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HNF1 and/or HNF3 may contribute to the tissue specific expression of glucokinase gene

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Abbreviations: GK, glucokinase; GLUT2, glucose transporter type 2; HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay

Abstract

A possible role of hepatocyte nuclear factor 1 (HNF1) or HNF3, a predominant trans-acting factors of hepatic or pancreatic β-cells, was examined on the tissue specific interdependent expression of glucokinase (GK) in liver, H4IIE, HepG2, HIT-T15 and MIN6 cell line. The tissues or cell lines known to express GK showed abundant levels of HNF1 and HNF3 mRNA as observed in liver, H4IIE, HepG2, HIT-T15 and MIN6 cells, whereas they were not detected in brain, heart, NIH 3T3, HeLa cells. The promoter of glucokinase contains several HNF3 consensus sequences and are well conserved in human, mouse and rat. Transfection of the glucokinase promotor linked with luciferase reporter to liver or pancreatic β cell lines showed high interacting activities with HNF1 and HNF3, whereas minimal activities were detected in the cells expressing very low levels of HNFs. The binding of HNF1 or HNF3 to the GK promoter genes was confirmed by electrophoretic mobility shift assay (EMSA). From these data, we propose that the expression of HNF1 and/or HNF3 may, in part, contribute to the tissue specific expression of GK

Keywords: GK, GLUT2, HNF1, HNF3

Introduction

Tissue specific expression of specific subsets of genes requires tightly controlled interplay of trans-acting factors. For these phenomena, general or ubiquitous factors as well as specific transcription factors are required for the initiation and maintenance. Recently, many trans-acting factors have been discovered acting on the liver or pancreas specific genes. Of these, HNFs are one of the trans-acting factors responsible for the expression of liver or pancreatic β-cell specific genes (De Simone *et al.*, 1991; Tronche *et al.*, 1997; Stoffel *et al.*, 1997; Duncan *et al.*, 1998). HNF1 is a homeodomain protein that plays a key role in the liver-specific expression of many genes during differentiation and development (Miura *et al.*, 1993). HNF1 is required for the expression of GLUT2 and other liver-specific genes such as albumin, α1-antitrypsin, and fibrinogen (Wang *et al.*, 1998; De Simone *et al.*, 1991; Lai *et al.*, 1991). HNF1α associates with the highly related HNF1β (vHNF1) to form homo- or heterodimers (Wang *et al.*, 1998). The expression of HNF1β dominant negative form resulted in reduction of GLUT2 gene expression (Mendel *et al.*, 1991). Mutation of HNF1β resulted in decreased transcription of GLUT2 and was ultimately related to familial type 2 diabetes mellitus (Tomura *et al.*, 1999).

HNF3, a member of the forkhead winged helix family, has been known to play a role in liver and gut development (Lai *et al.*, 1991). The HNF3 isotypes, namely, HNF3α, 3β, and 3γ bind to the same DNA sequence with different affinities. It was shown that HNF3 could activate the albumin gene expression by repositioning the nucleosomes in the albumin enhancer (McPherson *et al.*, 1993; Shim *et al.*, 1998).

Human GLUT2 promoter has binding sites for HNF1 and HNF1 and/or HNF3 at the −1030 bp region and +74 bp region, respectively (Tomura *et al.*, 1999; Cha *et al.*, 2000). Whereas it is well known that HNF1 and HNF3 plays important roles in tissue-specific expression of GLUT2, we do not know whether these transcription factors are able to mediate tissue specific expression of glucokinase which is mainly expressed in liver and pancreatic β-cells.

In this study, we have identified the *cis*-elements for HNF1 or HNF3 in the glucokinase gene promoters present in liver and pancreatic β-cells. Based on these results, we propose that the expression of GLUT2 or glucokinase in the liver or pancreatic β-cells may be governed by the action of HNF1 or HNF3 that are predominant transcriptional factors of these tissues.

Materials and Methods

Construction of plasmids

Rat glucokinase promoter spanning −1003/+196 of β cell specific gene and −1448/+127 of liver specific gene (Magnuson *et al.*, 1989) were cloned into pGL3 basic reporter vector (Promega, Madison, WI) and named pRGP-1003 and pRGL-1448, respectively. The sequences of constructs were confirmed by DNA sequencing. pRSV-HNF1 and pRSV-vHNF1 were given by Dr. M. Yaniv. pGem-HNF3α and pGem-HNF3β were kindly provided by Dr. R.H. Costa.

Preparation of nuclear extracts

Nuclear extracts from liver of male Sprague-Dawley rats or cell lines were prepared as described by Gorski *et al.* (Gorski *et al.*, 1986) or Dignam *et al.* (Dignam *et al.*, 1990). Protein concentration was determined according to Bradford (Bradford *et al.*, 1976). The extracts were frozen in aliquots and stored at −70°C.

Northern blot analysis

Total RNA was extracted from various tissues and cell lines using the TRIzol reagent™ (Life Technologies) following the manufacturer's protocol. Twenty micrograms of total RNA was separated on 1% agarose gels containing 0.66 M formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane (Schleicher & Schuell, Inc) by capillary transfer in the presence of 20x SSC. Then the filter which was UV-crosslinked with UV-crosslinker (Hoefer) was prehybridized and hybridized with ^{32}P-labeled HNF1α and HNF3β cDNA probes in Rapid-hyb buffer (Amersham Life Science) at 58°C overnight. After hybridization, the nylon membrane was rinsed with 2x SSC, 0.1% SDS followed by 0.2x SSC, 0.1% SDS and exposed to X-ray film at −70°C with an intensifying screen.

Cell culture and transient transfection

Cells used in this experiment were maintained as monolayer cultures and grown in appropriate media. Plasmid DNAs were purified on Qiagen Midiprep kit columns (Qiagen) at least twice independently. Cells were plated in six-well tissue culture plates at a density of 1×10^6 cells/well in 2 ml of medium. After a 20-h attachment period, transfections were performed with LipofectAMINE PLUS reagent (Life Technologies, Inc), according to the manufacturer's protocol. Briefly, 0.5 μg of each construct of GLUT2 promoter, 0.1 μg of pCMV-β-galactosidase and 4 μl of plus reagent and 2 μl of lipofectamine in 200 μl of OPTI-MEM I (Life Technologies, Inc) media lacking serum were mixed and added to cells. After 3 h, the medium containing the lipofectamine-DNA complex was removed and replaced by appropriate media (containing serum and antibiotics). Cells were then cultured further for 48 h and harvested in reporter lysis buffer (Promega). The lysed cells were centrifuged to remove cell debris and the supernatant was collected. Luciferase assays were conducted with 10 μl of cell extracts and 50 μl of luciferase assay reagent (Promega). β-Galactosidase activity was determined with 10 μl of cell extract and 190 μl assay reagent containing *O*-nitrophenol-β-$_D$-galactopyranoside in a colorimetric assay. Luciferase data were expressed as luciferase activity corrected by β-galactosidase activity in the cell lysate. Each transfection was performed in triplicate and repeated three to five times.

Electrophoretic mobility shift assay (EMSA) and supershift assay

Probes for gel-shift assays were labeled with ^{32}P in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled double-stranded oligonucleotides were prepared by mixing five-molar amounts of the complementary single-stranded DNAs in 50 mM NaCl, heating to 90°C for 5 min and then cooling to room temperature. The oligonucleotide used in these assays was as follows:

RGP2, 5'-GGCAAAGCACTTATTGATTAGATTCCCATC-3'

The oligonucleotides for HNF1 (Vaulont *et al.*, 1989) and HNF3 (Costa *et al.*, 1989) were synthesized, and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3') was purchased from Promega. The labeled probe (50,000 cpm) was combined with nuclear proteins in 25 mM Tris/HCl, pH 7.4, 80 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. The nonspecific competitor, 1.5 μg of poly (dI-dC), was added to each binding reaction. Binding reaction mixtures were incubated for 20 min on ice and resolved on a non-denatured (5% w/v) acrylamide gel (29 : 1 w/w acrylamide/bisacrylamide) in $0.5 \times$ TBE at 4°C. For competition assays, 100-fold molar excess of various unlabeled competitor DNAs were added to the reaction mixture prior to the addition of the labeled probe. The dried gels were exposed to X-ray film at −70°C with an intensifying screen.

Statistical analysis

All transfection studies were performed in three to five separate experiments, where triplicate dishes were transfected. The data were represented as mean ± standard deviation. Statistical analysis was carried out using Microsoft Excel® (Microsoft).

Results and Discussion

Northern blot analysis of the tissues or cell lines known to express GLUT2 or GK (liver, H4IIE, HepG2, HIT-T15, MIN6) showed abundant levels of HNF1 and HNF3 mRNA whereas their expressions were found to be low in the cells or tissues where GLUT2 or GK expression were low, *i.e.* brain, heart, NIH3T3, and HeLa (Jetton *et al.*, 1992; Miyazaki *et al.*, 1990) (Figure 1). GLUT2 was expressed only in renal proximal (convoluted) tubule of

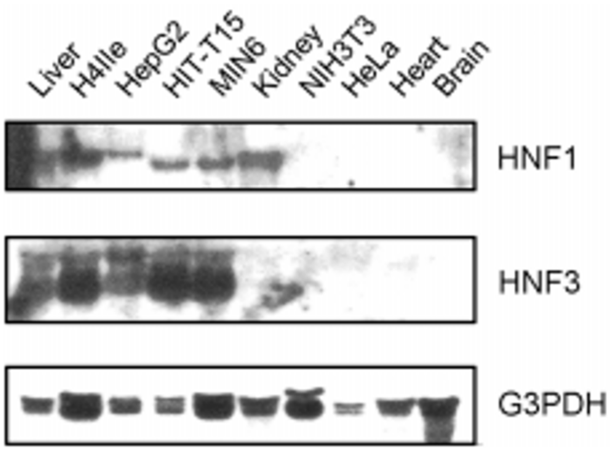

Figure 1. Northern blot analysis of HNF1 and HNF3 in variable tissues and cell lines. Twenty μg of total RNA was separated on 1% agarose gels and transfer onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled HNF1α and HNF3β cDNA probes. G3PDH gene served as an internal control for the different RNA samples.

kidney (Kamran *et al.*, 1997) and HNF1β, a major subtype of HNF1 in the kidney, was reported to suppress transcriptional activities of many liver genes (Song *et al.*, 1998). Thus, the highly expressed HNF1β but not HNF3 could likely have caused repression of GLUT2 or GK expression in the kidney. The northern blot results showed that the expression of HNF1 and HNF3 was correlated well with GLUT2 and GK levels in the tissues or cell lines tested in this study (Figure 1). Search for the consensus sequences for HNF1 or HNF3 in the GLUT2 or GK gene (Figure 2) showed that the promoter of GLUT2 contains HNF1 and HNF3 consensus sequences and they are well conserved in the human, mouse and rat promoters (Cha *et al.*, 2000). GK uses different promoters in liver and pancreas (Magnuson *et al.*, 1989), but both of them contain many HNF3 binding sites.

In order to explore whether HNFs are major effectors in tissue specific expression of GLUT2 or GK gene, the promoter region of these genes amplified by polymerase chain reaction (PCR) were linked to pGL3-luciferase vector and the effects of HNFs on the promoter activities were observed in the cells used for northern blot analysis (Figure 3). The high promoter activities of GLUT2 in liver or pancreatic β cells was reported (Cha *et al.*, 2000). The pancreatic promoter of GK gene showed high activities in the β cell lines, HIT-T15 and MIN6 (Figure 3a). The liver type GK promoter was activated in HepG2 cells (Figure 3b). However, this promoter showed minimal activities when the reporter constructs were transfected into cells expressing minimal HNFs (Figure 1, 3a and 3b).

To confirm experimentally whether such consensus sequences predicted from the database search were correct in its ability to bind HNF1 or HNF3, electrophoretic mobility shift assay (EMSA) was carried out. An oligonucleotide, covering the region between −14 and +10 of rat pancreatic GK promoter (RGP2) synthesized were used for the experiment. In RGP2 probe, one HNF3 binding site was detected (Figure 4). The absence of HNF1 consensus sequence on the GK promoter supported the observation that HNF1 dominant negative form couldn't suppress the GK gene expression in the beta cells (Wang *et al.*, 1998). Cha *et al.* showed that HNF1 and HNF3 could bind to the region between +87 and +132 of GLUT2 promoter. These data suggest that the expression of GLUT2 or GK is associated with the expression of HNF1 and/or HNF3 in the cells. And such association has to be important factors in overall regulation of GLUT2 or GK expression in the liver or pancreatic β cell along with the expected contributions and/or interactions with other transcription factors.

Acknowledgements

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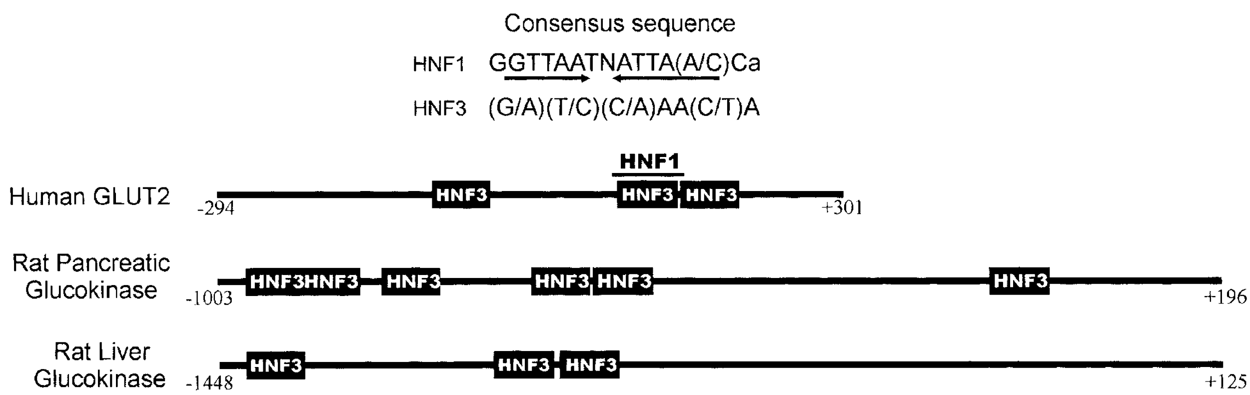

Figure 2. The consensus sequences for HNF1 and HNF3 in the human GLUT2, rat pancreatic glucokinase, and rat liver glucokinase promoters.

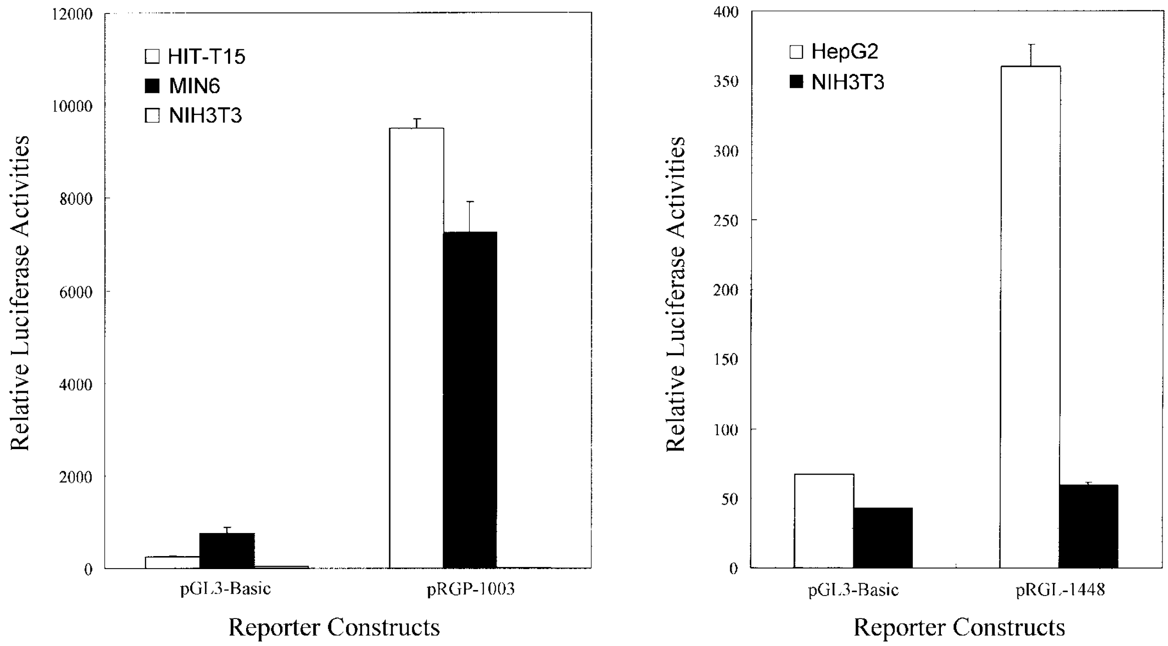

Figure 3. Transcriptional activities of rat glucokinase promoters. The reporter constructs containing rat pancreatic (A) and liver (B) glucokinase promoter were transfected to GK-expressing (HIT-T15, MIN6 or HepG2) and non-GK-expressing cells. Luciferase activities were normalized on the basis of β-galactosidase activity encoded by the co-transfected control plasmid. pCMV-β-galactosidase. Results are the mean ± S.D. of three independent experiments in triplicate.

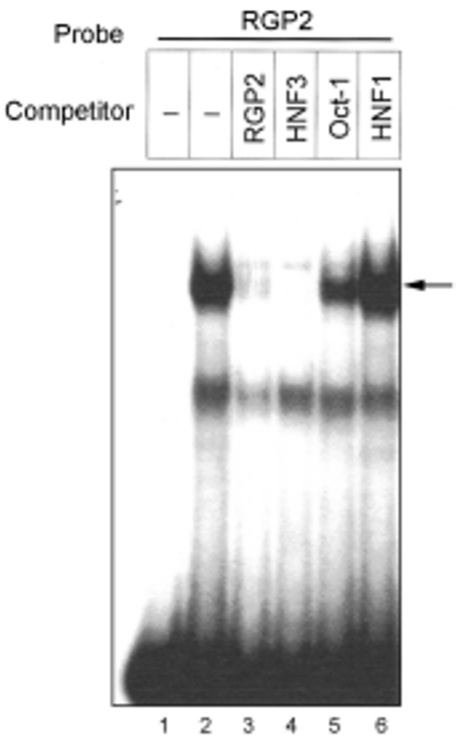

Figure 4. EMSA of the RGP2. ^{32}P-labeled RGP2 was incubated with rat liver nuclear extracts (5 μg) in the absence (lane 2) or presence of 50-fold molar excess of the indicated cold competitor: RGP2 (lane 3), HNF3 (lane 4), Oct-1 (lane 5), or HNF1 (lane 6). The band representing specific DNA-HNF3 complex was indicated.

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