Zeng S, Cladaras C, Ladias JAA: Functional domains of the nuclear receptor hepatocyte nuclear factor 4. *J Biol Chem* 272:539–550, 1997
46. Ek J, Hansen S, Lajer M, Nicot C, Boesgaard T, Pruhova S, Johansen A, Albrechtsen A, Yderstraede K, Lauenborg J, Parrizas M, Boj SF, Jorgensen T, Borch-Johnsen K, Damm P, Ferrer J, Lebl J, Pedersen O, Hansen T: A Novel −192c/g mutation in the proximal P2 promoter of the hepatocyte nuclear factor-4 α gene (HNF4A) associates with late-onset diabetes. *Diabetes* 55:1869–1873, 2006