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Functional analyses of the mutation nt-128 T→G in the hepatocyte nuclear factor-1 α promoter region in Chinese diabetes pedigrees

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Abstract

Aims—Hepatocyte nuclear factor-1 α (HNF-1 α) regulates the expression of genes encoding proteins involved in glucose metabolism and insulin secretion. Mutations in the HNF-1 α gene cause maturity-onset diabetes of the young Type 3. However, the mechanism leading to this disease has not been completely ascertained. Previously, we found a novel mutation in the regulatory element of the human HNF-1 α gene in two Chinese diabetes pedigrees. The nucleotide at position -128 T was substituted by G (nt-128 T→G). In this study, we analysed the functional defect of nt-128 T→G in HNF-1 α transcription activity.

Methods—Luciferase reporter gene assays were carried out to examine the functional characteristics of this mutant. Electrophoretic mobility shift assays and chromatin immunoprecipitation were performed to confirm the binding of nuclear proteins to oligonucleotides.

Results—The variant construct (nt-128 T→G) had a 1.65-fold increase in promoter activity compared with that of the wild-type construct in HepG2 cells and a 1.33-fold increase in MIN6 cells, respectively. The variant resided at a FOXA/ HNF-3 binding site identified by a series of competitive electrophoretic mobility shift assays and antibody supershift analyses. The assays showed a differential binding affinity in the wild-type and the nt-128 T→G mutant fragments by FOXA/ HNF-3. Chromatin immunoprecipitation indicated that FOXA/ HNF-3 bound to this region *in vivo*. One nucleotide substitution in the FOXA/ HNF-3 site in the human HNF-1 α regulatory element caused an increase of HNF-1 α transcriptional activity.

Conclusions—Our data suggested that this substitution in the promoter region affects DNA–protein interaction and HNF-1 α gene transcription. The mutant may contribute to the development of diabetes in these two nt-128 T→G pedigrees of Chinese.

Introduction

Maturity-onset diabetes of the young (MODY) is a genetically heterogeneous form of diabetes mellitus characterized by early onset autosomal dominant inheritance and a primary defect in insulin secretion. Molecular genetic analysis has shown that MODY is caused by mutations in genes encoding the glycolytic enzyme glucokinase (GCK) and hepatocyte nuclear factor (HNF) 4 α , HNF-1 α , insulin promoter factor 1 (IPF-1), HNF-1 β and neurogenic differentiation factor 1 (NeuroD1) [1–6]. In hepatocytes and pancreatic β -cells, these transcription factors are involved in a regulatory network that plays a critical role in maintenance and regulation of expression of genes encoding proteins implicated in glucose metabolism and insulin secretion [7–11]. The knowledge of the complicated processes of cross-regulation and auto-regulatory loops involved in such networks has been expanding.

Mutations in the HNF-1 α gene are the common cause of MODY in most populations [12]. HNF-1 α is expressed in various tissues, including liver, kidney and pancreas [13]. This protein functions as a regulator binding to the promoters of a variety of genes, including several liver-specific genes and genes involved in glucose transport and glucose metabolism [14–17]. There is a cross-regulatory loop between HNF-1 α and HNF-4 α in pancreatic cells [18]. HNF-4 α regulates the expression of the HNF-1 α gene in hepatocytes, pancreatic cells and embryonic endoderm, while HNF-1 α activates transcription of the HNF4 α gene through the P2 promoter in the pancreas [11]. Also, HNF-1 α is involved in glucose homeostasis through modulation of HNF transcription factor networks [19]. To date, more than two hundred mutations in the HNF-1 α gene have been identified in MODY, including the coding and non-coding domains. Mutations in the coding region could affect dimerization, DNA binding and transactivation domains of the HNF-1 α protein, leading to a loss of function [20,21]. However, mutations causing MODY in the HNF-1 α gene promoter have also been identified and characterized, and dual effects were observed [22–25]. For example, the nt-283A→C and nt-181 / 180GC→AA substitutions disrupted the binding to HNF-4 α and AP-1, respectively, contributing to the reduced transcriptional activity of HNF-1 α [22,23] whereas an increase in the HNF-1 α promoter activity was reported in the nt-124G→C mutation and -129 / -130insTTGGGG mutation detected from subjects with MODY, but the specific regions bound by potential factor(s) have not been identified [24,25].

In our previous studies, we screened for the HNF-1 α gene in the coding and putative promoter regions in probands of early-onset and / or multiplex diabetes pedigrees and we identified twenty variants in Chinese subjects [26,27]. Of these, nt-128 T→G in the HNF-1 α gene promoter region was found to be a novel mutation. More interestingly, this element is close to nt-124G→C and -129 / -130insTTGGGG mutations and may provide insights into the mechanism for relevant proteins binding to this specific region to influence the HNF-1 α gene transcription. The mutation co-segregated with diabetes in these two unrelated diabetes pedigrees was not observed in 80 healthy control subjects [26,27]. One of the probands with the nt-128 T→G mutant was a 42-year-old man who had been diagnosed with diabetes mellitus at the age of 39 years. He was treated with oral hypoglycaemic agents. The proband's father and one of his sibling, both with diabetes, were found to have the heterozygous mutation. Another proband was a 48-year-old man in a three-generation diabetic pedigree. Pedigree analysis revealed that it was consistent with autosomal dominant inheritance. The man's father, also carrying the nt-128 T→G mutant, was a patient with

diabetes. All the mutation carriers including proband's three siblings and one nephew were with diabetes, while the non-carriers were without diabetes.

In the present work, we explore the functional impact of this mutated promoter. Our results showed that the nt-128 T→G mutant increased the promoter activity. This mutation is located at a FOXA (previously called HNF-3) binding site and the nucleotide substitution affected the binding of FOXA / HNF-3 to its binding site, resulting in altered promoter activity.

Methods

Plasmid constructs

A 300-bp fragment of the human HNF-1 α gene promoter (corresponding to nucleotides -340 to -41 relative to the translation initiator codon) was generated by polymerase chain reaction (PCR) using human genomic DNA as the template. The PCR product was subcloned into the luciferase reporter vector pGL3-basic (Promega, Madison, WI, USA). The construct with an nt-128 T to G mutation was generated by site-directed mutagenesis according to the protocol as described by Kunkel *et al.* [28]. The oligonucleotide designed for site-directed mutagenesis was 5'-CCCCAACACCCCACTAGC-3'. The constructs were verified by DNA sequencing. FOXA1 / HNF-3 α cDNA subcloned in pBluescriptR was purchased from the Mammalian Gene Collection (NIH Institutes, Bethesda, MD, USA) and FOXA2 / HNF-3 β cDNA subcloned in pCR3.1 was a kind gift from Dr G. I. Bell (The University of Chicago, IL, USA). The FOXA1 / HNF-3 α and FOXA2 / HNF-3 β cDNAs were subcloned into the expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), respectively.

Cell culture, transient transfections and luciferase assay

The human hepatoma cell line HepG2 was grown as monolayer in minimum essential medium supplemented with 10% fetal bovine serum, 100 units / ml penicillin and 100 μ g / ml streptomycin (Invitrogen). The pancreatic β -cell line MIN6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 100 units / ml penicillin and 100 μ g / ml streptomycin. Cells were incubated at 37 °C in humidified air containing 5% carbon dioxide (CO₂). Transfection was performed with Lipofectamine Plus and Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. In brief, cells were grown at 70–80% confluence on the day of transfection. Either a wild-type or mutant HNF-1 α promoter-pGL3 DNA construct was transfected into HepG2 and MIN6 cells. Forty-eight hours after transfection, cells were harvested and luciferases were assessed using the Dual Luciferase Assay System (TD-20/20; Promega) according to manufacturers' protocols. The results were derived from three independent experiments performed in triplicate.

A computer-aided analysis of the HNF-1 α gene promoter

The HNF-1 α promoter region (from nt-154 to -105) was analysed to search for potential transcription factor binding sites by the Transcription Element Search System (TESS) (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>).

Electrophoretic mobility shift assays

Double-stranded oligonucleotides used as probes and competitors in this study were synthesized (Table 1) [29]. The probe of HNF-1 α contains the region between nt-139 and -114, encompassing nt-128. The probes of TTR and IGFBP-1 represent promoters of transthyretin and insulin-like growth factor-binding protein 1 genes containing the consensus FOXA / HNF-3 binding sites, respectively. Nuclear extracts from HepG2 cells

were prepared using the method of Dig-nam *et al.* [30]. Protein concentration was determined by using the bicinchoninic acid (BCA) assay and the nuclear extracts were stored at -80°C . Oligonucleotides were labelled by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (PerkinElmer Lifesciences, Boston, MA, USA) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). Probes were prepared by annealing oligonucleotides and purified by QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA, USA). For the electrophoretic mobility shift assay, nuclear extracts from HepG2 cells were pre-incubated in 20 μl reaction buffer containing 10 mM Tris-hydrochloride (HCl) (pH 7.5), 50 mM sodium chloride (NaCl), 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol and 2 μg of poly(deoxyinosinic-deoxycytidylic) [poly(dI-dC)]. After 5 min at room temperature (25°C), 20 fmol of labelled probes were added and incubation was continued for another 20 min. Protein–DNA complexes were separated from the free probe by electrophoresis on a 5% native polyacrylamide gel in $0.5 \times$ tris-borate-EDTA (TBE) buffer. After electrophoresis, the gel was dried and exposed to X-ray film. For the competition binding reactions, the unlabelled competitor in molar excesses of the labelled probe was included in the reaction. Quantification of the relative affinity of FOXA / HNF-3 binding to the HNF-1 α wild-type and mutant oligonucleotides was performed by analysing electrophoretic mobility shift assay using the Scion Image 4.0.3.2 program (Scion Corporation, Frederick, MD, USA). Supershift analysis was performed with a specific antibody against FOXA2 / HNF-3 β (sc-6554X; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody was added to the nuclear extracts for 20 min prior to the addition of the radio-labelled probe.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed by using a ChIP-IT kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, HepG2 and MIN6 cells were incubated for 10 min with 1% formaldehyde. After stopping the reaction with 0.1 M glycine, the chromatin DNA was sonicated into fragments of 200–1000 bp in length. The chromatin DNA was pre-cleared by adding protein G beads with salmon sperm DNA for 2 h at 4°C . After pre-clearing, 10 μl of sample was stored as input fraction at -20°C . The pre-cleared chromatin was incubated with 5 μg of antibody against FOXA2 / HNF-3 β (sc-6554X; Santa Cruz Biotechnology) and 2 μg of negative control immunoglobulin G (IgG) provided by this kit overnight at 4°C . The immunoprecipitated chromatin was washed and eluted from the protein G beads. The elution and input DNA was reverse cross-linked and removed RNA by incubating for 5 h at 65°C in 200 mM sodium chloride and 10 μg of RNase A. The recovered DNA was treated with proteinase K and purified by Qiagen columns (Qiagen). The purified DNA was amplified by PCR using the following primer pairs: HNF-1 α forward 5'-AGA-ATTTCCCCAGCTCCAAT-3' and HNF-1 α reverse 5'-CGGCAGACACAAACCAAACT-3'. PCR conditions were 95°C for 3 min, then 36 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s.

Western blot analysis

Western blot analysis was performed on whole cell lysates from HepG2 and MIN6 cells. Cells were lysed in ice-cold buffer containing 50 mmol / l HEPES (pH 7.6), 150 mmol / l sodium chloride, 20 mmol / l sodium pyrophosphate, 20 mmol / l β -glycerophosphate, 10 mmol / l sodium fluoride, 2 mmol / l sodium orthovanadate, 2 mmol / l EDTA, 10% octylpheno-xypolyethoxyethano (IGEPAL), 10% glycerol, 2 mmol / l phenylmethylsulphonyl fluoride (PMSF), 1 mmol / l magnesium chloride, 1 mmol / l calcium chloride, 10 μg / ml leupeptin and 10 μg / ml aprotinin. Cell lysates were clarified by centrifugation and the supernatants were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The expression levels of FOXA1 / HNF-3 α and FOXA2 / HNF-3 β in cell lysates were determined by western blotting with the

antibodies against FOXA1 / HNF-3 α (sc-9186; Santa Cruz Biotechnology) and FOXA2 / HNF-3 β (sc-6554; Santa Cruz Biotechnology).

Data analysis

Data were expressed as means $\pm$ sd. Statistical analysis with Student's *t*-test was used to determine significant differences in the wild-type and variant groups.

Results

The nt-128 T $\rightarrow$ G variation affects the HNF-1 α promoter activity in HepG2 cells and MIN6 cells

To analyse the functional effects of the nt-128 T $\rightarrow$ G variant on transcriptional activity *in vitro*, the variation was introduced into the wild-type reporter gene construct containing the promoter sequence of the HNF-1 α gene. Wild-type or nt-128 T $\rightarrow$ G reporter constructs were transiently transfected into HepG2 and MIN6 cells, together with the pRL-TK vector as an internal control that constitutively expresses the *Renilla* luciferase. Firefly luciferase activity, representing the promoter activation, was normalized to *Renilla* luciferase activity. As shown in Fig. 1, the nt-128 T $\rightarrow$ G variant construct showed a 1.65-fold increase in promoter activity compared with the wild-type construct in HepG2 cells and a 1.33-fold increase in MIN6 cells, respectively.

Identification of a potential FOXA / HNF-3 binding site in the human HNF-1 α promoter region

In view of the present finding that the nt-128 T $\rightarrow$ G variant affects the promoter activity, we suspected that the variation might contain functional transcriptional factor binding sites. Alignment of the human, rat and mouse sequences using information in public databases (<http://www.ncbi.nlm.nih.gov/gene?term=HNF1A>) showed that this element of the HNF-1 α promoter is highly homologous among these species. The nucleotide T at position -128 is conserved in the sequences of human, rat and mouse HNF-1 α genes. Transcription Element Search System analyses of the nt -154 to -105 region of the HNF1 α promoter revealed a potential binding site for FOX-A / HNF-3 (Fig. 2a).

Electrophoretic mobility-shift assays were performed to assess whether FOXA / HNF-3 was able to bind to the potential site in the HNF-1 α gene promoter. As shown in Fig. 2b, the wild-type HNF-1 α promoter-derived oligonucleotide formed a prominent complex with the nuclear extracts from HepG2 cells. The DNA-protein complex had mobility similar to those of complexes with the TTR or IGFBP-1 promoter-derived oligonucleotides as positive controls [29]. TTR and IGFBP-1 oligonucleotides containing the consensus site for FOX-A / HNF-3 have a similar core sequence to that of the HNF-1 α potential FOXA / HNF-3 binding site, but a distinct flanking sequence. Next, the cross-competition experiment was performed to see whether these three DNA-protein complexes competed with each other. The results showed that the complex of the HNF-1 α probe could be efficiently competed out with either unlabelled TTR or IGFBP-1 oligonucleotide, while the unlabelled HNF-1 α oligonucleotide partially competed for the formation of the complex of the TTR or IGFBP-1 probe, indicating that the HNF-1 α oligonucleotide binds to FOX-A / HNF-3, but the binding affinity of the HNF-1 α oligonucleotide was less than that of the TTR or IGFBP-1 oligonucleotide (Fig. 2c). To determine whether the band represented FOXA / HNF-3 bound to the HNF-1 α probe, anti-FOXA / HNF-3 antibody was used in electrophoretic mobility shift assay. As shown in Fig. 2d, the specific antibody against FOXA / HNF-3 prevented the formation of the DNA-protein complex, and the antibody also inhibited the formation of the TTR-protein complex, leading to the formation of a slow migrating band (lanes 2 and 5), whereas non-specific serum had no effect (lanes 3 and 6). Thus, these gel retardation assays

supported our hypothesis that this sequence was bound by FOXA / HNF-3. Like the wild-type HNF-1 α probe, the mutant HNF-1 α probe also bound to HepG2 nuclear proteins and the binding could be blocked by addition of excess unlabelled mutant HNF-1 α oligonucleotide (Fig. 2e). The antibody against FOXA / HNF-3 prevented the complex formation of nuclear extracts with the mutant HNF-1 α probe, resulting in a weak supershift band (Fig. 2d, lane 8). To investigate the impact of the nt-128 T→G mutant on DNA–protein formation, both the unlabelled wild-type and mutant oligonucleotides were used as competitors. The DNA–protein complex was competed by adding increasing amounts of the unlabelled oligonucleotides, but the unlabelled mutant oligo-nucleotide competed for this binding to a lesser extent compared with the unlabelled wild-type oligonucleotide (Fig. 2f). Quantification of the relative affinity of the DNA–protein complex showed that, in the presence of 20-, 40- and 80-fold excess unlabelled wild-type oligonucleotide, the DNA–protein complex intensity relative to the uncompetited complex was ~38, 23 and 8%, respectively. However, in the presence of the same amount of excess unlabelled mutated oligonucleotide, the DNA–protein complex intensity relative to the uncompetited complex was ~81, 40 and 28%, respectively ($P < 0.05$) (Fig. 2g), indicating that the FOXA / HNF3 bound to the mutated oligonucleotide with reduced affinity in comparison with the wild-type sequence. To test whether FOXA / HNF3 interacts directly with this region *in vivo*, we performed chromatin immunoprecipitation assays. Anti-FOXA / HNF3 antibody immunoprecipitated protein–DNA complexes were recovered and the purified DNA was used as a template for PCR using primers corresponding to the potential FOXA / HNF3 binding site. A 153-bp PCR product was amplified from the DNA fragment immunoprecipitated by anti-FOXA antibody (Fig. 3, lane 3). The result indicated that FOXA bound to the potential FOXA / HNF-3 site in the HNF-1 α gene promoter encompassing nt-128 *in vivo*.

The nt-128 T→G mutation alters the effect of FOXA / HNF-3 on reporter gene expression

Previous studies revealed that FOXA1 / HNF-3 α and FOXA2 / HNF-3 β were expressed in liver and pancreas and regulated hepatic and / or pancreatic gene expression related to glucose metabolism such as HNF-1 α , HNF-4 α and IPF-1 [8,31–34]. Our western blot assay also showed that FOXA1 / HNF-3 α and FOXA2 / HNF-3 β expressed in HepG2 and MIN6 cells (Fig. 4). As the nt-128 T→G mutation located in the FOXA / HNF-3 binding site altered the HNF-1 α promoter activity, we further investigated which isoform of FOXA / HNF-3 could be involved in the regulation of the HNF-1 α gene transcription in the wild-type and mutant manners. The wild-type HNF-1 α promoter reporter construct and expression vector encoding either FOXA1 / HNF-3 α or FOXA2 / HNF-3 β were co-transfected into HepG2 cells. As shown in Fig. 5a, FOXA2 / HNF-3 β increased approximately 1.7-fold of the wild-type reporter activity, whereas FOXA1 / HNF-3 α repressed the wild-type promoter activity compared with the control group transfected by the wild-type HNF-1 α promoter reporter construct and empty expression vector (pcDNA3.1). To further examine the effect of FOXA / HNF-3 on the nt-128 T→G mutation, the wild-type or mutant reporter construct was co-transfected with the indicated amount of expression vectors encoding FOXA1 / HNF-3 α and FOXA2 / HNF-3 β into HepG2 cells. As Fig. 5b illustrated, the inhibitory effect of FOXA1 / HNF-3 α on the reporter gene expression was significantly reduced and the ability of FOXA2 / HNF-3 β to induce the expression of the reporter gene was slightly decreased in the mutated nt-128 T→G reporter construct in comparison with the wild-type construct. These results indicated that one nucleotide substitution at the FOXA / HNF-3 binding domain within the human HNF-1 α regulatory region resulted in alternation of HNF-1 α gene transcription levels.

Discussion

HNF-1 α regulates a number of liver and β -cell specific genes; for example albumin, glucose transporter-2, L-type pyruvate kinase, aldolase B, insulin and IPF-1 [14–17]. As an essential component of the regulatory network, this protein works cooperatively with other transcription factors such as HNF-4 α , HNF-6 in liver and in pancreatic islets [19]. Besides HNF-1 α dimerization, DNA binding and transcriptional activation potential, accurate HNF-1 α expression is also required for normal function of liver and pancreatic islets. Both reduced and extreme expressions of HNF-1 α are all associated with diabetes [12,24,25,35].

Several variants have been previously identified in the HNF-1 α promoter that both positively and negatively affect transcriptional activity. It was reported that the nt-283A $\rightarrow$ C, nt-218T $\rightarrow$ C and nt-181 / 180GC $\rightarrow$ AA mutations in the promoter region diminished the binding affinity for HNF-4 α , NF-Y and AP-1, and the transcriptional activities of these mutant constructs were decreased by 30, 70 and 20% compared with the wild-type construct, respectively [22,23]. In contrast, our current study has shown that introduction of the nt-128T $\rightarrow$ G nucleotide substitution increased the HNF-1 α transcriptional activity in HepG2 and MIN6 cells. In accordance with this observation, two mutations immediately surrounding the nt-128T $\rightarrow$ G mutation, namely nt-124G $\rightarrow$ C and nt-129 / -130insTTGGGG found in patients with MODY, were also associated with an increase in the promoter activity [24,25]. On the one hand, the functional studies indicated that the nt-124G $\rightarrow$ C mutant promoter construct had 42–75% more activity than that of the wild-type construct. The transcriptional activity of the nt-129 / -130insTTGGGG mutant was 1.6–2.0 times higher than that of the wild-type. On the other hand, HNF-1 α mutations in the coding regions potentially causing MODY have shown a loss of function. For instance, the mutant L12H disrupts the ability to form an HNF-1 α dimer, Y122C has a reduced protein stability coupled with a decrease in DNA binding and P379fsdelCT is defective in *trans*-activation potential [36–38]. The normal function and expression of HNF-1 α are essential for glucose homeostasis and normal β -cell function. Both haploinsufficiency and over expression potentially cause abnormal glucose tolerance. The nt-283A $\rightarrow$ C, L12H and P379fsdelCT mutations mentioned above were identified in patients with a clinical diagnosis of MODY [20,35]. These mutations cause diabetes through haploinsufficiency [22,38]. However, increased HNF-1 α could also be deleterious for glucose homeostasis. The nt-124G $\rightarrow$ C and -129 / -130insTTGGGG were diabetes-associated mutations. These two mutations led to increased promoter activity of the HNF-1 α gene and thus might contribute towards the higher than normal levels of HNF-1 α expression, which subsequently altered the transcription of its target gene expression involved in glucose metabolism. Furthermore, the transgenic mice with supraphysiological levels of HNF-1 α developed diabetes by a severe reduction of cellular proliferation, increased apoptosis and β -cell depletion [39]. Our study suggests that the nt-128T $\rightarrow$ G mutation leads to an increase of the promoter activity in HepG2 and in MIN6 cells, resulting in a baneful influence on glucose homeostasis. As the transcription regulation of the HNF-1 α gene causing MODY has not been completely understood, the mechanisms that increased levels of HNF-1 α protein may result in diabetes should be further investigated.

By alignment of the nucleotide sequences and prediction of transcription factors, we found the sequence encompassing the nt-128 within a FOXA / HNF-3 binding site and this FOXA / HNF-3 site is well conserved in human, mouse and rat. FOXA / HNF-3 is a member of the forkhead gene family. The FOXA subfamily of forkhead transcription factors consists of three distinct transcription factors, FOXA1, FOXA2 and FOXA3 [40]. The FOXA / HNF3-binding site consensus sequence is VAWTRTTTKRYTY (where V = A, C or G; W = A or T; K = G or T; Y = C or T; R = G or A) [41]. In this study, electrophoretic mobility shift assays demonstrated that the FOXA / HNF-3 bound to the sequence

encompassing nt-128, but the wild-type and nt-128 T→G mutant oligonucleotides exhibited a differential affinity for FOXA / HNF-3. Because the forkhead family possesses a conserved 100-amino-acid winged helix DNA-binding domain and comprises more than 100 members in humans, classified from FOXA to FOXQ on the basis of sequence similarity [40,42,43], other forkhead members such as FOXO might also bind to this region. Therefore, through the antibody supershift analysis and chromatin immunoprecipitation, we confirmed that the FOXA / HNF-3 bound to the region of the HNF-1 α promoter harbouring the -128 nucleotide *in vitro* and *in vivo*. Previous gene expression studies showed that FOXA1 / HNF-3 α and FOXA2 / HNF-3 β were critical regulators of HNF-1 α , HNF-4 α , and IPF-1 [8,34]. FOXA1 / HNF-3 α and FOXA2 / HNF-3 β are encoded by two unlinked genes. These two proteins share over 90% of amino acid identities in their DNA binding domains and bind to the same DNA recognition sequences as monomers with different affinities [40]. By co-transfecting FOXA1 / HNF-3 α or FOXA2 / HNF-3 β expression plasmids and the HNF-1 α promoter reporter constructs, we found that FOXA1 / HNF-3 α negatively, but FOXA2 / HNF-3 β positively regulated the HNF-1 α gene transcription. The result was supported by the study of embryonic bodies with inactivated FOXA1 / HNF3 α or FOXA2 / HNF3 β alleles. It revealed that the expression of HNF1 α was regulated by FOXA1 / HNF3 α and FOXA2 / HNF3 β . FOXA2 / HNF3 β acts as an activator, whereas FOXA1 / HNF3 α was a repressor [8]. In the present study, we showed that FOXA2 / HNF-3 β increased and FOXA1 / HNF-3 α repressed the wild-type promoter activity. The nt-128 T→G mutation reduced the repressive effect of FOXA1 / HNF3 α on the HNF1 α promoter activation significantly and had a slight influence on the active effect of FOXA2 / HNF3 β on the HNF1 α transcription. Taken together, we deduced that the repressive effect of FOXA1 / HNF3 α on the HNF1 α promoter activation in the case of the nt-128 T→G mutation might be a result, in part, of the alteration in the binding ability of FOXA / HNF3 for the FOXA / HNF3 site. It was interesting that the nt-128 T→G and two other reported mutations (nt-124G→C and -129 / -130insTTGGGG) all lay in the FOXA / HNF-3 binding site identified in the present study. Moreover, this FOXA / HNF-3 binding site overlapped the A-site, which was reported previously by Kuo *et al.* [10]. The transcription factor binding to the A-site has not been known and deletion of the A-site was associated with the increased activity. On the one hand, it was speculated that this region is important for the promoter activity of the HNF-1 α gene, and that these variations of HNF-1 α would lead to the decreasing effects of FOXA / HNF-3 and thus result in the increased expression of HNF-1 α . On the other hand, an additional FOXA / HNF-3 binding site in the murine HNF-1 α promoter identified by Kuo *et al.* [10] is presented in the corresponding region of the reporter gene constructs containing the promoter sequence of the human HNF-1 α gene used in this study. This could be a potential influence of the co-transfection experiments, but it might have less effect on the comparison of the regulation of the wild-type and mutant reporter gene constructs by FOXA / HNF-3, as both wild-type and mutant promoter constructs encompass the additional FOXA / HNF-3 binding site. As other proteins such as C / EBP α might also bind to this region, we do not exclude the possibility that interactions between FOXA / HNF-3 and other associated proteins could play a role in mediating the effects on the regulation of HNF-1 α expression.

In conclusion, we have shown evidence that nt-128 T→G in the HNF-1 α gene promoter had a functional effect *in vitro*. This substitution in the promoter region affected the DNA–protein interaction and the promoter activity of the HNF-1 α gene. The mutant may contribute to the development of diabetes in these two nt-128 T→G pedigrees of Chinese.

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FIGURE 1.

Effect of the nt-128 T→G HNF-1 α promoter variant on transcriptional activity. HepG2 cells (a) and MIN6 cells (b) were transiently transfected with the wild-type or nt-128 T→G reporter constructs. Forty-eight hours after transfection, cells were harvested and luciferase activity was determined. Wild-type and nt-128 T→G constructs were indicated by filled and open boxes, respectively. Relative luciferase activity was expressed as means $\pm$ sd in fold of activity obtained with the wild-type construct. All data were presented as means of three individual transfection experiments ($n = 9$, * $P < 0.05$).

FIGURE 2.

Electrophoretic mobility shift assay. (a) Comparison of the sequences of the HNF-1α gene promoter regions of the human, rat and mouse where nt-128 T→G is located. The number refers to the translation initiator codon. The potential FOXA / HNF-3 binding site is boxed and the T→G substitution at nt-128 is indicated by an arrow. (b) The labelled wild-type HNF-1α oligonucleotide encompassing nt-128, TTR and IGFBP-1 oligonucleotides containing the consensus site for FOXA / HNF-3 were incubated in the absence or presence of nuclear extracts from HepG2 cells. The specific DNA–protein complexes are indicated by the arrow. (c) For the cross-competition, the unlabelled competitors in 200-fold molar excesses of the labelled probe were included in the reaction for 5 min prior to the addition of the labelled probes. The specific DNA–protein complexes are indicated by the arrow. (d) Nuclear extracts from HepG2 cells were incubated with the antibody against FOXA / HNF-3 or serum for 20 min before the addition of the labelled wild-type HNF-1α oligonucleotide or mutant HNF-1α oligonucleotide as well as TTR oligonucleotide and then incubated for another 20 min at room temperature (25 °C). The arrow indicates supershift band. The arrowhead shows the DNA–protein complex. (e) Nuclear extracts from HepG2 cells were incubated in the absence or in the presence of 100- and 200-fold excess of either the unlabelled wild-type or mutant HNF-1α oligonucleotides before addition of the labelled wild-type and mutant HNF-1α probes. (f) Nuclear extracts from HepG2 cells were incubated with the labelled wild-type HNF-1α oligonucleotide. Increasing amounts (20-,

40-, 80- and 100-fold excess) of unlabelled wild-type or mutant oligonucleotides were used as competitors. (g) Determination of the relative of FOXA / HNF-3 binding to the HNF-1 α wild-type and mutant oligonucleotides. *A P -value < 0.05 was considered statistically significant. MUT, mutation; WT, wild-type.

FIGURE 3.

Chromatin immunoprecipitation analysis was performed as described under Methods. Lane 3 shows DNA isolated through the antibody against FOXA / HNF-3 chromatin immunoprecipitation. Lane 4 shows DNA isolated through chromatin immunoprecipitation using the negative control immunoglobulin G (IgG). Lane 5 shows the diethylpyrocarbonate-treated water (DEPC H₂O) used as a template was amplified by HNF-1 α primers. Lane 6 shows input DNA amplified by HNF-1 α primers.

FIGURE 4.

Expressions of FOXA1 / HNF-3 α and FOXA2 / HNF-3 β . The lysates of HepG2 and MIN6 cells were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the expression levels of FOXA1 / HNF-3 α and FOXA2 / HNF-3 β in cell lysates were determined by western blotting with antibody against FOXA1 / HNF-3 α and FOXA2 / HNF-3 β . Equal loading of protein in cell lysates was determined by western blotting using an anti- β -actin antibody.

FIGURE 5.

The effect of FOXA / HNF-3 on reporter gene expression. HepG2 cells were transiently co-transfected with the wild-type or nt-128 T→G reporter constructs and either the expression vector encoding FOXA1 / HNF-3 α or FOXA2 / HNF-3 β . Forty-eight hours after transfection, cells were harvested and luciferase activity was determined. Wild-type and nt-128 T→G constructs were indicated by filled and open boxes, respectively. Relative luciferase activity was expressed as means $\pm$ sd in fold of activity obtained with the wild-type or mutant reporter construct and empty expression vector. All data were presented as means of three individual transfection experiments ($n = 9$, $*P < 0.05$).

Table 1

Sequences of the sense strands of oligonucleotides used in this study

Oligonucleotide	Sequence ^{*†}
HNF-1α	–139 5′-GCTAGTGGGGTTTGGGGGG GCAGTG-3′–114
Mutation HNF-1α	–139 5′-GCTAGTGGGGTgTTGGGGGG GCAGTG-3′–114
TTR	–111 5′-GTTGACTAAGTCAATAATCA GAATCAG-3′–85 [29]
IGFBP-1	–125 5′-GCACTAGCAAAACAACCTTA TTTGAACACG-3′–95 [29]

* The substitution mutation is represented as a lower-case letter.

† Core FOXA / HNF-3 binding sites are underlined.