Activation of glucokinase gene expression by hepatic nuclear factor 4α in primary hepatocytes

Ulrike ROTH, Kurt JUNGERMANN[1] and Thomas KIETZMANN[2]

Institut für Biochemie und Molekulare Zellbiologie, Georg-August-Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, Germany

Glucokinase (GK) is a key enzyme for glucose utilization in liver and shows a higher expression in the perivenous zone. In primary rat hepatocytes, the GK gene expression was activated by HNF (hepatic nuclear factor)-4α via the sequence $-52/-39$ of the GK promoter. Venous pO_2 enhanced HNF-4 levels and HNF-4 binding to the GK–HNF-4 element. Thus, HNF-4α could play the role of a regulator for zonated GK expression.

Key words: glycolysis, hypoxia, insulin, maturity-onset diabetes of the young, metabolic zonation, nuclear receptor.

INTRODUCTION

The mammalian glucokinase (GK) is a key enzyme of glucose utilization, playing a crucial role in maintaining blood glucose homoeostasis. In contrast with the other hexokinase family members (hexokinases I–III), the GK (hexokinase IV) has a lower affinity for glucose with sigmoidal kinetics and is not inhibited by its reaction product glucose 6-phosphate [1–5]. GK expression is restricted to hepatocytes [6], the pancreatic β-cells and some neuroendocrine cells of the gastrointestinal tract [7].

In the liver, the glycolytic enzymes GK and pyruvate kinase show a slight predominance in the less aerobic, perivenous zone, whereas the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose 6-phosphatase were found in the more aerobic, periportal zone [8].

The GK gene can be transcribed from two different promoters. An extrahepatic expression is initiated from the β-cell-specific promoter which is localized approx. 20 kb upstream of the GK gene, whereas the hepatocytic expression is controlled by the liver-specific promoter adjacent to the GK gene [9,10]. A number of binding sites for transcription factors were found in the liver-specific GK promoter by DNase-I footprint assays. However, not all the regulatory transcription factors regulating the GK gene expression are known yet, except for the upstream stimulatory factor 1 [11] and the sterol regulatory element binding protein 1c [12]. The P1 region $-54/-35$ [11] of the GK promoter identified by footprint analysis with nuclear proteins from rat liver contains a direct repeat-like sequence of an hexanucleotide consensus nuclear receptor binding site [13], which could be a target for the hepatic nuclear factor 4 (HNF-4).

HNF-4 is a member of the steroid/thyroid superfamily and exists in various isoforms [13–18]. In addition to the liver, HNF-4α is expressed in the kidney, pancreas, small intestine, testis and colon, whereas HNF-4γ was not detectable in the liver [15]. The active HNF-4 binds DNA as a homodimer and, at present, there is no evidence that HNF-4 forms heterodimers with other members of the nuclear receptor family [13,19].

It has been demonstrated that HNF-4α is critical for embryonic development: targeted disruption of the mouse HNF-4α gene has been found to be lethal [20]. HNF-4 is known to be important for glucose and lipid homoeostasis [21–25], because mutations in the HNF-4 gene can cause maturity-onset diabetes of the young (MODY-1).

Since HNF-4 is important for the appropriate expression of genes regulating glucose and lipid metabolism and since the P1 site contains a putative HNF-4 element, the aim of the present study was to investigate the role of HNF-4α as a factor involved in the regulation of GK expression in primary cultured rat hepatocytes. We demonstrate that HNF-4α may function as a transcriptional activator for the GK expression and that, together with the O_2 gradient, HNF-4α may regulate the zonated GK gene expression in liver.

EXPERIMENTAL

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers.

Animals

Male Wistar rats (200–260 g) were kept on a 12 h day–night rhythm (light from 07:00 to 19:00 h) with free access to water and food. Rats were anaesthetized with pentobarbital (60 mg/kg body weight) before the preparation of hepatocytes between 08:00 and 09:00 h.

Plasmid constructs

The reporter plasmid rat GK-1430 LUC (luciferase) was constructed by inserting a *Bgl*II–*Hin*dIII GK promoter fragment ($-1430/+21$) into pGL3 basic from Promega (Heidelberg, Germany). The reporter plasmid GK-101 LUC was constructed by inserting a *Sma*I–*Xho*I promoter fragment ($-101/+79$) [26] into pGL3 basic. The plasmid GK-101 HNFm LUC was

Abbreviations used: DIG, digoxigenin; EMSA, electrophoretic mobility-shift assay; EPO, erythropoietin; GK, glucokinase; HNF, hepatic nuclear factor; LUC, luciferase; MODY, maturity-onset diabetes of the young; PK_L, pyruvate kinase type liver; WT, wild-type.

[1] Deceased 10 May 2002

[2] To whom correspondence should be addressed (e-mail tkietzm@gwdg.de).

constructed by PCR, using the reporter plasmid GK-101 LUC and the oligonucleotide primer pair HNFm (5′-CCCTGGCCC-**TGA**at**T**cG**TGACAC**T-3′) containing a mutated HNF-4 sequence together with the RV3 (Promega) or GL2 (Promega) primer to amplify 150 and 200 bp fragments. With a second PCR using the RV3 and GL2 primer, a 350 bp fragment was generated which was cloned into the *Sma*I and *Hin*dIII sites of pGL3 basic. All constructs were verified by sequencing in both directions.

The expression plasmid pCMX-HNF-4 containing the full-length rat HNF-4α1 cDNA was described previously [27,28].

Cell transfection and LUC assay

Liver cells were isolated by collagenase perfusion and were maintained under standard conditions in an atmosphere of periportal pO_2 (16 % O_2/79 % N_2/5 % CO_2, by vol.) in medium M199 containing 1 nM of insulin added as a growth factor for culture maintenance, 100 nM dexamethasone required as a permissive hormone and, until the first change of medium after 5 h, 4 % (v/v) neonatal-calf serum. Transfections were essentially performed as described in [29]: in brief, 2.5 μg of DNA was precipitated in 150 μl of transfection buffer [7.5 μl $CaCl_2$/75 μl of 2 × Hepes (pH 7.05)/67.5 μl H_2O] and was added to 10^6 freshly isolated hepatocytes for 5 h. In HNF-4α co-transfection assays, 2 μg of the LUC constructs were transfected with 500 ng of pCMX-HNF-4, whereas in controls they were transfected with 500 ng of the empty vector. After 5 h, the medium was changed and the cells were cultured under periportal pO_2 for up to 24 h. Then, the medium was changed again, and the cells were further cultured for another 24 h under periportal (16 % O_2) or perivenous (8 % O_2/87 % N_2/5 % CO_2, by vol.) O_2 tensions.

RNA preparation and Northern-blot analysis

Total RNA was prepared from 3×10^6 cells as described previously [30]. RNA (15 μg) was denatured using formaldehyde and used in a typical Northern-blot experiment. Digoxigenin (DIG)-labelled antisense GK and β-actin RNA served as hybridization probes; they were generated by *in vitro* transcription from pBS-GK1, pBS-β-actin using T3 RNA polymerase and DIG–UTP. Hybridizations were carried out with 50 ng/ml transcript at 68 °C for 6 h according to the manufacturer's application notes for the DIG–nucleic acid detection kit (Roche, Mannheim, Germany). Detection of hybrids was performed as described previously [30]. Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen, Germany).

Western-blot analysis and GK enzyme-activity assay

For Western-blot analysis, protein from primary-cultured hepatocytes and transiently transfected hepatocytes was isolated as described previously [31]. The protein content was determined using the Bradford method. Protein (50 μg) was dissolved in a 27 μl SDS sample buffer [32], loaded on a SDS–10 % polyacrylamide gel and after electrophoresis blotted on nitrocellulose membranes. Non-specific binding was blocked with a blocking buffer [10 mM Tris/HCl (pH 7.5), 100 mM NaCl, 0.1 % (v/v) Tween 20, 10 % (w/v) milk powder]. Blots were incubated with a primary goat antibody raised against amino acids 2–20 of the human GK (Santa Cruz, Heidelberg, Germany) in a 1:200 dilution or with a 1:1000 dilution of the goat antibody raised against the 18 C-terminal amino acids of human HNF-4α (Santa Cruz) in blocking buffer overnight at 4 °C. Washing was performed with blocking buffer without milk powder. The secondary antibodies were an anti-goat IgG (Dako, Hamburg, Germany) used in a 1:2000 dilution for 1 h. After washing for 30 min, the ECL® Western-blotting system (Amersham, Freiburg, Germany) was used for detection. Under these conditions, GK was visible as a band of 53 kDa and HNF-4α as a band of 54 kDa.

For determining the GK enzyme activity, cells were washed with 0.9 % NaCl, scraped in a buffer containing 50 mM Hepes (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM dithioerythritol, 10 mg/ml BSA and homogenized with an Ultraturrax (Jahnke and Kunkel, Stauffen). GK activity was then essentially recorded as in [33] and DNA was estimated as described previously [34].

Preparation of nuclear extracts

Nuclear extracts were prepared by modification of a standard protocol [35,36] with buffers A and C containing 0.5 mM dithioerythritol (Sigma, Taufkirchen, Germany), 0.4 mM PMSF (Serva, Heidelberg, Germany), 2 μg/ml leupeptin and pepstatin (Roche), 2 μg/ml aprotinin (Bayer, Leverkusen, Germany), 1 mM sodium vanadate (Sigma) and the 'complete' protease inhibitor cocktail tablets (Roche), essentially as described in [37].

Electrophoretic mobility-shift assay (EMSA)

The sequence of the GK–HNF-4-WT (WT, wild-type) oligonucleotide was 5′-CCCTGGCCCTGACCTTGTGACACT-3′, whereas the sequence of the GK–HNF-4-m oligonucleotide contains a 3 bp exchange to generate the sequence 5′-CCCTGG-CCCTGAatTcGTGACACT-3′. Equal amounts of complementary oligonucleotides were annealed, and labelled by 5′-end labelling with [γ-^{32}P]ATP (Amersham, Freiburg, Germany) and T4 polynucleotide kinase (MBI, St. Leon-Rot, Germany). They were purified with the Nucleotide Removal Kit (Qiagen, Hilden, Germany). Binding reactions were carried out in a total volume of 20 μl, containing 50 mM KCl, 1 mM $MgCl_2$, 1.1 mM EDTA, 5 % (v/v) glycerol, 10 μg of nuclear extract, 250 ng poly d(I-C) and 5 mM dithioerythritol. After preincubation for 5 min at room temperature, 1 μl of the labelled probe (10^4 c.p.m.) was added and the incubation was continued for an additional 10 min. For supershift analysis, 2 μl of HNF-4α antibody was added to the EMSA reaction which was then incubated at 4 °C for 2 h. The electrophoresis was then performed with a 5 % (w/v) non-denaturing polyacrylamide gel in TBE (89 mM Tris/89 mM boric acid/5 mM EDTA) buffer at 200 V. After electrophoresis the gels were dried and exposed to a PhosphorImager screen.

RESULTS

Enhancement of GK mRNA, enzyme activity and protein levels by perivenous pO_2 and overexpression of HNF-4α in rat hepatocytes

Primary hepatocytes were investigated to discover whether the transfection of rat HNF-4α expression vectors could enhance the GK mRNA, protein and enzyme activity level under periportal and perivenous pO_2. For comparison, GK mRNA and enzyme activities were measured after treatment of hepatocytes with 1 nM insulin. Under arterial pO_2, in the absence of insulin, GK mRNA was nearly undetectable, whereas under perivenous pO_2, GK mRNA was detectable. Insulin elevated GK mRNA within 3 h, maximally to approx. 700 % under venous pO_2 and to a lower level of approx. 400 % under arterial pO_2 (Figure 1). This positive modulation of GK mRNA induction was in line with a previous study [30]. The transfection of hepatocytes with the HNF-4α expression vector enhanced GK mRNA levels to approx. 600 % under both arterial and venous pO_2 when compared with the perivenous control (Figure 1).

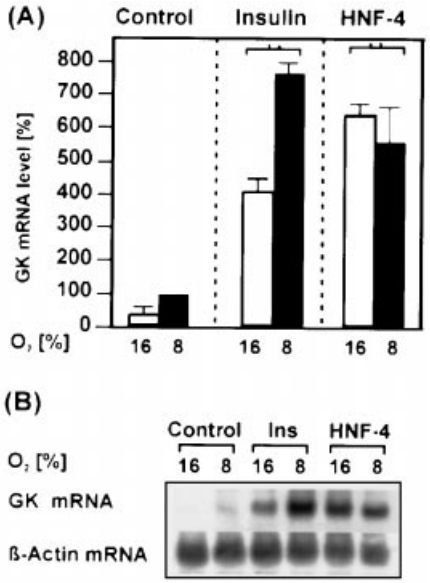

Figure 1 Induction of GK mRNA levels by overexpression of HNF-4α under periportal and perivenous pO_2 in rat hepatocyte cultures

(**A**) Hepatocytes were transfected with an expression plasmid for HNF-4α (cf. Materials and methods section) and cultured for 24 h under arterial pO_2. When indicated GK mRNA was induced at 24 h without changing the culture medium by adding 1 nM insulin for 3 h under arterial and venous pO_2. In each experiment, the highest induction of GK mRNA under perivenous pO_2 measured by Northern blotting (**B**) was set equal to 100%. Values are means ± S.E.M. of culture experiments, each performed in duplicate with hepatocytes from three different preparations. Student's *t* test for paired values: *significant differences 16% O_2 versus 8% O_2, $P \leqslant 0.05$; **significant differences insulin treated or HNF-4 transfected at 16% O_2 or 8% O_2 versus controls at 16% O_2 or 8% O_2, $P \leqslant 0.05$. (**B**) Representative Northern blot: 20 μg of total RNA prepared from the cultured hepatocytes was hybridized to DIG-labelled GK and β-actin antisense RNA probes (cf. Materials and methods section). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry.

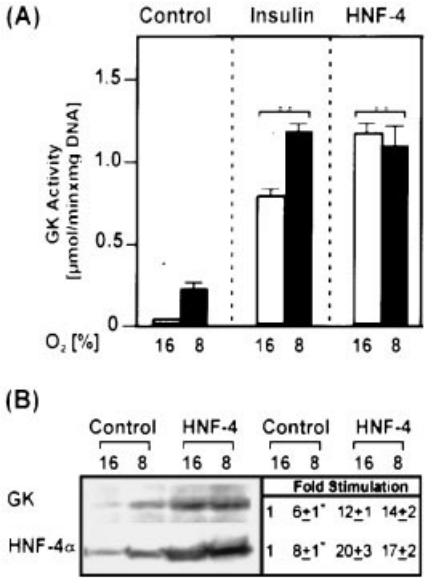

Figure 2 Induction of GK enzyme activity and protein expression by overexpression of HNF-4α under both periportal and perivenous pO_2 in rat hepatocyte cultures

(**A**) Hepatocytes were transfected with an expression plasmid for HNF-4α (cf. Materials and methods section) and cultured for 24 h under arterial pO_2. The medium was then changed; where indicated, 1 nM of insulin was added and the culturing was continued for another 24 h under arterial or venous pO_2. GK enzyme activity values are means ± S.E.M. of culture experiments each performed in duplicate with hepatocytes from three different preparations. Student's *t* test for paired values: *significant differences 16% O_2 versus 8% O_2, $P \leqslant 0.05$; **significant differences insulin treated or HNF-4 transfected at 16% O_2 or 8% O_2 versus controls at 16% O_2 or 8% O_2, $P \leqslant 0.05$. (**B**) Western-blot analysis of GK and HNF-4α protein in cultured hepatocytes transfected with an expression plasmid for HNF-4α. Total cellular protein (50 μg) was subjected to Western-blot analysis with an antibody raised against rat GK or rat HNF-4α respectively (cf. Materials and methods section). Blots were scanned by videodensitometry, and in each experiment, the GK level and HNF-4α level measured under periportal pO_2 was set equal to 1. The values represent means ± S.E.M. of culture experiments with hepatocytes from three different preparations. Student's *t* test for paired values: *significant differences 16% O_2 versus 8% O_2, $P \leqslant 0.05$.

The HNF-4-mediated GK mRNA induction was followed by an increase in GK enzyme activity on the basis of enhanced GK protein levels. In the controls under arterial pO_2, GK enzyme activity was in the range of 0.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ DNA, whereas under venous pO_2, GK enzyme activity was enhanced to approx. 0.25 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ DNA. Insulin enhanced GK activity to approx. 0.7 and 1.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ DNA under arterial and venous pO_2 respectively, in line with a previous study [38]. The overexpression of HNF-4 increased GK activity to 1.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ DNA under arterial pO_2 and to 1.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ DNA under perivenous pO_2. The increases in GK enzyme activity were due to the enhanced GK protein levels. In the untransfected cells, GK protein was enhanced by approx. 6-fold under perivenous pO_2 compared with periportal pO_2. After transfection of the HNF-4α expression vector, the GK protein level was enhanced by approx. 12-fold under periportal pO_2 and by approx. 14-fold under perivenous pO_2, compared with the untransfected control under arterial pO_2 (Figure 2).

Interestingly, the HNF-4α levels were increased by approx. 8-fold under perivenous pO_2 (8% O_2). The transfection of the HNF-4α vector enhanced the HNF-4 level by approx. 20-fold under periportal pO_2 and by approx. 17-fold under perivenous pO_2 compared with the untransfected control under arterial pO_2 (Figure 2).

Thus, in rat hepatocytes, transfection of HNF-4α expression vectors increased GK mRNA, protein and enzyme activity levels and mimicked perivenous pO_2, displaying a pattern of zonation observed in the liver acinus.

Activation of transfected rat GK promoter LUC gene constructs by HNF-4α in rat hepatocytes

As the next step, the ability of HNF-4α to activate the liver-specific GK promoter was tested by co-transfection of rat liver GK promoter LUC gene constructs together with an expression vector encoding the rat HNF-4α1 isoform.

The co-transfection of GK-1430 LUC, a construct containing the entire liver-specific GK promoter and the HNF-4α vector induced LUC activity by approx. 5-fold (Figure 3). Then, a largely shortened 101 bp fragment of the GK promoter that still contained the potential HNF-4 element, was used to drive the LUC gene in pGL3 basic (GK-101 LUC) (Figure 3). In comparison with GK-1430 LUC, hepatocytes transfected with GK-101 LUC displayed approx. 2-fold higher basal LUC activity which is in line with a previous study [26]. But again, after co-transfection of hepatocytes with GK-101 LUC and HNF-4α, the LUC activity was enhanced by approx. 2-fold. In contrast, after co-transfection of the GK-101 HNFm LUC containing a mutation inside the HNF-4 recognition site HNF-4α did not activate LUC activity (Figure 3).

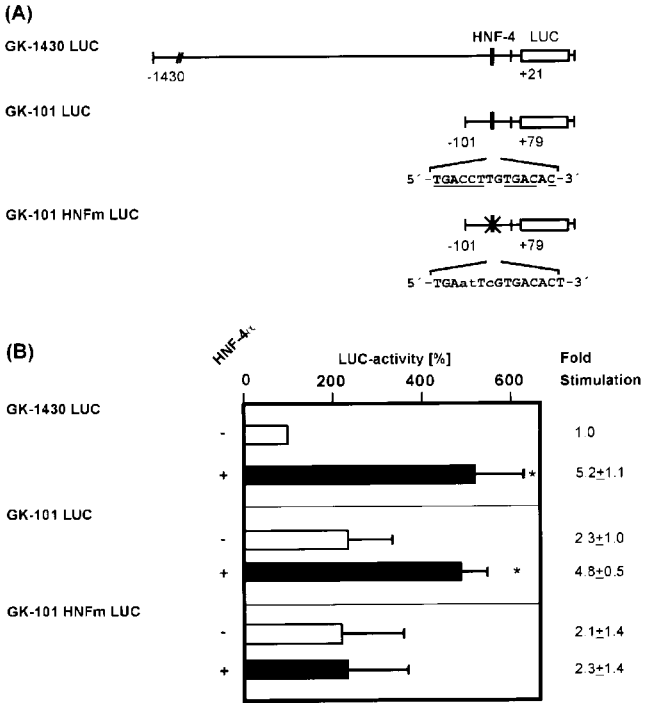

Figure 3 Activation of GK promoter-controlled LUC expression by overexpression of HNF-4α via the HNF-4 binding element in rat hepatocyte cultures

(**A**) LUC gene constructs with rat liver-specific GK promoter regions: a WT 1430 bp rat GK promoter fragment (GK-1430 LUC), a 101 bp rat GK promoter fragment (GK-101 LUC) or the 101 bp promoter mutated at the HNF-4 binding element (GK-101 HNFm LUC). The WT HNF-4 binding sequences are underlined and mutated bases are shown in lower-case letters. (**B**) Hepatocytes were transiently co-transfected with pCMX-neo HNF-4 containing the full-length rat HNF-4 isoform α1 cDNA and LUC gene constructs driven by a WT 1430 bp rat GK promoter (GK-1430 LUC) or a WT 101 bp rat GK promoter (GK-101 LUC) or the 101 bp promoter mutated at the HNF-4 site (GK-101 HNFm LUC). After 18 h under periportal pO_2 (16% O_2), the medium was changed and the transfected cells were cultured for another 24 h under periportal pO_2. In each experiment, the percentage of LUC activity was determined relative to the GK-1430 LUC control (−), which was set equal to 100% or, for the determination of the fold stimulation of LUC activity, to 1. The values represent means ± S.E.M. of culture experiments, each performed in duplicate with hepatocytes from three different preparations. Student's *t* test for paired values: *significant differences control (−) versus HNF-4α (+), $P \leqslant 0.05$; open bars, transfected with control empty vector; solid bars, transfected with HNF-4α expression vector.

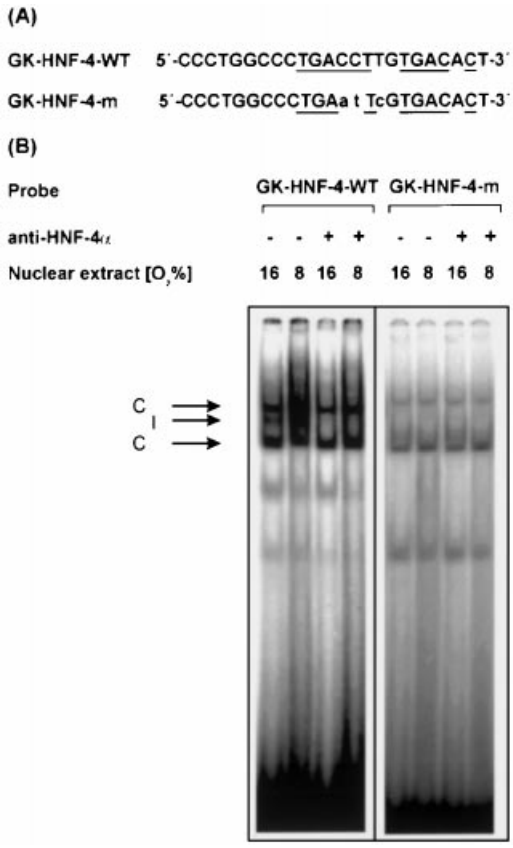

Figure 4 Binding of HNF-4α to the rat GK–HNF-4 element

(**A**) Oligonucleotides. The sense strand of the rat GK promoter oligonucleotides is shown. Bases matching the HNF-4 binding element are underlined, mutated bases are shown in lower-case letters. (**B**) EMSA: the ^{32}P-labelled GK–HNF-4-WT (left panel) and GK–HNF-4-m (right panel) oligonucleotides were incubated with 10 μg of protein from nuclear extracts of periportal (16% O_2) or perivenous (8% O_2) cells as indicated (see the Materials and methods section). In EMSA with the antibody, the nuclear extracts were preincubated with 2 μl of HNF-4α antibody for 2 h at 4 °C before adding the labelled probe. The DNA protein binding was analysed by electrophoresis on 5% native polyacrylamide gels. C, constitutive complex; I, induced complex.

These results demonstrated that the GK promoter can be activated by HNF-4α via the HNF-4 binding element inside the P1 region.

Binding of HNF-4α to the HNF-4 binding element of the rat GK promoter

To corroborate further the conclusion from the transfection experiments that HNF-4α interacts with the HNF-4 binding element of the GK promoter (GK–HNF), the binding of nuclear proteins to the GK–HNF was examined by EMSA. The GK–HNF-4-WT oligonucleotide (−61/−38) (Figure 4) was able to bind three complexes. In addition to the largest and the smallest constitutive protein complex, a hypoxia-inducible nuclear protein complex was detectable (Figure 4). To investigate the presence of HNF-4α in these complexes, an antibody against HNF-4α was included in the binding reaction. Addition of the HNF-4α antibody to the EMSA reaction strongly reduced the formation of the hypoxia-inducible DNA complex with the GK–HNF-4-WT oligonucleotide (Figure 4). Furthermore, this hypoxia-induced DNA protein complex was no longer detectable by using the mutated GK–HNF-4-m oligonucleotide, whereas the constitutive complexes were still formed. These complexes were not affected in the presence of anti-HNF-4α (Figure 4). These results indeed corroborate the transfection studies and indicate that HNF-4α can interact at the HNF-4 binding element in the GK promoter.

DISCUSSION

In the present study, it has been shown that in primary hepatocytes the GK expression can be activated by HNF-4α (Figure 1). Transient transfection of GK promoter LUC gene constructs GK-1430 LUC, GK-101 LUC and GK-101 HNFm LUC together with the HNF-4α expression vector show that HNF-4α can enhance LUC activity via the HNF element (−52/−39) in primary rat hepatocytes (Figure 3). The interaction of HNF-4α at the GK–HNF-responsive element can be demonstrated by EMSA (Figure 4). Thus it has been shown for

the first time that HNF-4α can influence the GK gene activation via the HNF-4 site in the P1 region.

HNF-4 as a regulator of developmental and nutrient-dependent gene expression

HNF-4 responsive elements have been identified in genes, the expression of which is essential for lipid–glucose homoeostasis, e.g. apolipoprotein genes *ApoB*, *ApoCIII*, *ApoAII*, glucose transporter 2 and the glycolytic enzyme genes aldolase B, glyceraldehyde-3-phosphate dehydrogenase and the pyruvate kinase type liver (PK_L). HNF-4 is also essential for the expression of gene products which are responsible for the proliferation and differentiation of red blood cells such as erythropoietin (EPO) [21–25,27]. Several of these genes contain HNF-4 responsive elements which only matched the HNF-4 consensus sequence [13] in 9 or 11 of 12 positions, but they were all functionally active [27,39–41]. Moreover, the GK–HNF-4 responsive element (−52/−39) in the rat GK promoter matched the HNF-4 consensus site in 11 of 12 positions and was functionally active. However, the HNF-4 responsive element of the GK promoter does not appear to be an optimal HNF-4 responsive element, since the direct repeats of the element are separated by only two nucleotides. The influence of different nucleotide numbers between the HNF-4 repeats was tested in EMSAs by using nuclear extracts from COS cells transfected with an HNF-4 WT expression vector [42]. It was demonstrated that HNF-4 WT bound with high affinity to a response element consisting of a direct repeat separated by one nucleotide, whereas a direct repeat separated by two nucleotides reduced binding of HNF-4 WT and the direct repeat without spacer almost completely suppressed binding of HNF-4 WT. Thus, despite the fact that GK–HNF-4 element does not represent a high-affinity binding site, it remained functionally active.

GK is a key enzyme in glucose utilization and the results from embryonic stem cells lacking the HNF-4 gene have shown the importance of HNF-4 for the expression of genes, products of which are involved in glucose metabolism. Thus, the expression of genes encoding glucose transporter 2, and the glycolytic enzymes aldolase B, glyceraldehyde-3-phosphate dehydrogenase and the PK_L is reduced in HNF-4α-deficient (−/−) embryonic stem cells [25,43]. In the study with HNF-4 −/− embryonic stem cells, the GK gene expression appeared to be unaffected. The reason for this could be that GK first appears in the liver of rats 2 weeks after birth and after weaning [44,45].

Both, GK and HNF-4α have been shown to be responsible for MODY. Both, MODY1 with a defective HNF-4 gene and MODY2 caused by GK gene mutations, are autosomal-dominant inherited diseases and characterized by defective insulin secretion of the β-cells of the pancreas [24,46,47]. Thus in MODY1 patients, hepatic glucose utilization can be impaired due to reduced GK expression caused by the defective HNF-4.

The 5′-flanking region of the PK_L promoter contains an HNF-4 responsive element, called L3 box. It has been shown that overexpression of HNF-4 results in activation of the PK_L promoter [39]. The PK_L gene is positively regulated in the presence of insulin by glucose and negatively regulated by glucagon via cAMP [48,49]. In this way, HNF-4 is phosphorylated by protein kinase A suppressing the binding of HNF-4 to the L3 box [50]. These results indicate that HNF-4 is phosphorylated by protein kinase A and therefore HNF-4 might be involved in the transcriptional inhibition of liver genes, e.g. by glucagon via cAMP. Thus, it appears that HNF-4 is a possible candidate regulating the inhibition of insulin-mediated GK induction by glucagon.

HNF-4 as a cofactor regulating the O_2-dependent gene expression

The findings of this study, namely that perivenous pO_2 enhanced the levels and the binding of HNF-4 to the GK–HNF-4 element clearly indicate that HNF-4 might act as a regulatory factor mediating the perivenous zonated GK expression in the liver acinus. This is also supported by the results of a study [51], which showed that HNF-4 mRNA levels were enhanced in perivenous cell lysates compared with periportal cell lysates and that a higher HNF-4 content was detected in the less aerobic perivenous region of the rat liver. Furthermore, it was demonstrated that HNF-4 bound as a transcriptional activator to a region within the hypoxia-sensitive EPO enhancer, thereby enhancing the hypoxia-induced EPO gene expression [27]. These findings together with the results of our study suggest that HNF-4α might play a role in the O_2-mediated zonated GK expression in rat liver.

This study was supported by the Deutsche Forschungsgemeinschaft SFB 402, TP A1 and GRK 335. We thank Professor P. B. Iynedjian (Division of Clinical and Diabetes Research, University of Geneva School of Medicine, Geneva, Switzerland) and Professor F. Sladek (Department of Cell Biology and Neuroscience, University of California, Riverside, CA, U.S.A.) for the gift of plasmids.

REFERENCES

1 Parry, M. J. and Walker, D. G. (1966) Purification and properties of adenosine 5′-triphospate-D-glucose 6-phosphotransferase from rat liver. Biochem. J. **99**, 266–274
2 Vandercammen, A. and Van Schaftingen, E. (1991) Competitive inhibition of liver glucokinase by its regulatory protein. Eur. J. Biochem. **200**, 545–551
3 Vandercammen, A. and Van Schaftingen, E. (1990) The mechanism by which rat liver glucokinase is inhibited by the regulatory protein. Eur. J. Biochem. **191**, 483–489
4 Printz, R., Magnuson, M. A. and Granner, D. (1993) Mammalian glucokinase. Annu. Rev. Nutr. **13**, 463–496
5 Iynedjian, P. B. (1993) Mammalian glucokinase and its gene. Biochem. J. **293**, 1–13
6 Iynedjian, P. B., Pilot, P. R., Nouspikel, T., Milburn, J. L., Quaade, C., Hughes, S., Ucla, C. and Newgard, C. B. (1989) Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. Proc. Natl. Acad. Sci. U.S.A. **86**, 7838–7842
7 Jetton, T. L., Liang, Y., Pettepher, C. C., Zimmerman, E. C., Cox, F. G., Horvath, K., Matschinsky, F. M. and Magnuson, M. A. (1994) Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut. J. Biol. Chem. **269**, 3641–3654
8 Jungermann, K. and Kietzmann, T. (1996) Zonation of parenchymal and nonparenchymal metabolism in liver. Annu. Rev. Nutr. **16**, 179–203
9 Magnuson, M. A. and Shelton, K. D. (1989) An alternate promoter in the glucokinase gene is active in the pancreatic β cell. J. Biol. Chem. **264**, 15936–15942
10 Postic, C., Niswender, K., Decaux, J., Parsa, R., Shelton, K., Gouhot, B., Pettepher, C., Granner, D., Girard, J. and Magnuson, M. A. (1995) Cloning and characterization of the mouse glucokinase gene locus and identification of distal liver-specific DNase I hypersensitive sites. Genomics **29**, 740–750
11 Iynedjian, P. B. (1998) Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. Biochem. J. **333**, 705–712
12 Foretz, M., Guichard, C., Ferre, P. and Foufelle, F. (1999) Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. Proc. Natl. Acad. Sci. U.S.A. **96**, 12737–12742
13 Sladek, F. M., Zhong, W. M., Lai, E. and Darnell-JE, J. (1990) Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. **4**, 2353–2365
14 Chartier, F. L., Bossu, J. P., Laudet, V., Fruchart, J. C. and Laine, B. (1994) Cloning and sequencing of cDNAs encoding the human hepatocyte nuclear factor 4 indicate the presence of two isoforms in human liver. Gene **147**, 269–272
15 Drewes, T., Senkel, S., Holewa, B. and Ryffel, G. U. (1996) Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. Mol. Cell Biol. **16**, 925–931
16 Hata, S., Inoue, T., Kosuga, K., Nakashima, T., Tsukamoto, T. and Osumi, T. (1995) Identification of two splice isoforms of mRNA for mouse hepatocyte nuclear factor 4 (HNF-4). Biochim. Biophys. Acta **1260**, 55–61
17 Kritis, A. A., Argyrokastritis, A., Moschonas, N. K., Power, S., Katrakili, N., Zannis, V. I., Cereghini, S. and Talianidis, I. (1996) Isolation and characterization of a third isoform of human hepatocyte nuclear factor 4. Gene **173**, 275–280

18 Holewa, B., Zapp, D., Drewes, T., Senkel, S. and Ryffel, G. U. (1997) HNF4β, a new gene of the HNF4 family with distinct activation and expression profiles in oogenesis and embryogenesis of *Xenopus laevis*. Mol. Cell Biol. **17**, 687–694
19 Jiang, G., Nepomuceno, L., Hopkins, K. and Sladek, F. M. (1995) Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. Mol. Cell Biol. **15**, 5131–5143
20 Chen, W. S., Manova, K., Weinstein, D. C., Duncan, S. A., Plump, A. S., Prezioso, V. R., Bachvarova, R. F. and Darnell-JE, J. (1994) 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
21 Ladias, J. A., Hadzopoulou, C. M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V. and Cladaras, C. (1992) Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3. J. Biol. Chem. **267**, 15849–15860
22 Mietus, S. M., Sladek, F. M., Ginsburg, G. S., Kuo, C. F., Ladias, J. A., Darnell-JE, J. and Karathanasis, S. K. (1992) Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol. Cell Biol. **12**, 1708–1718
23 Metzger, S., Halaas, J. L., Breslow, J. L. and Sladek, F. M. (1993) Orphan receptor HNF-4 and bZip protein C/EBPα bind to overlapping regions of the apolipoprotein B gene promoter and synergistically activate transcription. J. Biol. Chem. **268**, 16831–16838
24 Yamagata, K., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., Fajans, S. S., Signorini, S., Stoffel, M. and Bell, G. I. (1996) Mutations in the hepatocyte nuclear factor-4α gene in maturity-onset diabetes of the young (MODY1) (see comments). Nature (London) **384**, 458–460
25 Stoffel, M. and Duncan, S. A. (1997) The maturity-onset diabetes of the young (MODY1) transcription factor HNF4α regulates expression of genes required for glucose transport and metabolism. Proc. Natl. Acad. Sci. U.S.A. **94**, 13209–13214
26 Iynedjian, P. B. (1998) Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. Biochem. J. **333**, 705–712
27 Galson, D. L., Tsuchiya, T., Tendler, D. S., Huang, L. E., Ren, Y., Ogura, T. and Bunn, H. F. (1995) The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol. Cell Biol. **15**, 2135–2144
28 Sladek, F. M. (1993) Orphan receptor HNF-4 and liver-specific gene expression [published erratum appears in Receptor 1994 Spring; 4(1): following 63]. Receptor **3**, 223–232
29 Immenschuh, S., Hinke, V., Ohlmann, A., Gifhorn, K. S., Katz, N., Jungermann, K. and Kietzmann, T. (1998) Transcriptional activation of the haem oxygenase-1 gene by cGMP via a cAMP response element/activator protein-1 element in primary cultures of rat hepatocytes. Biochem. J. **334**, 141–146
30 Kietzmann, T., Roth, U., Freimann, S. and Jungermann, K. (1997) Arterial oxygen partial pressures reduce the insulin-dependent induction of the perivenously located glucokinase in rat hepatocyte cultures: mimicry of arterial oxygen pressures by H_2O_2. Biochem. J. **321**, 17–20
31 Wiesener, M. S., Turley, H., Allen, W. E., Willam, C., Eckardt, K. U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W. et al. (1998) Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1α. Blood **92**, 2260–2268
32 Laemmli, U. K. (1970) A factor preventing the major head protein of bacteriophage T4 from random aggregation. Nature (London) **227**, 680–685
33 Brinkmann, A., Katz, N., Sasse, D. and Jungermann, K. (1978) Increase of the gluconeogenic and decrease of the glycolytic capacity of rat liver with a change of the metabolic zonation after partial hepatectomy. Hoppe Seylers. Z. Physiol. Chem. **359**, 1561–1571
34 Oliver, I. T., Edwards, A. M. and Pitot, H. C. (1978) Hormonal regulation of phosphoenolpyruvate carboxykinase in primary cultures of adult-rat liver parenchymal cells. Eur. J. Biochem. **87**, 221–227
35 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**, 1475–1489
36 Semenza, G. L. and Wang, G. L. (1992) A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. **12**, 5447–5454
37 Kietzmann, T., Roth, U. and Jungermann, K. (1999) Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia inducible factor-1 in rat hepatocytes. Blood **94**, 4177–4185
38 Probst, I., Schwartz, P. and Jungermann, K. (1982) Induction in primary culture of 'gluconeogenic' and 'glycolytic' hepatocytes resembling periportal and perivenous cells. Eur. J. Biochem. **126**, 271–278
39 Diaz, G. M., Bergot, M. O., Martinez, A., Cuif, M. H., Kahn, A. and Raymondjean, M. (1993) Functional characterization of the L-type pyruvate kinase gene glucose response complex. Mol. Cell Biol. **13**, 7725–7733
40 Hall, R. K., Sladek, F. M. and Granner, D. K. (1995) The orphan receptors COUP-TF and HNF-4 serve as accessory factors required for induction of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. Proc. Natl. Acad. Sci. U.S.A. **92**, 412–416
41 Fraser, J. D., Martinez, V., Straney, R. and Briggs, M. R. (1998) DNA binding and transcription activation specificity of hepatocyte nuclear factor 4. Nucleic Acids Res. **26**, 2702–2707
42 Bogan, A. A., Dallas-Yang, Q., Ruse, M. D. J., Maeda, Y., Jiang, G., Nepomuceno, L., Scanlan, T. S., Cohen, F. E. and Sladek, F. M. (2000) Analysis of protein dimerization and ligand binding of orphan receptor HNF4α. J. Mol. Biol. **302**, 831–851
43 Li, J., Ning, G. and Duncan, S. A. (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4α. Genes Dev. **14**, 464–474
44 Bossard, P., Parsa, R., Decaux, J. F., Iynedjian, P. and Girard, J. (1993) Glucose administration induces the premature expression of liver glucokinase gene in newborn rats. Relation with DNase-I-hypersensitive sites. Eur. J. Biochem. **215**, 883–892
45 Postic, C., Leturque, A., Printz, R. L., Maulard, P., Loizeau, M., Granner, D. K. and Girard, J. (1994) Development and regulation of glucose transporter and hexokinase expression in rat. Am. J. Physiol. **266**, E548–E559
46 Fajans, S. S. (1989) Maturity-onset diabetes of the young (MODY). Diabetes Metab. Rev. **5**, 579–606
47 Vionnet, N., Stoffel, M., Takeda, J., Yasuda, K., Bell, G. I., Zouali, H., Lesage, S., Velho, G., Iris, F., Passa, P. et al. (1992) Nonsense mutation in the glucokinase gene causes early-onset non-insulin-dependent diabetes mellitus. Nature (London) **356**, 721–722
48 Decaux, J. F., Antoine, B. and Kahn, A. (1989) Regulation of the expression of the L-type pyruvate kinase gene in adult rat hepatocytes in primary culture. J. Biol. Chem. **264**, 11584–11590
49 Vaulont, S., Munnich, A., Decaux, J. F. and Kahn, A. (1986) Transcriptional and post-transcriptional regulation of L-type pyruvate kinase gene expression in rat liver. J. Biol. Chem. **261**, 7621–7625
50 Viollet, B., Kahn, A. and Raymondjean, M. (1997) Protein kinase A-dependent phosphorylation modulates DNA-binding activity of hepatocyte nuclear factor 4. Mol. Cell Biol. **17**, 4208–4219
51 Lindros, K. O., Oinonen, T., Issakainen, J., Nagy, P. and Thorgeirsson, S. S. (1997) Zonal distribution of transcripts of four hepatic transcription factors in the mature rat liver. Cell Biol. Toxicol. **13**, 257–262

Received 28 February 2002/4 April 2002; accepted 12 April 2002
Published as BJ Immediate Publication 12 April 2002, DOI 10.1042/BJ20020340