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Liver glucokinase gene expression is controlled by the onecut transcription factor hepatocyte nuclear factor-6

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Abstract

Aims/hypothesis. Glucokinase plays a key role in glucose homeostasis and the expression of its gene is differentially regulated in pancreatic beta cells and in the liver through distinct promoters. The factors that determine the tissue-specific expression of the glucokinase gene are not known. Putative binding sites for hepatocyte nuclear factor (HNF)-6, the prototype of the ONECUT family of transcription factors, are present in the hepatic promoter of the glucokinase gene and in diabetic *hnf6* knockout mice. We hypothesized that HNF-6 controls the activity of the hepatic glucokinase promoter.
Methods. We tested the binding of recombinant HNF-6 to DNA sequences from the mouse hepatic glucokinase promoter in vitro and the effect of HNF-6 on promoter activity in transfected cells. We investigated in vivo the role of HNF-6 in mice by examining the effect of inactivating the *hnf6* gene on glucokinase gene-specific deoxyribonuclease I hypersensitive sites in liver chromatin and on liver glucokinase mRNA concentration.
Results. HNF-6 bound to the hepatic promoter of the glucokinase gene and stimulated its activity. Inactivation of the *hnf6* gene did not modify the pattern of deoxyribonuclease I hypersensitive sites but was associated with a decrease of liver glucokinase mRNA to half the control value.
Conclusions/interpretation. Although HNF-6 is not required to open chromatin of the hepatic promoter of the glucokinase gene, it stimulates transcription of the glucokinase gene in the liver. This could partly explain the diabetes observed in *hnf6* knockout mice. [Diabetologia (2002) 45:1136–1141]

Keywords Glucokinase, HNF-6, MODY, OC-2, onecut transcription factors, Type II diabetes mellitus.

In the liver, glucokinase catalyses the first reaction for the storage of glucose as glycogen or for its utilisation through the glycolytic and pentose phosphate pathways. In the endocrine pancreas, glucokinase serves as a glucose sensor in beta cells [1]. Thus, glucokinase plays a key role in glucose homeostasis. Indeed mutations in the glucokinase gene are associated with a subtype of MODY, MODY2 [2, 3] and inactivation of the glucokinase gene causes Type II (non-insulin-dependent) diabetes mellitus in the mouse [4, 5].

Liver glucokinase is coded by a mRNA which differs in its 5'portion from that coding for beta-cell glucokinase [6, 7]. These two mRNAs originate from distinct promoters of the same gene, different first exons being spliced to the same nine common exons which code for the catalytic region of the protein [8]. Because they differ in cis-acting sequences, tissue-specific promoters allow for a distinct expression and transcriptional regulation of the same enzyme in different tissues. One way to delineate promoter boundaries is to evaluate their accessibility to transcriptional activators in chromatin by analysing DNase I hyper-

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Abbreviations: Hss, Hypersensitive sites; HNF, hepatocyte nuclear factor; OC-2, onecut-2; PFK-2, 6-phosphofructo-2-kinase; EMSA, electrophoretic mobility shift assay.

sensitive sites (Hss), which are indicative of nucleosome disruption. This technique has shown that, both in rats and in mice, the hepatic promoter of the glucokinase gene encompasses about 7 kb of 5′ flanking sequence [9, 10, 11]. The factors that determine the liver-specific transcription of the hepatic glucokinase mRNA have not been identified.

No DNase I Hss sites are found on the hepatic glucokinase gene promoter in chromatin from tissues other than the liver. The hepatic glucokinase gene promoter is in a closed chromatin conformation outside the liver. In liver, a hepatocyte-specific factor would bind to the hepatic glucokinase gene promoter and disrupt nucleosomes to render it accessible to the transcriptional machinery. A candidate is the hepatocyte nuclear factor (HNF)-6 [12]. This transcription factor is the prototype of the ONECUT class of homeodomain proteins, the members of which possess two DNA-binding domains, a cut domain and a divergent homeodomain [13]. Two members of this class are present in mammalian liver, namely HNF-6 and Onecut-2 (OC-2) [14]. HNF-6 is expressed in the embryonic liver and pancreas [15, 16] and it plays an important role in the development of these organs [17]. In the adult, HNF-6 is involved in the liver-specific control of several genes of glucose metabolism, e.g. 6-phosphofructo-2-kinase (PFK-2) [12], phosphoenolpyruvate carboxykinase [18] and glucose-6-phosphatase [19]. Moreover, the hepatic promoter region of the glucokinase gene contains two sequences that are putative binding sites for HNF-6 [12]. Our aim was to evaluate the role of HNF-6 in the transcriptional control of liver glucokinase and to test whether HNF-6 is required to open chromatin of the hepatic promoter.

Materials and methods

Plasmid constructions. pECE-HNF6α, pECE-HNF6β and pXJ42-OC2 have been described [12, 13, 14]. The pXP2-GKS1 firefly luciferase reporter vector contains the hepatic promoter of the mouse glucokinase gene (–987 to +19) cloned in the *Sac*I and *Bgl*II sites of pXP2. This gene fragment contains the proximal (–868 to –877) HNF-6 binding site. Plasmid pXP2-GKS10 is identical except that it contains a shorter fragment (–520 to +19) of the glucokinase promoter, which is devoid of the proximal HNF-6 binding site. This 539-bp fragment was amplified with the oligonucleotide primer pair 5′-CCAGAGCTCCTGAATCTTCTCCAGC-3′ and 5′-AGGAGATCTAGGACTTCTGCACTAATGGGTC-3′. Plasmid pXP2-GKS9 is identical to pXP2-GKS1 except for an additional fragment containing bp –6000 to –7800 of the glucokinase gene cloned in the *Sac*I site of pXP2. This fragment includes the distal (–7613 to –7622) HNF-6 binding site. The internal control vector pRL-138 coding *Renilla* luciferase contains the *pfk-2* promoter (-138 to +86) cloned in pRLnull (Promega, Leiden, The Netherlands) [13].

Transfections and cell extracts. Rat hepatoma FTO-2B cells were grown in DMEM/Ham's F-12 medium supplemented with 10% FCS. Cells (1×10^5 cells per well on 24-well plates) were transfected in medium without serum by lipofection using lipofectamine-PLUS (Life Technologies, Invitrogen, Merelbeke, Belgium), 400 ng of pXP2-GK10, pXP2-GK1 or pXP2-GK9, 30 ng of pECE-HNF-6α or pECE-HNF-6β or pXJ42-OC2 and 15 ng of pRL-138 as internal control. After 4 h the cells were washed with phosphate-buffered saline and further incubated for 45 h in DMEM/Ham's F-12 medium plus 10% serum before measuring luciferase activities with the dual luciferase kit and TD-20/20 luminometer (Promega). Luciferase activities were expressed as the ratio of reporter activity (firefly luciferase) to internal control activity (*Renilla* luciferase). COS-7 cells (3×10^5 cells per 6-cm dish) were transfected in DMEM without serum by lipofection using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-triethylammonium methylsulfate (DOTAP, Boehringer-Mannheim, Mannheim, Germany) and 5 µg of expression vector. Fortyeight hours after transfection, the cells were washed with phosphate-buffered saline and harvested in 1 ml of 40 mmol/l Tris-Cl (pH 7.5), 1 mmol/l EDTA, 150 mmol/l NaCl. The cells were pelleted and resuspended in 60 µl of 50 mmol/l Tris-Cl (pH 7.9), 500 mmol/l KCl, 0.5 mmol/l EDTA, 2.5 µg/ml leupeptin, 1 mmol/l dithiothreitol, 0.1% (vol/vol) Nonidet-P40, 1 mmol/l phenylmethylsulfonyl fluoride and 20% (vol/vol) glycerol. After three freeze-thaw cycles, the lysates were centrifuged and the supernatants collected.

Electrophoretic mobility shift assays (EMSA). COS-7 cells lysates (5 µl) were incubated on ice for 20 min in a final volume of 20 µl containing 10 mmol/l HEPES (pH 7.6), 1 mmol/l dithiothreitol, 1 mmol/l $MgCl_2$, 0.5 mmol/l EGTA, 50 mmol/l KCl, 10% (vol/vol) glycerol, 4 µg of poly(dI-dC) and the ^{32}P-labelled probe (30,000 cpm). The samples were loaded on a 7% acrylamide gel (acrylamide/bisacrylamide ratio was 29:1) in 25 mmol/l Tris-borate, 0.25 mmol/l EDTA and electrophoresed at 200 V. The double-stranded oligonucleotide probes used in EMSA were as follows (the HNF-6 binding site is underlined): hepatic GK proximal, 5′-TTTGGGAAAGTGATCAATCGTGTCAAGGGG-3′ (–887 to –858 of the mouse hepatic glucokinase promoter); hepatic GK distal, 5′-TCCTATTTTGAAATCAATATGGAATCTCAA-3′ (–7603 to –7632 of the mouse hepatic glucokinase promoter).

Animals. hnf6 null mice were selected in our laboratory and treated according to the principles of laboratory animal care of the University animal welfare committee. These mice have a defective development of the endocrine pancreas and they are diabetic [17]. As the penetrance of this phenotype is variable, the severity of diabetes was determined by a glucose tolerance test. Mice (5 to 16 week-old) fasted overnight were injected intraperitoneally with 2 g of glucose per kg of body weight. Blood glucose (tail vein) was measured with an Elite glucometer (Bayer, Leverkusen, Germany) immediately before and 20, 60 and 120 min after the injection. As *hnf6+/–* mice appeared to be normal they served as controls together with *hnf6+/+* mice.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the liver or pancreas of male and female fed mice, with TriPure reagent and following the manufacturer's instructions (Boehringer). cDNA was synthesized using Moloney leukaemia virus reverse transcriptase and random hexamer primers (Gibco-BRL, Merelbeke, Belgium). These cDNAs provided templates for PCR using specific primers in the presence of α^{32}P-dCTP and *taq* DNA polymerase. The following forward and reverse primers were used for specific amplification: hepatic glucokinase, 5′-GCTGTGGATACTACAAGGAGG-3′ and 5′-ACCAGCATCACCCTGAAGTTG-3′; OC-2, 5′-GCCACGCCGCTGGGCAAC-3′ and

5′-CAGCTGCCCGGACGTGGC-3′; β-*actin*, 5′-GGCATCGTGATGGACTCCG-3′ and 5′-GCTGGAAGGTGGACAGCGA-3′. Reactions without reverse transcriptase served as negative controls.

Statistical analysis. Differences in means between groups were compared by the paired Student's *t* test. A *p* value of less than 0.05 was considered significant.

DNase hypersensitives sites. Nuclei for chromatin studies were isolated from fresh mouse liver as described [20] and stored at –80°C until use. DNase I digestion has been described [10]. The probe used for analysis of the DNase I hypersensitive sites was a 350-bp *Bam*HI-*Sph*I fragment from the genomic clone λ40 of mouse glucokinase [10]. This fragment is located at the beginning of the first intron of the gene. The probe was labelled by random priming and the blot was washed as described [9] before autoradiography.

Results

HNF-6 binds to the hepatic promoter of the glucokinase gene. Examination of the mouse hepatic glucokinase gene promoter showed that it contains a proximal sequence on the sense strand (–877) TGATCAATCG (–868) and a distal sequence on the antisense strand (–7613) AAATCAATAT (–7622) that are compatible with the 10-bp consensus for binding HNF-6 [13]. We found that these sequences are also present in the rat and human glucokinase genes, which suggests their functional importance. The binding of HNF-6 to these two sequences was investigated by EMSA. Oligonucleotides corresponding to the proximal or distal sequence were incubated in the presence of extracts of cells that are devoid of endogenous HNF-6 and that had been transfected with expression vectors for the two isoforms of HNF-6. These isoforms originate from differential splicing of the same primary transcript and differ by a linker of 26 residues between the cut domain and the homeodomain. This linker is present in HNF-6β but not in HNF-6α. These two isoforms were tested because they have different DNA-binding specificities depending on the DNA target [13]. Despite weak binding of proteins present in extracts from nontransfected cells, there was clear binding of HNF-6α and HNF-6β to both the proximal and the distal sequences (Fig. 1). We also tested the binding of OC-2, as this homologue of HNF-6 is also present in liver and it is known to bind to some, but not all, HNF-6 binding sites [14]. The data in Fig. 1 show that OC-2 bound to both the proximal and the distal HNF-6 sites in the hepatic glucokinase gene promoter. No HNF-6 binding site was found in the pancreatic promoter of the glucokinase gene.

Liver chromatin over the promoter is decondensed even in absence of HNF-6. We tested whether HNF-6 is involved in opening chromatin in the region of the hepatic glucokinase gene promoter in vivo. To

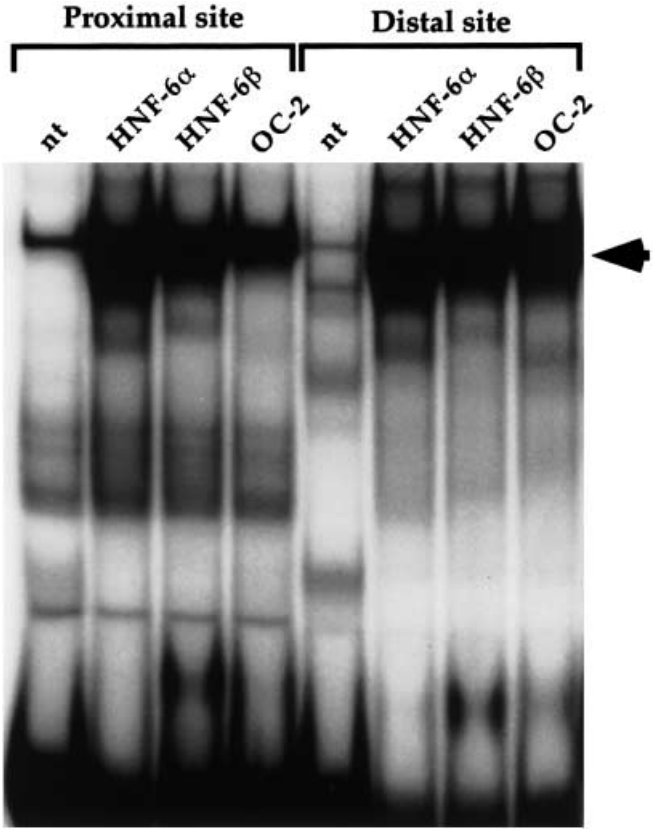

Fig. 1. HNF-6 and OC-2 bind to the hepatic promoter of the glucokinase gene. EMSA were carried out with extracts from nontransfected COS-7 cells (nt) or from COS-7 cells transfected with an expression vector for HNF-6α, HNF-6β or OC-2 as indicated. The radioactive probes used are indicated above the lanes and described in Materials and Methods. The *arrowhead* points to specific complexes

this end, we first mapped the DNase I Hss on this promoter in mouse liver. Six DNase I Hss were detected, as expected [10] (Fig. 2). Although the distal HNF-6 binding site does not correspond to a DNase I Hss, the proximal HNF-6 binding site coincides with Hss 2. Therefore, we hypothesized that Hss 2 and perhaps also other liver-specific DNase I Hss might disappear in the absence of HNF-6. To investigate this possibility, we repeated the experiment with liver from mice in which we have inactivated the *hnf6* gene by homologous recombination [17]. We found earlier that inactivation of the *hnf6* gene leads to incomplete maturation of beta cells and to perturbed structure of the islets of Langerhans [17]. This leads to diabetes mellitus in the mice that survive until adulthood. These diabetic mice fail to increase their basal insulin secretion in response to glucose administration [17]. The phenotype of the *hnf6*–/– mice is not fully penetrant (our unpublished observations) and 20 to 25% of the mice do not show glucose intolerance. As expression of the glucokinase gene is stimulated by insulin [21, 22], we separated non-diabetic from diabetic *hnf6*–/– mice based on glucose tolerance tests. We found that, both in the

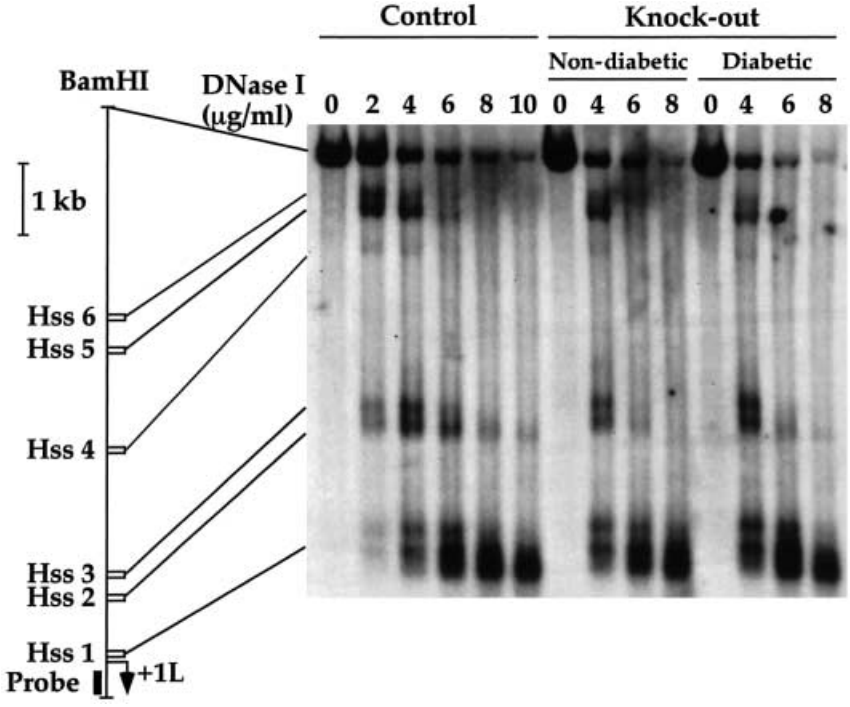

Fig. 2. Detection and mapping of DNase I hypersensitive sites in the mouse hepatic glucokinase gene promoter. Nuclei isolated from the liver of four *hnf6*+/+ mice and from two non-diabetic and two diabetic *hnf6*–/– mice were digested with the indicated concentration of DNase I. The DNA was extracted and restricted with *Bam*HI and the fragments were separated in a 1% agarose gel. The blot was hybridised with the radioactive probe indicated and autoradiographed to reveal the non-digested fragment (8.5 kb) and fragments corresponding to Hss1 at –0.1 kb, Hss2 at –0.8 kb, Hss3 at –1.25 kb, Hss4 at –3 kb, Hss5 at –4.5 kb and Hss6 at –5 kb

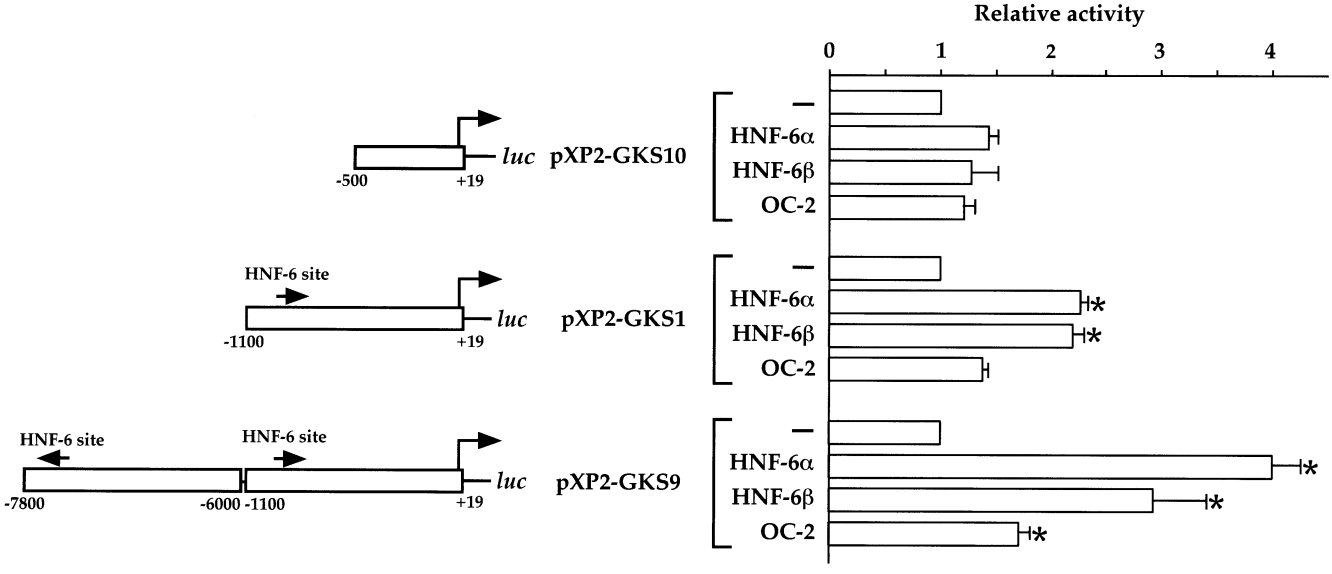

Fig. 3. HNF-6, but not OC-2, stimulates the activity of the hepatic promoter of the glucokinase gene. FTO-2B cells were transiently transfected with the firefly luciferase reporter constructs indicated without (–) or with expression vectors for HNF-6α, HNF-6β or OC-2 and an internal control plasmid coding for *Renilla* luciferase. The relative promoter activity is the normalised ratio of firefly to *Renilla* luciferase activities. Data are means ± SEM for four independent experiments. * Denotes a statistically significant difference with data for cells transfected with the pXP2-GKS10 expression vector

non-diabetic and in the diabetic *hnf6*–/– mice, the DNase I Hss corresponding to the proximal HNF-6 binding site (Hss 2) was still present. The overall pattern of the DNase I Hss map was not affected by inactivation of the *hnf6* gene. We concluded that liver-specific factors other than HNF-6 open the chromatin on the hepatic glucokinase gene promoter.

HNF-6 stimulates liver glucokinase gene expression in vivo. To determine the influence of HNF-6 on the

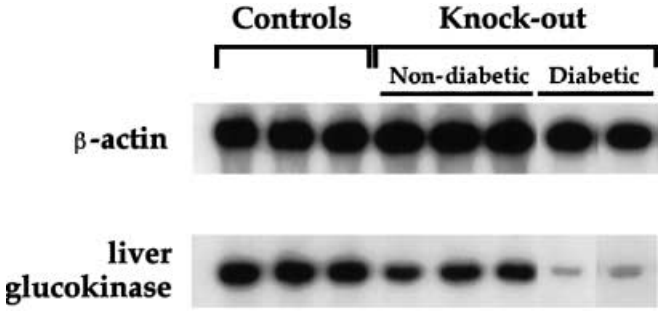

Fig. 4. Liver glucokinase mRNA concentration is decreased in *hnf6–/–* mice. Liver glucokinase mRNA was measured by semi-quantitative RT-PCR, with β-actin mRNA as a reference. Representative individual experiments are shown for control (*hnf6*+/+ or *hnf6*+/–) mice and for non-diabetic or diabetic *hnf6–/–* mice

activity of the open hepatic glucokinase promoter, we made reporter constructs in which the luciferase gene is cloned downstream of the proximal and distal HNF-6 binding regions of the hepatic glucokinase promoter. These reporters were transiently cotransfected in cells with expression vectors for HNF-6α or β. Under these conditions, reporter plasmids are not integrated into chromatin and are therefore accessible to transcription factors. HNF-6 had no effect on the reporter construct devoid of HNF-6 binding site (Fig. 3). In contrast, a two to threefold stimulation was observed with HNF-6α and with HNF-6β when the proximal promoter region contained the HNF-6 binding sequence. The stimulation reached three to fourfold when the region that contains the distal HNF-6 binding site was added to the proximal region. We concluded that HNF-6 can stimulate directly the activity of the hepatic promoter of the glucokinase gene.

To demonstrate that HNF-6 controls the liver expression of the glucokinase gene in vivo, we measured the amount of liver glucokinase mRNA in *hnf6–/–* mice. We found that liver glucokinase mRNA concentration in nondiabetic *hnf6–/–* mice was reduced to 46% (±4 SEM) (n=8) of control values (n=7) (Fig. 4). A further drop to 15% (±5 SEM) (n=7) of control values was seen in diabetic mice (Fig. 4), as expected from their insulinopenia. Therefore, in vivo HNF-6 is required for full expression of the glucokinase gene in liver independently of insulin. In contrast, glucokinase mRNA concentration in the pancreas was not affected by the inactivation of the *hnf6* gene [17]. This is consistent with our failure to find HNF-6 binding sites in the pancreatic promoter of the glucokinase gene.

As OC-2 binds to the HNF-6 sites in the hepatic glucokinase gene promoter (Fig. 1), the drop in liver glucokinase mRNA observed in *hnf6–/–* mice was surprising because OC-2 was expected to compensate for the lack of HNF-6. Indeed, OC-2 gene expression was not only maintained but it was actually up-regulated in the liver of *hnf6–/–* mice (data not shown), suggesting that HNF-6 exerts a negative feed-back on OC-2 gene expression. We were left with the hypothesis that, although OC-2 binds to the hepatic glucokinase promoter, it does not activate it. To test this, we cotransfected cells with an OC-2 expression vector and the glucokinase promoter-luciferase reporter constructs that had been used to test the activity of HNF-6. Contrary to what was seen with HNF-6, the stimulatory effect of OC-2 on the hepatic glucokinase promoter was negligible (Fig. 3). We have shown that coactivator recruitment by HNF-6 depends on the target gene [23]. Failure of OC-2 to recruit the appropriate coactivator when it binds the hepatic glucokinase promoter could explain why OC-2 does not compensate in this particular case for the lack of HNF-6.

Discussion

In humans heterozygous mutations in the glucokinase gene are associated with MODY2 [2]. These mutations decrease the affinity of glucokinase for glucose and MODY2 seems to result from decreased glucose sensitivity and insulin secretion by the beta cell and from decreased liver glucose metabolism [4, 5]. The *hnf6–/–* phenotype is not fully penetrant since 20 to 25% of the mice are not diabetic. These mice show a 50% decrease in liver glucokinase mRNA but have normal pancreatic expression of the glucokinase gene. This is consistent with our finding that HNF-6 binds to the hepatic glucokinase promoter but not to the pancreatic promoter. These non-diabetic *hnf6–/–* mice can therefore be compared with humans who show mutations in the hepatic promoter of the glucokinase gene that reduce glucokinase expression by about 50%, but are not associated with glucose intolerance [24]. In diabetic *hnf6–/–* mice, which constitute the majority of the *hnf6–/–* mice, insulinopenia resulting from impaired development of the endocrine pancreas and poor differentiation of the beta cells induces glucose intolerance [17]. In these mice, liver glucokinase gene expression is reduced to barely detectable levels, presumably because the lack of stimulation of the liver glucokinase promoter by HNF-6 is combined with the lack of insulin stimulation of the promoter. This liver defect in turn is expected to contribute to glucose intolerance [5], so that the diabetes mellitus of *hnf6–/–* mice would result from a defective function of the pancreas as well as of the liver.

Our previous work on a Danish population failed to show an association between MODY or late-onset Type II (non-insulin-dependent) diabetes mellitus and mutations in the *HNF-6* gene [25]. However, we show that HNF-6 plays a key role in the control of liver glucose homeostasis. Mutations in the HNF-6 binding sites in the glucokinase gene could therefore contribute to diabetes.

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