The EMBO Journal Vol.17 No.22 pp.6701–6713, 1998

Dominant-negative suppression of HNF-1α function results in defective insulin gene transcription and impaired metabolism–secretion coupling in a pancreatic β-cell line

Haiyan Wang, Pierre Maechler, Kerstin A.Hagenfeldt and Claes B.Wollheim[1]

Division de Biochimie Clinique et de Diabétologie Expérimentale, Centre Médical Universitaire, CH-1211 Geneva 4, Switzerland

[1]Corresponding author
e-mail: claes.wollheim@medicine.unige.ch

Mutations in the hepatocyte nuclear factor-1α (HNF-1α) have been linked to subtype 3 of maturity-onset diabetes of the young (MODY3), which is characterized by a primary defect in insulin secretion. The role of HNF-1α in the regulation of pancreatic β-cell function was investigated. Gene manipulation allowed graded overexpression of HNF-1α and controlled dominant-negative suppression of HNF-1α function in insulinoma INS-1 cells. We show that HNF-1α is essential for insulin gene transcription, as demonstrated by a pronounced decrease in insulin mRNA expression and in insulin promoter activity under dominant-negative conditions. The expression of genes involved in glucose transport and metabolism including glucose transporter-2 and L-type pyruvate kinase is also regulated by HNF-1α. Loss of HNF-1α function leads to severe defects in insulin secretory responses to glucose and leucine, resulting from impaired glucose utilization and mitochondrial oxidation. The nutrient-evoked ATP production and subsequent changes in plasma membrane potential and intracellular Ca^{2+} were diminished by suppression of HNF-1α function. These results suggest that HNF-1α function is essential for maintaining insulin storage and nutrient-evoked release. The defective mitochondrial oxidation of metabolic substrates causes impaired insulin secretion, indicating a molecular basis for the diabetic phenotype of MODY3 patients.

Keywords: HNF1α/insulin secretion/insulin transcription/mitochondria/MODY3

Introduction

Maturity-onset diabetes of the young (MODY) is an early-onset form of non-insulin-dependent diabetes mellitus, which is characterized by autosomal dominant inheritance. MODY phenotypes have now been linked to mutations in three genes, namely hepatocyte nuclear factor-4α (HNF-4α) on chromosome 20q for MODY1 (Yamagata *et al*., 1996a), glucokinase on chromosome 7p for MODY2 (Froguel *et al*., 1993) and HNF-1α on chromosome 12q for MODY3 (Yamagata *et al*., 1996b). More recently, a MODY4 pedigree has been associated with mutations in a pancreatic homeodomain transcription factor PDX-1 (IPF-1, IDX-1, STF-1), which regulates the development of pancreas and the expression of insulin and other β-cell-specific genes (Stoffers *et al*., 1997). The transcription factor HNF-1α is known to be expressed in liver, kidney and intestine, and in the β-cells of the endocrine pancreas (Frain *et al*., 1989; Emens *et al*., 1992; Miquerol *et al*., 1994). The phenotypic analysis of MODY3 pedigrees, which revealed hyperglycaemia resulting from severe defects in glucose-stimulated insulin secretion, suggest that β-cell dysfunction plays an important pathophysiological role in the development of MODY3 (Byrne *et al*., 1996; Lehto *et al*., 1997).

The HNF-1α gene is comprised of 10 exons that span ~23 kb and codes for a 628-amino acid protein that is composed of three functional domains: a short N-terminal dimerization domain encoded by exon 1; a homeobox DNA-binding domain encoded by exons 2, 3 and 4; and a C-terminal transactivation domain encoded by exons 5–10 (Frain *et al*., 1989; Nicosia *et al*., 1990; Kaisaki *et al*., 1997). Mutations have been reported in MODY3 pedigrees affecting the dimerization domain, the DNA-binding domain or the transactivation domain (Yamagata *et al*., 1996b; Glucksmann *et al*., 1997). Mutations in any one of these three domains could lead to diminished amounts of functional HNF-1α by either haploinsufficiency or a dominant-negative mechanism. HNF-1α regulates the expression of many liver-specific genes by direct binding to their promoter and/or enhancer regions (Nicosia *et al*., 1990; Yanuka-Kashles *et al*., 1994). Some of these genes such as glucose transporter 2 (GLUT-2) and L-type pyruvate kinase (L-PK) are also expressed in pancreatic β-cells (Emens *et al*., 1992; Miquerol *et al*., 1994). In addition, a consensus binding site for HNF-1α has been identified in the promoter of the rat insulin I gene, and HNF-1α has been proposed to regulate the expression of the rat insulin I gene in concert with other transcription factors (Emens *et al*., 1992).

To explore the molecular basis by which HNF-1α deficiency causes β-cell dysfunction, we established the β-cell-derived insulinoma cell lines capable of over-expressing HNF-1α and its dominant-negative mutant, termed DNHNF-1α, under the tight control of a doxycycline-dependent transcriptional activator. DNHNF-1α, also referred to as SM6 by Nicosia *et al*. (1990), represents a substitution mutation of 83 amino acids in the HNF-1α DNA-binding domain and forms non-functional heterodimers with wild-type HNF-1α. The expression of HNF-1α, as well as DNHNF-1α, could be maximally induced to a level >100-fold that of endogenous HNF-1α, and the induction level could be pin-pointed within precise brackets by particular doxycycline doses and time intervals. We demonstrate that the levels of GLUT-2 and L-PK mRNA are rapidly reduced following the induction of DNHNF-1α. Maximal induction of DNHNF-1α resulted in a dramatic decrease in HNF-1α binding to double-

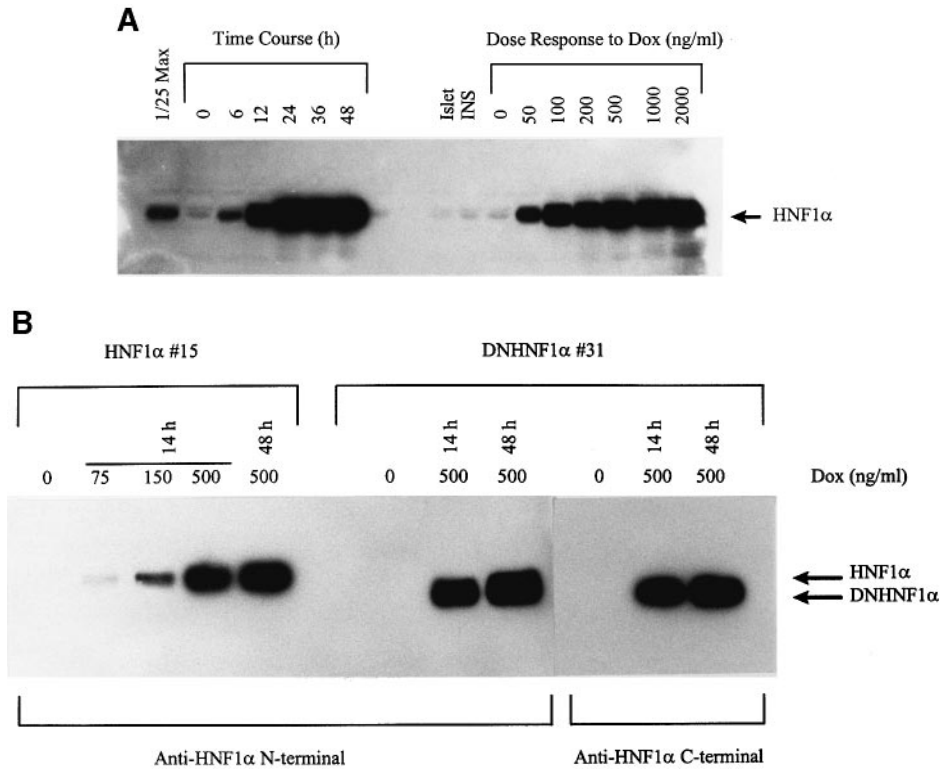


Fig. 1. Induction of HNF-1α and DNHNF-1α protein by doxycycline. (**A**) Time course and dose–response of doxycycline effect on HNF-1α expression in HNF-1α#15 cells. For studying time course, cells were cultured in medium containing 1000 ng/ml doxycycline and harvested for nuclear extract at the indicated times. For studying dose–response, cells were cultured with the indicated doses of doxycycline (Dox) for 48 h. 10 μg nuclear extract protein was resolved in 9% SDS–PAGE, transferred to nitrocellulose, and immunoblotted with antibody against the N terminus of HNF-1α. The left-most lane received 0.4 μg (1/25) nuclear extract from cells cultured for 48 h with 1000 ng/ml doxycycline. (**B**) Protein levels of HNF-1α and DNHNF-1α used in the present study. Cells were cultured in medium containing the indicated doses of doxycycline for 14 or 48 h before harvesting. All lanes were loaded with 10 μg of nuclear extract protein. Antisera against N-terminal or C-terminal sequences of HNF-1α were used in the Western blot.

stranded oligonucleotides corresponding respectively to the FLAT element of the rat insulin-1 enhancer (Emens *et al.*, 1992) and to the L1 element of the L-PK promoter (Miquerol *et al.*, 1994). This dominant-negative suppression of HNF-1α function led to a pronounced reduction in the expression of insulin mRNA and the activity of the rat insulin-1 promoter. Remarkably, insulin biosynthesis and glucose- or leucine-stimulated insulin secretion are diminished in INS-1 cells overexpressing DNHNF-1α. The suppression of HNF-1α function in INS-1 cells also inhibited glucose and leucine metabolism, which resulted in reduced cellular ATP generation as well as decreased membrane potential depolarization and intracellular Ca^{2+} ($[Ca^{2+}]_i$) rises. These findings suggest that metabolism–secretion coupling is defective in β-cells deficient in HNF-1α function and elucidate the molecular basis of the impairment of insulin secretion in MODY3 patients.

Results

Establishment of insulinoma cells overexpressing HNF-1α and DNHNF-1α using an inducible system

The INS-1-derived INS-r3 cell line (Wang and Iynedjian, 1997), which carries the reverse tetracycline/doxcycycline-dependent transactivator (Gossen *et al.*, 1995) was secondarily transfected with plasmids encoding either the wild-type HNF-1α or its dominant-negative mutant DNHNF-1α driven by a minimal cytomegalovirus promoter placed under control of the tetracycline operator. Eight out of 60 hygromicin-resistant clones showed >100-fold overexpression of HNF-1α with maximal induction. Six out of 50 resistant clones positively expressed DNHNF-1α at a similar level. The clones, termed HNF-1α#15 and DNHNF-1α#31, were randomly selected for the present study. The time-course of doxycycline effect on HNF-1α expression is represented by immunoblotting in Figure 1A. The transcription factor (88 kDa) was induced time-dependently and reached a maximal level of >100-fold over the endogenous protein after 24 h. The dose dependence for doxycycline after 48 h culture is also illustrated in Figure 1A. The increase in HNF-1α expression was directly correlated with the doxycycline concentration and maximal levels were reached at a dose of 500 ng/ml. Moreover, the leakage of this tetracycline/doxycycline-controlled promoter is negligible, as HNF-1α expression in non-induced HNF-1α#15 cells was comparable with that in parental INS-1 cells and in rat pancreatic islets (Figure 1A). Therefore, the level of HNF-1α expression can be tightly controlled within precise brackets by culturing the cells with well-selected inducer dosage over defined time periods.

The induction levels of HNF-1α and DNHNF-1α used in the following experiments are shown in Figure 1B. Stepwise increase in HNF-1α expression was achieved with rising doses of doxycycline. This graded overexpression is remarkably reproducible with fixed doxycycline

doses and time intervals. DNHNF-1α (SM6; Nicosia *et al*., 1990) represents a long-substitution mutation of HNF-1α, in which an essential part of the DNA-binding domain (amino acids 73–155) was replaced. Consequently, the mutant protein DNHNF-1α migrates slightly faster than the glycosylated wild-type protein (Lichtsteiner and Schibler, 1989). The mutant retains an intact N-terminal dimerization domain and C-terminal activation domain, as supported by reactions with antibodies against both N- and C-terminal sequences of HNF-1α (Figure 1B). DNHNF-1α is expected to heterodimerize with endogenous HNF-1α and to prevent its DNA binding.

DNHNF-1α prevents the binding of endogenous HNF-1α to its recognition site in the promoters of the rat insulin I and L-PK genes

The high level of induction of DNHNF-1α by doxycycline may overcome the stabilization that occurs when the native transcription factor or factors (De Simone *et al*., 1991; Rey-Campos *et al*., 1991) bind(s) to DNA, thereby acting in a dominant-negative manner. We therefore used gel shift assays to analyse HNF-1α binding activity in nuclear extracts from HNF-1α$^{\#}$15 and DNHNF-1α$^{\#}$31 cells, cultured for 48 h in the presence or absence of doxycycline. The rat insulin I FLAT element (Emens *et al*., 1992) and the rat L-PK L1 element (Miquerol *et al*., 1994), which contain the HNF-1α binding sequence, were used as probes. Antibody supershift assays and cold-probe competition experiments allowed qualitative characterization of HNF-1α–DNA complexes. The relative amount of protein in nuclear extracts was semi-quantified by immunoblotting with respect to the ubiquitous TFIIE-α transcription factor.

Nuclear extracts from both HNF-1α$^{\#}$15 and DNHNF-1α$^{\#}$31 cells cultured in the absence of doxycycline contained similar amounts of HNF-1α binding activity (Figure 2A), corresponding to endogenous HNF-1α levels present in parental INS-1 cells (Figures 1A and 2C). The specificity of HNF-1α binding to the insulin I and L-PK probes was demonstrated by supershift assays with an antibody raised against the C terminus of HNF-1α (Figure 2A) and by competition experiments with an excess of the corresponding unlabelled oligonucleotide duplex (Figure 2B). In cross-competition analyses, the unlabelled L-PK probe, which does not contain a consensus sequence for the transcription factor PDX-1, did not compete with the radioactive insulin I oligonucleotide for PDX-1 binding, but specifically eliminated the retarded HNF-1α complex (Figure 2B). The disappearance of the PDX-1 complex in HNF-1α-overexpressing cells might be due to co-binding by a large excess of HNF-1α rather than by lack of expression of the PDX-1 protein (Figure 2C). Overexpression of wild-type HNF-1α resulted in a significant increase in its binding activity (Figure 2A). On the other hand, induction of DNHNF-1α almost completely eradicated endogenous HNF-1α binding to the insulin probe, and also significantly blocked its binding to the L-PK probe (91% and 55% decrease, respectively, as estimated by densitometry and corrected for the amount of TFIIE-α present in the nuclear extracts) (Figure 2C). The difference of HNF-1α binding activity to distinct probes affected by expression of the dominant-negative form is possibly due to some competition based on the limited amounts and different affinities of HNF-1α for the sites. It therefore can be concluded that DNHNF-1α acts in a dominant-negative manner, as predicted for a transcription factor that retains an intact dimerization domain but has lost the ability to bind DNA (Nicosia *et al*., 1990).

HNF-1α regulates the gene expression of Glut-2, L-PK and insulin and activates the insulin promoter

The L-PK transcription is induced in response to an increase of extracellular glucose concentration in INS-1 and native β-cells (Marie *et al*., 1993; Kennedy *et al*., 1997) and this was used as a model in the present study to explore whether HNF-1α is involved in glucose-regulated gene expression. HNF-1α$^{\#}$15 and DNHNF-1α$^{\#}$31 cells were cultured with or without indicated doses of doxycycline for 14 or 48 h with 2.5 mM glucose, followed by an additional 8 h in culture medium with 2.5, 6, 12 and 24 mM glucose. One of two independent experiments is presented in Figure 3A and B. GLUT-2 transcripts which share strikingly similar expression patterns with L-PK mRNAs (Antoine *et al*., 1997) were responsive to glucose (Figure 3A). The glucose-stimulated expression of GLUT-2 and L-PK was not augmented in an additive manner by graded overexpression of HNF-1α. At low glucose (2.5 mM), however, when the glucose-responsive transcription factors were depressed, the expression of GLUT-2 and L-PK was enhanced by overexpression of HNF-1α. In comparison, the dominant-negative suppression of HNF-1α in DNHNF-1α$^{\#}$31 cells resulted in reduced gene expression of GLUT-2 and L-PK (Figure 3B). At high glucose concentration (24 mM), on the other hand, the impact of repressed HNF-1α function on GLUT-2 and L-PK expression could be overcome by glucose-induced or -activated transcription (Figure 3B). In conclusion, HNF-1α does not seem to mediate the glucose-responsiveness of these genes, but it plays an important part in the regulation of GLUT-2 and L-PK transcription in combination with other transcription factors. As reported (Marie *et al*., 1993), the β-cell-specific glucokinase gene (β-GK) was not responsive to the rise of glucose concentration in this β-cell-derived INS-1 cell line (Figure 3A and B). The mRNA levels of β-GK were not affected by overexpression of HNF-1α or DNHNF-1α. Note that even a slight increase in β-GK mRNAs was seen in DNHNF-1α$^{\#}$31 cells after 48 h induction (Figure 3B). This change was not specific as it also occurred in the expression of β-actin and of Beta-2/NeuroD, a transcription factor implicated in insulin gene transcription (Naya *et al*., 1997) (Figure 3B). Therefore, it can be concluded that the expression of β-GK is not regulated directly by HNF-1α, which is in agreement with previous analysis of the β-GK promoter (Shelton *et al*., 1992; Jetton *et al*., 1994).

The expression of the insulin gene in DNHNF-1α$^{\#}$31 cells was decreased by $>$80% after dominant-negative suppression of HNF-1α for 48 h (Figure 3B). Recently, we have also established a similar cell line expressing a MODY3 mutant Pro291insC (Glucksmann *et al*., 1997) which has been proven to act in a dominant-negative manner (Yamagata *et al*., 1998). We found that overexpression of human mutant Pro291insC in INS-1 cells has similar consequences on gene expression of GLUT2, L-PK and insulin as the induction of DNHNF-1α (unpublished

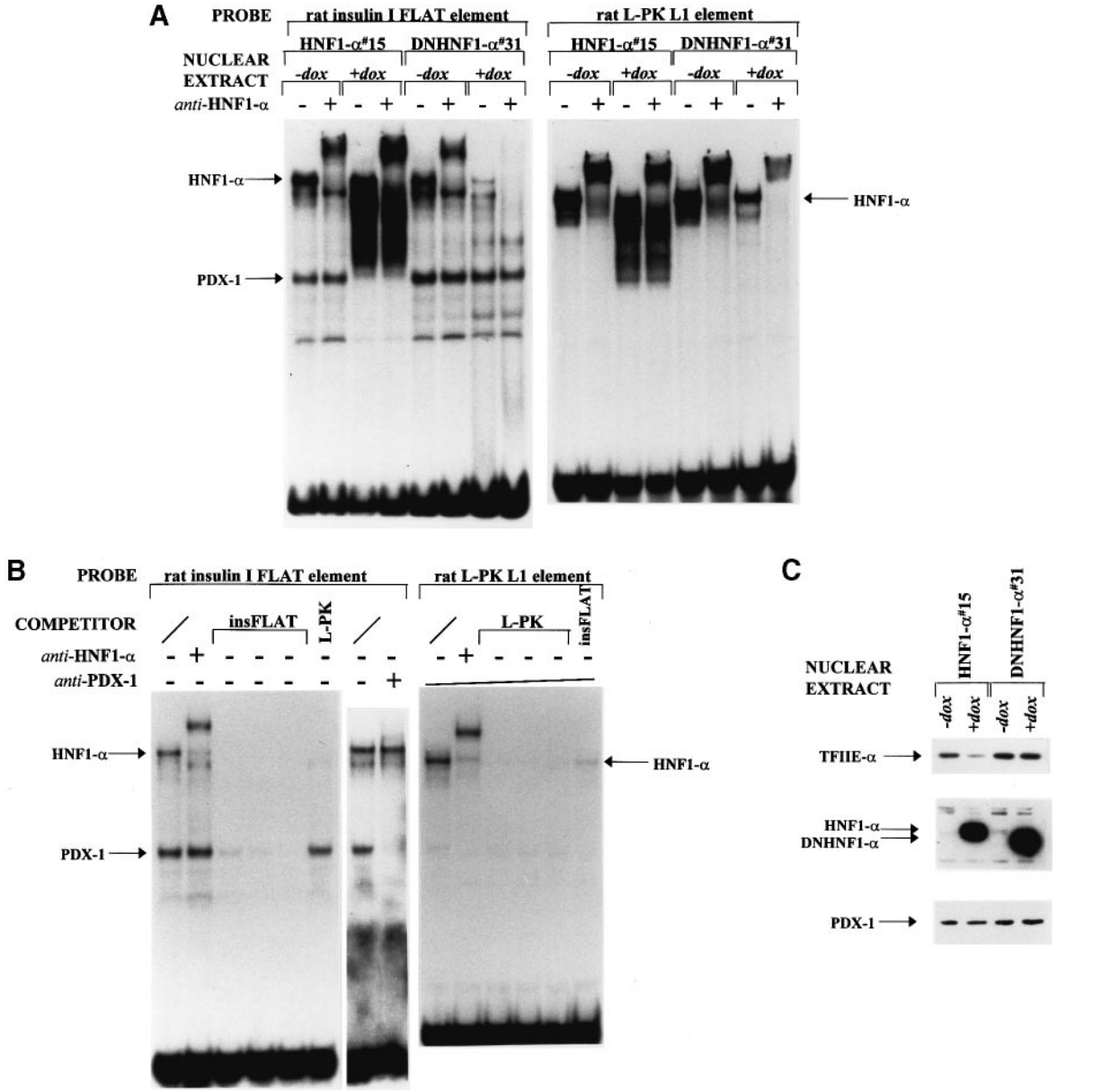


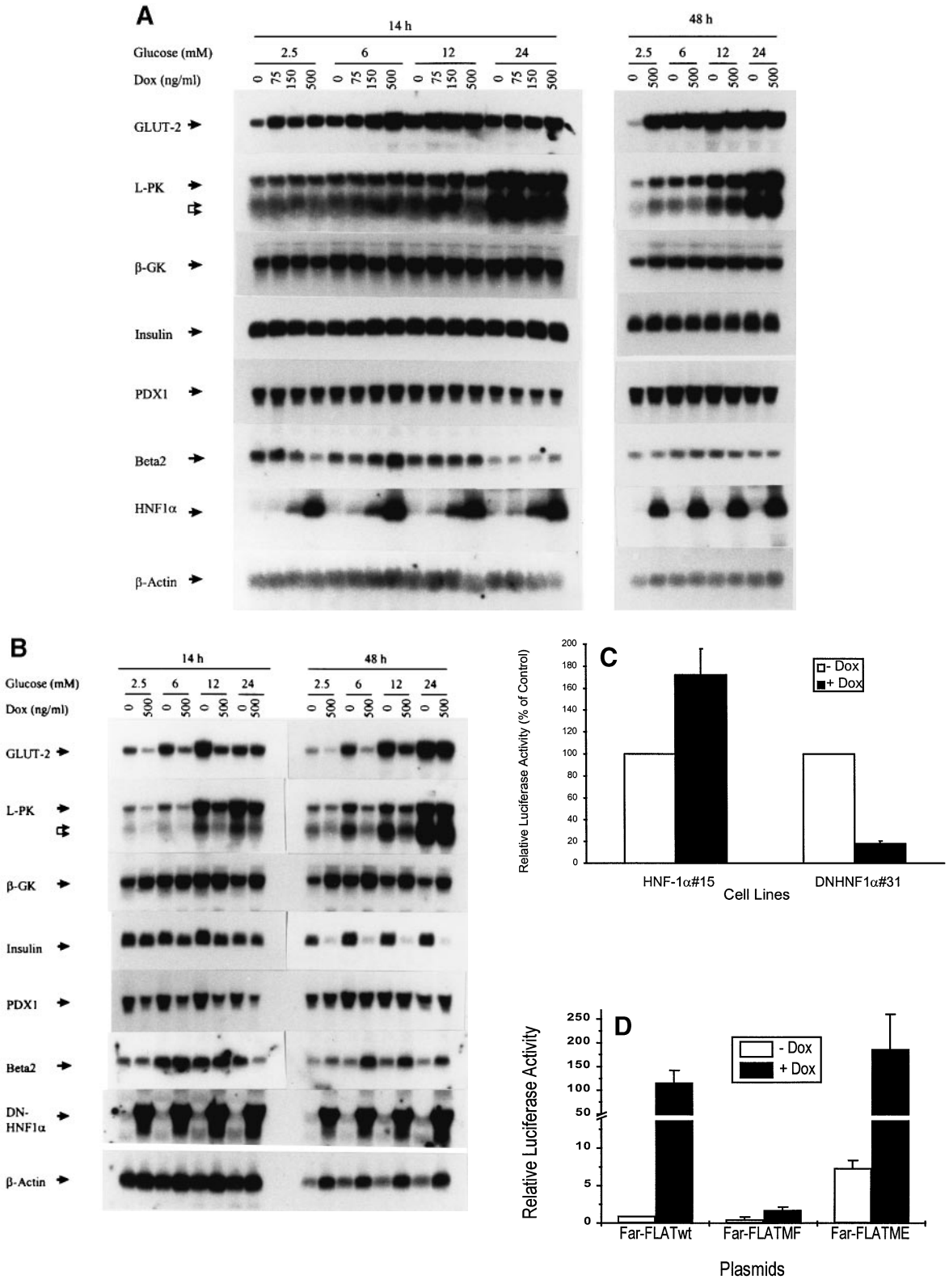
Fig. 2. DNHNF-1α acts as a dominant-negative mutant by preventing the endogenous HNF-1α binding to DNA. (**A**) Gel shift assay with the oligonucleotide probes corresponding to the rat insulin I FLAT element and the rat L-PK L1 element, respectively. Eight micrograms of crude nuclear extracts from non-induced HNF-1α#15 and DNHNF-1α#31 cells and doxycycline-induced DNHNF-1α#31 cells were used in the binding reactions. To balance the signal, 2 μg of nuclear extract from doxycycline-induced HNF-1α#15 cells was used. 1 μl of undiluted antibody raised against the C terminus of HNF1-α was added to the reaction for supershift experiments. (**B**) Competition with unlabelled oligonucleotide probes. Formation of the HNF1-α binding complex was specifically competed for by an excess of cold probes (50-, 100- and 200-fold molar excess, respectively, from left to right). The retarded HNF1-α complex (marked by arrow), but not PDX-1 complex, formed by the ^{32}P-labelled insulin probe was eliminated by an excess of the cold L-PK probe. The identity of the PDX-1 complex (see arrow) was confirmed by supershifting with anti-PDX-1. (**C**) Western blot analysis and quantification of the nuclear extracts. 20 μg of nuclear extract was loaded on each lane. The amount of ubiquitous TFIIE-α protein present in the nuclear extracts, as revealed by blotting with anti-TFIIE-α (top panel, indicated by arrow), was used to quantify the proteins in the nuclear extracts.

data). The mRNA level of PDX1, another transactivator regulating insulin gene transcription, was reduced by 14 h induction of DNHNF-1α, but recovered after 48 h (Figure 3B). As the expression patterns of the transcription factors PDX1 and Beta-2 did not match those of insulin (Figure 3B), they do not appear to be involved in the suppression of the insulin gene. The level of insulin transcripts was not altered in HNF-1α#15 cells by overexpression of wild-type HNF-1α protein (Figure 3A).

To confirm that HNF-1α regulates insulin gene transcription, we transiently transfected DNHNF-1α#31 cells with plasmid –410INSLuc encoding a luciferase reporter gene driven by the rat insulin I promoter containing the HNF-1α-binding sequence (German *et al*., 1992a; Sander and German, 1997). As shown in Figure 3C, the rat insulin I promoter activity was reduced by >5-fold after induction of the dominant-negative mutant for 48 h. In contrast, the maximal induction of wild-type HNF-1α led to a slight (1.7-fold) increase in the luciferase reporter enzyme activity in HNF-1α#15 cells transiently transfected with plasmid –410INSLuc.

In order to conclude that HNF-1α has a direct effect

on the rat insulin I promoter activity, HNF-1α#15 and DNHNF-1α#31 cells were transiently transfected with three luciferase constructs containing multimerized segments of the rat insulin I minienhancer: wild-type (Far-FLAT*wt*), HNF-1α binding site-mutated (Far-FLAT*MF*) and E-element-mutated (Far-FLAT*ME*) (German *et al*., 1992a,b). The rat insulin I promoter contains at least two regulatory elements, the Far element (–239 to –228) and the FLAT element (–222 to –208), which function synergistically to confer both tissue-specificity and glucose-responsiveness of the promoter (German *et al*., 1992a). The FLAT element itself is composed of two functionally distinct elements: a positive locus, FLAT-F that predominantly binds HNF-1α (Emens *et al*., 1992);



and the adjacent negative locus, FLAT-E that predominantly binds other homeodomain proteins (German *et al*., 1992a). As shown in Figure 3D, the induction of HNF-1α enhanced the luciferase activity by >100-fold in the HNF-1α#15 cells transfected with Far-FLAT*wt* plasmid. The HNF-1α activation requires an intact FLAT-F site since minienhancer with F element mutated (Far-FLAT*MF*) is not activated by induction of HNF-1α (Figure 3D). It has been demonstrated that the very same mutation of this F element also abolished the HNF-1α binding activity (Emens *et al*., 1992). In contrast, the HNF-1α activation was well preserved in doxycyline-induced HNF-1α#15 cells transfected with Far-FLAT*ME* that contains the E element mutation (Figure 3D). The basal luciferase activity was significantly higher in non-induced HNF-1α#15 cells transfected with Far-FLAT*ME* than those transfected with Far-FLAT*wt* or Far-FLAT*MF*, which is in agreement with previous observations (German *et al*., 1992b). Therefore, HNF-1α is indeed capable of mediating transactivation of the rat insulin I promoter through the FLAT-F site, as proposed by Emens *et al*. (1992). The transient transfection experiments performed in DNHNF-1α#31 cells further supported the role of HNF-1α in the regulation of the rat insulin minienhancer. Induction of DNHNF-1α resulted in 72.3 ± 25.2% and 90.3 ± 3% (n = 3) decreases of luciferase activity in DNHNF-1α#31 cells transfected with Far-FLAT*wt* and Far-FLAT*ME* respectively, but had no inhibitory effects on the minienhancer with the HNF-1α site mutated (Far-FLAT*MF*).

Insulin secretory defect induced by loss of HNF-1α function

Mutations in HNF-1α, which are linked to the MODY3 subtype, lead to hyperglycaemia because of severe derangement of glucose-stimulated insulin secretion from the pancreatic β-cells. We investigated the impact of controlled overexpression and dominant-negative suppression of HNF-1α on insulin secretory responses to glucose, leucine and K^+. These secretagogues were selected for their action at different and specific levels of the signal transduction cascade: glucose at the primary step, leucine at the mitochondria downstream of glycolysis, and K^+ at the late depolarization event. Cells were cultured with or without doxycycline for either 14 h (Figure 4A and B) or 48 h (Figure 4C and D) in medium containing 2.5 mM glucose. Thereafter, insulin secretion was measured over a period of 30 min with indicated stimulators. Non-induced cells exhibited a typical insulin secretory response to glucose (Figure 4A–C), as seen in parental INS-1 cells (Asfari *et al*., 1992). Induction of wild-type HNF-1α resulted in a leftward shift of the dose–response curve, but the maximal insulin response did not exceed that of non-induced cells (Figure 4A). This enhancement of insulin secretory response was not due to increased insulin biosynthesis, since the insulin content was not altered in HNF-1α#15 cells cultured with 500 ng/ml doxycycline over a time period of 14–48 h (data not shown). In agreement with the reduced insulin transcription, insulin content in DNHNF-1α#31 cells was time-dependently decreased by induction of DNHNF-1α: the values (expressed as % reduction) were 3.7 ± 8.6, 25.8 ± 14.0 and 66.9 ± 12.1 after 14, 30 and 48 h, respectively. The insulin content of non-induced cells was 56.3 ± 5.9 ng/μg DNA. The overexpression of HNF-1α mutated in the DNA-binding domain in DNHNF-1α#31 cells led to a dramatic decrease in the insulin secretory response to glucose (Figure 4B and C). Insulin secretion stimulated by 12 and 24 mM glucose was inhibited by 40% following 14 h induction of DNHNF-1α (Figure 4B), when the insulin content was not affected. In DNHNF-1α#31 cells, the glucose stimulatory effect was completely abolished after 48 h culture with doxycycline (Figure 4C), and the 67% reduction of basal insulin secretion at 2.5 mM glucose (Figure 4C and D) exactly paralleled the decrease in cellular insulin content. Insulin secretion caused by another nutrient stimulus, leucine, was inhibited by 57% in cells exposed to doxycycline for 48 h (Figure 4D). The secretory response to 20 mM K^+, which does not require generation of ATP and other metabolic coupling factors in contrast to the nutrient stimuli (Wollheim *et al*., 1996), was also decreased—albeit to a lesser extent when compared with the effects of glucose and leucine (Figure 4D). When secretion is related to insulin content rather than to DNA content, the leucine-stimulated insulin secretion fell from 3.7-fold in non-induced cells to 1.6-fold in induced cells, while the K^+ stimulatory action was reduced from 2.6- to 1.9-fold under the same conditions. There is thus a marked inhibition of insulin secretion in response to glucose and leucine, which is not merely a reflection of the reduction of insulin content, but suggests impaired nutrient metabolism.

Impaired glucose and leucine metabolism led to reduced ATP production in INS-1 cells deficient in HNF-1α function

Glucose-stimulated insulin secretion is controlled by glucose metabolism comprising glycolysis and mitochondrial oxidation (Matschinsky, 1996). The glycolytic flux was investigated by following the production of [^{3}H]water from D-[5-^{3}H]glucose and the rate of oxidation was

Fig. 3. HNF-1α is required for the gene expression of GLUT-2, L-PK and insulin. Northern blot analysis of gene expression in (**A**) HNF-1α#15 and (**B**) DNHNF-1α#31 cells induced with indicated doses of doxycycline and cultured at given concentrations of glucose. Cells were cultured with indicated doses of doxycycline at 2.5 mM glucose for 14 or 48 h. Culture was continued for 8 h at 2.5 mM glucose or at the indicated glucose concentrations before cells were extracted for total RNA. RNA samples were analysed by hybridizing with the indicated cDNA probes. (**C**) Effect of HNF-1α dysfunction on rat insulin I promoter activity. DNHNF-1α#31 cells were transiently transfected with plasmid –410INSLuc by calcium phosphate–DNA co-precipitation. After 48 h culture with or without 500 ng/ml doxycycline, cells were collected, and 20 μg cytosolic protein was assayed for luciferase activity. Luciferase activity measured in non-induced cells was defined as 100%. Data are the mean ± SEM of six independent experiments. HNF-1α#15 cells, which were subjected to the same procedures, served as controls to eliminate the possibility that the luciferase activity was affected non-specifically by doxycycline treatment. (**D**) HNF-1α activates the insulin promoter through the minienhancer FLAT-F element. HNF-1α#15 cells were transiently transfected with three luciferase constructs containing multimerized segments of the insulin minienhancer: wild-type (Far-FLAT*wt*), HNF-1α binding site-mutated (Far-FLAT*MF*) and E-element-mutated (Far-FLAT*ME*). After 48 h culture with or without 500 ng/ml doxycycline, cells were collected, and 20 μg cytosolic protein was assayed for luciferase activity. Luciferase activity measured in non-induced cells transfected with plasmid Far-FLAT*wt* was arbitrarily set at 1.0. Data are the mean ± SEM of three independent experiments.

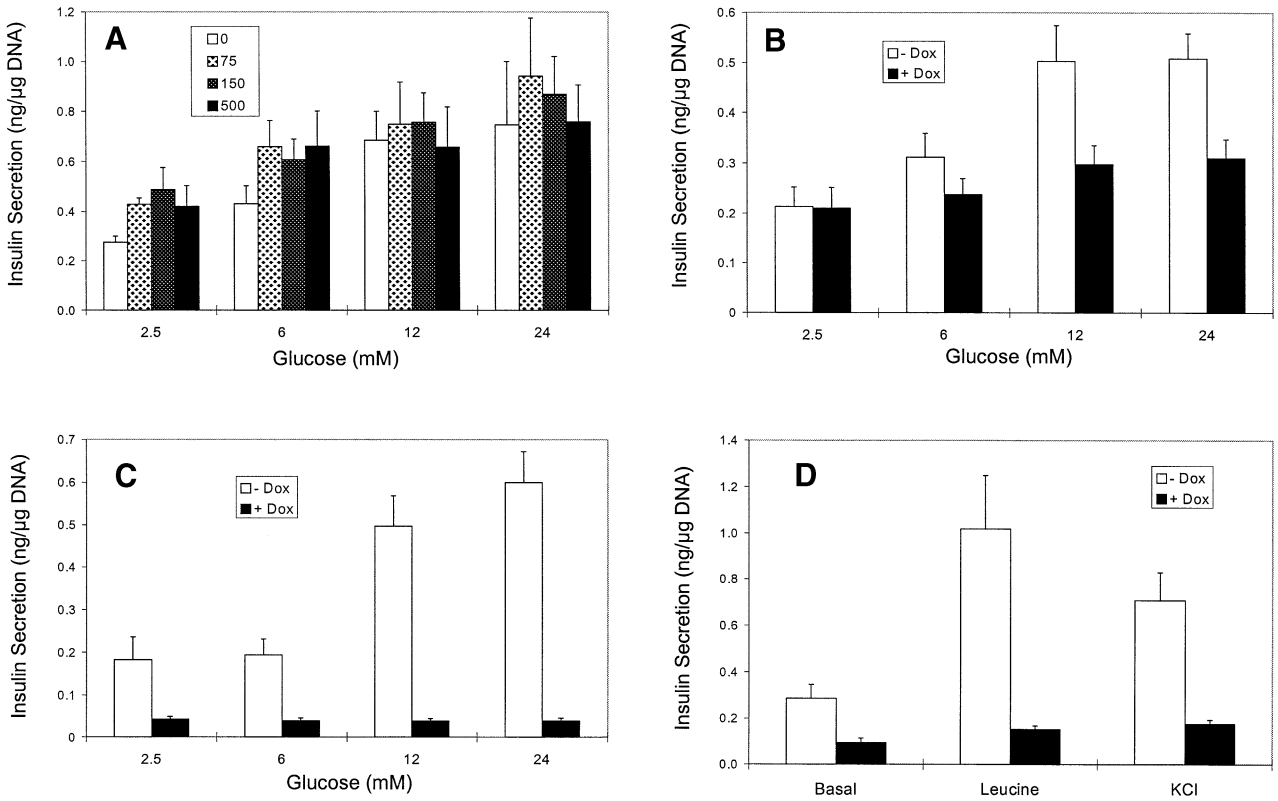


Fig. 4. Effect of defective HNF-1α function on insulin secretory responses to stimulators. Cells were cultured at 2.5 mM glucose in the presence or absence of specified doses of doxycycline for 14 or 48 h as indicated. Cells were then incubated in KRBH with specified stimulators for 30 min. Insulin released in KRBH was quantified by radioimmunoassay and normalized by cellular DNA content [measured as described by Wang and Iynedjian (1997)]. (**A**) Glucose-stimulated insulin secretion in HNF-1α#15 cells induced with indicated doses of doxycycline for 14 h. Data represent the mean ± SEM of six separate experiments. Statistical significance between doxcycline-induced and non-induced cells was obtained at 2.5 and 6 mM glucose ($P < 0.01$, all data in the present study were analysed by unpaired Student's *t*-test). (**B**) Glucose-stimulated insulin secretion in DNHNF-1α#31 cells induced with 500 ng/ml doxycycline for 14 h. Data show the mean ± SEM of six independent experiments. Statistical significance between doxycycline-induced and non-induced cells was observed at 12 and 24 mM glucose ($P < 0.01$). (C) Glucose-stimulated insulin secretion in DNHNF-1α#31 cells induced with 500 ng/ml doxycycline for 48 h. Data are the mean ± SEM of six separate experiments. (**D**) Leucine- and K^+-stimulated insulin secretion in DNHNF-1α#31 cells induced with 500 ng/ml doxycycline for 48 h. Insulin secretion was measured during 30 min incubation with 20 mM leucine or 20 mM KCl in KRBH containing 2.5 mM glucose. Data represent the mean ± SEM of three independent experiments.

estimated from the conversion of D-[^{14}C(U)]glucose and L-[1-^{14}C]leucine to $^{14}CO_2$. Cells of the HNF-1α#15 and DNHNF-1α#31 lines were cultured with or without 500 ng/ml doxycycline for 48 h in medium containing 2.5 mM glucose. The rate of glycolysis was measured over a time period of 30 min, at extracellular glucose concentrations of 2.5, 6, 12 and 24 mM. Under non-induced conditions, the glycolytic rate in both HNF-1α#15 and DNHNF-1α#31 cells increased as expected over the range of glucose concentrations (Figure 5A and B). Overexpression of HNF-1α had no significant impact on the glycolytic flux (Figure 5A). However, at 12 and 24 mM glucose concentrations, the dominant-negative suppression of HNF-1α resulted in a 40% reduction ($P < 0.001$, $n = 6$) in the rate of glycolysis (Figure 5B).

Glucose and leucine oxidation was then investigated in DNHNF-1α#31 cells. After the 48 h induction by doxycycline (500 ng/ml), formation of $^{14}CO_2$ from 12.8 mM D-[^{14}C(U)]glucose was found to be decreased by 45% ($P < 0.02$, $n = 8$) in cells overexpressing DNHNF-1α compared with non-induced cells (Figure 5C). As seen in Figure 5C, when L-[1-^{14}C]leucine (20 mM) was provided as the ^{14}C donor, this inhibition reached 74% ($P < 0.01$, $n = 8$), indicating that the mitochondrial metabolism was predominantly impaired.

This contention was borne out by measurements of cellular ATP levels (Figure 5D). In non-induced cells, both glucose and leucine caused 2-fold increases of ATP after 8 min incubation, whereas K^+ had no effect. By contrast, the generation of ATP by glucose and leucine was completely abolished following suppression of HNF-1α function. It is noteworthy that the basal ATP levels at 2.5 mM glucose remain unaltered. These results further defined defective mitochondrial metabolism as the primary consequence of the loss of HNF-1α function.

Changes in plasma membrane potential and $[Ca^{2+}]_i$ after suppression of HNF-1α function

To characterize further the mechanism underlying the impairment of insulin secretion, the effects of glucose and leucine on plasma membrane potential and $[Ca^{2+}]_i$ were monitored in DNHNF-1α#31 cells. DNHNF-1α was induced by doxycycline (500 ng/ml) for 48 h prior to the spinner culture period for measurements on cell suspensions. The addition of 10 mM glucose (to yield a final concentration of 12.5 mM) evoked a clear depolariz-

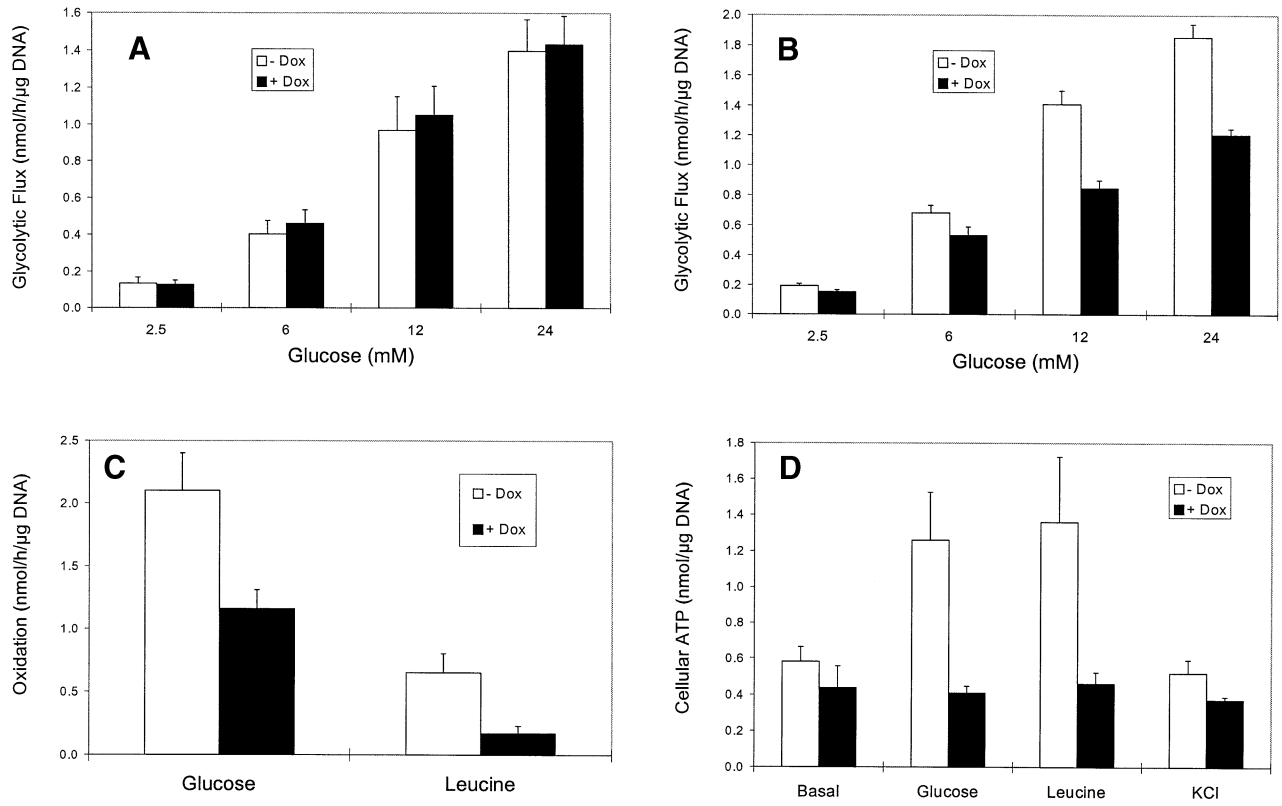


Fig. 5. Glycolytic flux, mitochondrial oxidation and ATP levels after disruption of HNF-1α function. Cells were cultured with or without 500 ng/ml doxycycline at 2.5 mM glucose for 48 h before experiments. DNA content was used to normalize cell number variations between wells. The glycolytic flux in (**A**) HNF-1α#15 and (**B**) DNHNF-1α#31 cells was measured during 30 min incubation with indicated concentrations of glucose and a constant specific activity of tracer glucose. Data are mean ± SEM from six independent experiments. (**C**) Glucose and leucine oxidation: $^{14}CO_2$ formation during 1 h incubation with 12.8 mM D-[^{14}C(U)]glucose or 20 mM L-[1-^{14}C]leucine were both significantly inhibited by DNHNF-1α. (**D**) Mitochondrial ATP production measured for 8 min in DNHNF-1α#31 cells. Data are mean ± SEM from six separate experiments.

ation of the plasma membrane in non-induced control cells, whereas the changes were only minor in DNHNF-1α-overexpressing cells (Figure 6A). Similar results were obtained when the mitochondrial substrate L-leucine (20 mM) was added to induce cell depolarization (Figure 6B). This lack of membrane depolarization was metabolism-dependent, since a full response to 20 mM KCl was recorded for both cell preparations and nutrients at the end of each trace (Figure 6A and B).

As expected from the membrane potential recordings, nutrient-induced $[Ca^{2+}]_i$ rises were inhibited by induction of DNHNF-1α (Figure 6C and D). As seen in Table I, the basal $[Ca^{2+}]_i$ was unchanged in doxycycline-induced cells compared with control cells. In contrast, at 5 min after the addition of 10 mM glucose (12.5 mM final) or 20 mM L-leucine, the incremental rises in $[Ca^{2+}]_i$ were significantly inhibited by 56% and 45%, respectively, by induction of DNHNF-1α. This was not due to impairment of L-type Ca^{2+} channel function, as simple depolarization of the membrane by 20 mM KCl caused similar $[Ca^{2+}]_i$ increases in both control and DNHNF-1α-overexpressing cells, both at the transient peak just after the addition of the depolarizing agent and at the subsequent plateau (see Table I and Figure 6C and D).

Discussion

Establishment of a cellular model for MODY3

β-cell dysfunction appears to be the primary cause of the diabetic syndrome in MODY3 patients. To investigate the consequences of impaired HNF-1α function, we have established β-cell-derived insulin-secreting cell lines, in which HNF-1α function could be suppressed in a controlled and dominant-negative manner. Many mutations in HNF-1α have been reported in MODY3 patients, and most of these are believed to act as dominant-negative mutants by forming non-functional heterodimers with the product of the normal HNF-1α allele. In our INS-1-derived stable cell line, DNHNF-1α, which is mutated in the DNA-binding domain, could be induced to extremely high levels capable of eliminating the function of endogenous HNF-1α through competitive dimerization. The dominant-negative effect was verified by the reduction of HNF-1α binding to its recognition sequences in the insulin enhancer and the L-PK promoter. Under non-induced conditions, DNHNF-1α is not detectable and the expression of HNF-1α represents the endogenous level of parental INS-1 cells and pancreatic islets. During the 48 h induction period, the cellular morphology and replication (DNA content) were unaltered (data not shown).

HNF-1β, which is closely related to HNF-1α and is able to heterodimerize with HNF-1α, overlaps the expression pattern of HNF-1α (De Simone *et al.*, 1991; Rey-Campos *et al.*, 1991; Pontoglio *et al.*, 1996). Both proteins transactivate the targeted gene by forming a homodimer or heterodimer and binding to the same recognition sequences. Most HNF-1α mutant proteins identified in MODY3 pedigrees would result in loss of function of both HNF-1α and HNF-1β by a dominant-

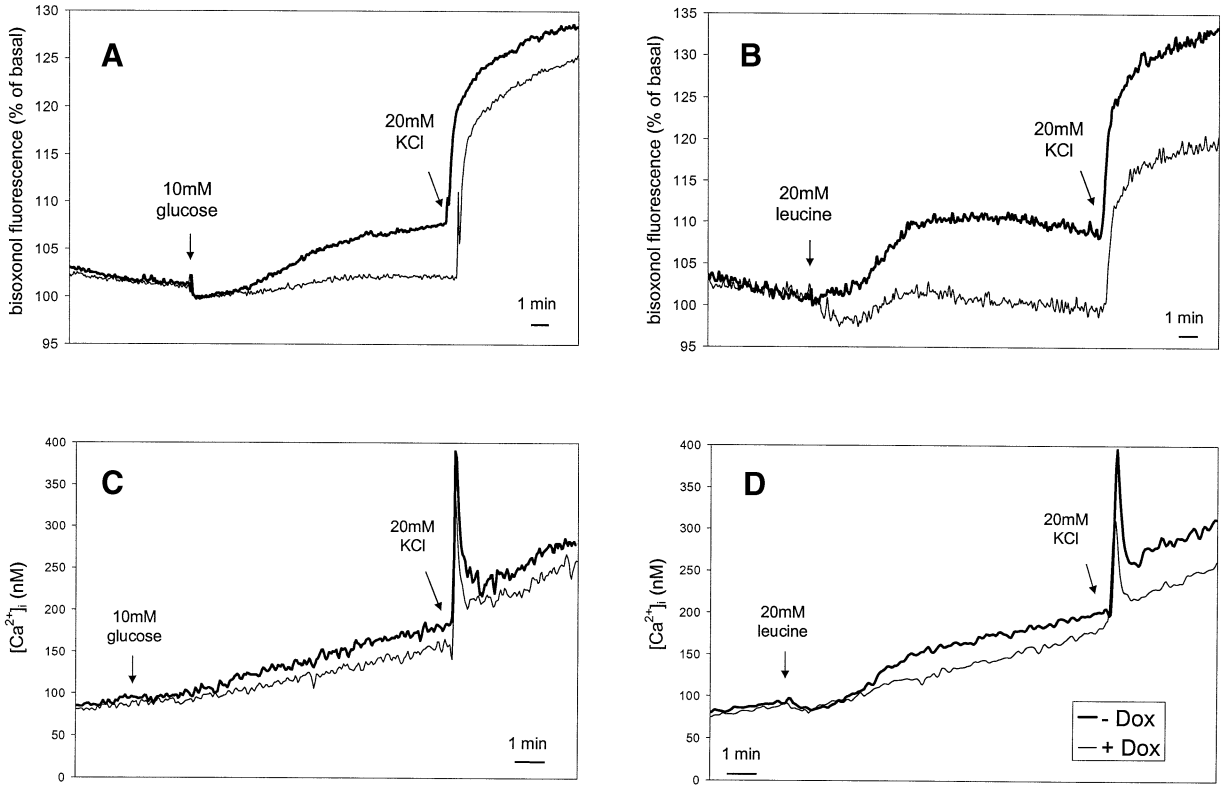


Fig. 6. Plasma membrane potential and $[Ca^{2+}]_i$ monitored in DNHNF-1α#31 cells. Bisoxonol fluorescence was used to measure plasma membrane potential. Glucose-induced membrane depolarization was inhibited in DNHNF-1α#31 (+Dox) cells compared with control cells (–Dox) (**A**). Similar effects were observed using L-leucine as nutrient stimulus (**B**). Rises in $[Ca^{2+}]_i$ in response to glucose (**C**) or L-leucine (**D**) were also attenuated in DNHNF-1α cells and are analysed further in Table I.

Table I. Effects of glucose, leucine and KCl on $[Ca^{2+}]_i$ (nM) in DNHNF-1α#31 cells

	Basal	Glucose	Δ	Leucine	Δ	KCl-peak	Δ	KCl-plateau	Δ
– Dox	95 ± 4	153 ± 46	58 ± 15	171 ± 27	76 ± 14	348 ± 72	253 ± 20	253 ± 43	158 ± 12
+Dox	94 ± 5	119 ± 15	25 ± 5	136 ± 9	42 ± 4	303 ± 52	209 ± 14	244 ± 38	150 ± 11
% change	–1	–22	–56	–20	–45	–17	–17	–5	–5
P	NS	<0.02	<0.05	<0.05	<0.05	NS	NS	NS	NS
n	13	9		4		13		13	

DNHNF-1α was induced by doxycycline for 48 h prior to the spinner culture and Fura-2 AM loading. $[Ca^{2+}]_i$ was then measured: just before the addition of substrates (basal); 5 min after the addition of 10 mM glucose (12.5 mM final) or 20 mM L-leucine; at the highest value after the addition of 20 mM KCl (KCl-peak); and 5 min after KCl (KCl-plateau). Values are mean ± SEM (see Figure 6C and D for representative traces).

negative mechanism (Yamagata *et al*., 1996b, 1998; Glucksmann *et al*., 1997). Mutations in HNF-1β have also been linked to the MODY3 phenotype (Lindner *et al*., 1997; Nishigori *et al*., 1998). Mutations in one allele of the HNF-1α gene led to impaired insulin secretory response and diabetes, whereas the heterozygous HNF-1α (+/–) mice appeared normal (Pontoglio *et al*., 1996; Lee *et al*., 1998). This is probably due to the latter lacking the dominant-negative function on insulin secretion. In addition, the upregulated expression of HNF-1β seen in HNF-1α-null mice may have a compensatory effect (Pontoglio *et al*., 1996). In the present study, the induction of DNHNF-1α is expected to heterodimerize with both HNF-1α and HNF-1β and diminish their function, providing a model which resembles more closely the β-cell phenotype of MODY3 patients than that of the HNF1α-null mice (Pontoglio *et al*., 1996, 1998; Lee *et al*., 1998). Strictly speaking, neither HNF-1α knockout mice nor cell lines overexpressing dominant-negative mutations reflect the 20-year period during which the MODY3 patients develop the diabetic syndrome. It must be assumed that moderate expression of dominant-negative mutants of HNF-1α will require long time periods before impact on β-cell function becomes apparent. Therefore, we chose high levels of overexpression of the dominant-negative DNHNF-1α to achieve rapid effects. However, the dominant-negative action is not the only mechanism leading to MODY3. Mutations in the promoter region (Gragnoli *et al*., 1997) and dimerization domain (Glucksmann *et al*., 1997) of HNF-1α, which result in reduced HNF-1α gene dosage, have also been associated with the MODY3 phenotype.

HNF-1α is necessary for insulin gene transcription

The nucleotide sequence of the insulin gene, as well as the deduced amino acid sequences, have been remarkably

conserved throughout evolution. Most species carry a single copy of the insulin gene, except the rat and mouse which functionally express two non-allelic insulin genes, termed insulin I and II. Evolutionarily conserved promoter sequences control the expression of the insulin gene that represents one of the hallmarks of β-cell differentiation in the developing pancreas. The exact length of the 5′-flanking region sufficient for basal and regulated transcriptional activity remains unclear, but sequences at least 4 kb upstream are proposed to regulate transcription (Fromont-Racine *et al*., 1990). The best-defined 400 bp of the insulin proximal promoter, which confers β-cell-specific expression, is composed of multiple *cis*-acting DNA elements interacting with distinct classes of transcription factors including PDX1, BETA2 and HNF-1α (reviewed by Sander and German, 1997).

Emens *et al*. (1992) reported that expression of HNF-1α cDNA in COS cells resulted in transactivation of a chloramphenicol acetyltransferase (CAT) reporter gene driven by the thymidine kinase promoter placed under the control of multimerized HNF-1α binding segments of the rat insulin I enhancer, and suggested that HNF-1α may be involved in cell-specific and physiological regulation of insulin gene transcription. A recent report by Lee *et al.* (1998) showed that the amount of immunohistochemically stained insulin peptide was significantly reduced in the pancreas of HNF-1α knockout mice. MODY3 patients exhibit diminished plasma insulin concentrations both under basal and glucose-stimulated conditions, and often require insulin replacement therapy (Byrne *et al*., 1996; Lehto *et al*., 1997). This suggests reduced expression of insulin in patients carrying a mutant allele of HNF-1α. To our knowledge, the present study provides the first demonstration at the molecular level that HNF-1α is essential for insulin gene transcription. There was a drastic reduction in HNF-1α binding to its recognition site in the rat insulin minienhancer when DNHNF-1α was induced. Mutation of FLAT-F has been shown to eliminate the HNF-1α DNA-binding activity (Emens *et al*., 1992) and the present study clearly demonstrates that the same mutation also abolished its transactivation of luciferase reporter constructs containing multimerized segments of the rat insulin I minienhancer (Figure 3D). Furthermore, the dominant-negative suppression of HNF-1α function resulted in >80% decreases in both the expression of endogenous insulin mRNA and the activity of rat insulin I promoter. The marked time-dependent decrease in cellular insulin content is an expected consequence of the reduced insulin gene transcription. A 40% reduction in pancreatic insulin content (adjusted for pancreas weight) has also been reported by Pontoglio *et al*. (1998) in the HNF-1α knockout mice, which agrees with immunofluorescence studies on another transgenic mouse model (Lee *et al*., 1998). However, the unaltered insulin mRNA levels observed by Pontoglio *et al*. (1998) remain intriguing. In contrast to the altered insulin secretion and $[Ca^{2+}]_i$ rises which were measured in islets of 2-month-old mice, Northern blotting of insulin was performed on 2-week-old animals. Nevertheless, the decreased insulin content does not explain the impaired insulin secretory response to nutrient stimuli in cells deficient in HNF-1α function.

HNF-1α regulates expression of genes involved in glucose transport and metabolism

Glucose is the predominant regulator of insulin secretion (Newgard and McGarry, 1995; Matschinsky, 1996; Prentki, 1996; Wollheim *et al*., 1996) and biosynthesis. In the latter case, the action on translation is acute, while it is more chronic on transcription and mRNA stability (Dumonteil and Philippe, 1996). All these effects require glucose metabolism via glycolysis; especially in the case of secretion, mitochondrial oxidation is of crucial importance (Prentki, 1996; Maechler *et al*., 1997). In β-cells and in INS-1 cells, expression of the high-capacity and low-affinity glucose transporter GLUT-2 and the high K_m glucose-phosphorylating enzyme, glucokinase, are considered necessary for normal glucose sensing, although glucokinase rather than GLUT-2 constitutes the rate-limiting step for glycolytic flux (Sekine *et al*., 1994; Newgard and McGarry, 1995; Matschinsky, 1996; Wang and Iynedjian, 1997). Furthermore, knockout of GLUT-2 only impairs the acute phase of glucose-stimulated insulin release (Guillam *et al*., 1997). It is known that the GLUT-2 promoter contains a putative HNF-1α-binding site (Emens *et al*., 1992), but the role of HNF-1α in the regulation of GLUT-2 gene expression has not been proven by experimental evidence until now. We demonstrate that defective HNF-1α function results in diminished expression of GLUT-2 transcripts. The reduced expression of GLUT-2 as shown by Northern blotting (and by Western blotting, ~40% decrease; data not shown) may contribute in part to the diminished glycolysis, but is not sufficient to explain the impaired glucose metabolism. This conclusion is supported by the even more pronounced inhibition of leucine oxidation compared with glucose oxidation observed in DNHNF-1α-overexpressing cells. Leucine is a physiological stimulator of insulin secretion, which acts downstream of glycolysis and depends exclusively on mitochondrial oxidation for its signal generation (Prentki, 1996). The reduction of glycolytic flux following suppression of HNF-1α function is probably secondary to diminished mitochondrial oxidation, as it is well established that there is a tight coupling between aerobic and non-aerobic glucose metabolism in the β-cell (Sekine *et al*., 1994 and references therein).

We found that the expression of L-PK, which like GLUT-2 is a glucose-responsive gene (Marie *et al*., 1993; Antoine *et al*., 1997), is also regulated by HNF-1α. However, the activity of this enzyme is not flux-determining for glycolysis in the β-cell (MacDonald, 1995). In contrast, the glucose-sensing enzyme, glucokinase, is not affected by manipulations of HNF-1α function. Thus, alterations in glucokinase activity are unlikely to contribute to the severe impairment of insulin secretion in MODY3 patients (Byrne *et al*., 1994) in contrast to its involvement in the mild secretory defect in MODY2 patients (Froguel *et al*., 1993). The HNF-1α deficiency may also lead to defective liver glucose metabolism, since HNF-1α is known to regulate many genes important for liver function, such as GLUT2, phosphoenolpyruvate carboxykinase (Yanuka-Kashles *et al*., 1994), L-PK and insulin-like growth factor I (IGF-I; Lee *et al*., 1998). Gene knockout studies have shown that HNF-1α-deficient mice develop liver dysfunction (Pontoglio *et al*., 1996).

Defective mitochondrial function as an explanation for impaired nutrient-stimulated insulin secretion in β-cells deficient in HNF-1α function

Mitochondrial substrate oxidation in the β-cell plays a critical role in the generation of factors coupling glucose and leucine metabolism to insulin secretion (Prentki, 1996). This is most clearly demonstrated in patients with mutations in the mitochondrial genome who often suffer from a severe, maternally inherited diabetes syndrome characterized by impaired insulin secretion and neurosensory deafness (Maassen and Kadowaki, 1996). The mitochondrial DNA encodes 13 polypeptides which are subunits of the respiratory chain complexes, the majority of the subunits being encoded by nuclear DNA (Wallace *et al*., 1995). Decreased cytochrome *c* oxidase activity has been demonstrated in islets in such a diabetic patient post-mortem (Kobayashi *et al*., 1997). Elimination of mitochondrial DNA in cell culture has been shown to abolish completely glucose-stimulated insulin secretion (Soejima *et al*., 1996; Kennedy *et al*., 1998), whereas K^+ depolarization, which also increases $[Ca^{2+}]_i$, continued to promote insulin secretion (Kennedy *et al*., 1998). Thus, functional mitochondria are required for signal generation implicated in glucose-induced insulin secretion.

Suppression of HNF-1α function leads to profound inhibition of glucose- and leucine-induced insulin secretion. The underlying mechanism is revealed by the strong reduction of mitochondrial oxidation of these two nutrients. This is further emphasized by the abrogation of ATP production, which for glucose occurs predominantly and for leucine exclusively in the mitochondria. Mitochondrial ATP generation leads to membrane depolarization and voltage-dependent Ca^{2+} influx. Increased $[Ca^{2+}]_i$ triggers exocytosis of insulin (Wollheim *et al*., 1996). Mitochondrial metabolism also generates other coupling factors in a $[Ca^{2+}]_i$-dependent manner (Maechler *et al*., 1997). Therefore, the inhibition of the membrane depolarization and $[Ca^{2+}]_i$ rises evoked by glucose and leucine suggests a primary defect in the production of ATP and other metabolic coupling factors by mitochondria. L-type Ca^{2+} channel function and the downstream process of insulin exocytosis (Wollheim *et al*., 1996) are not affected by loss of HNF-1α function, as K^+-induced $[Ca^{2+}]_i$ rises and insulin secretion were largely preserved. In agreement with our results, Pontoglio *et al*. (1998) have recently reported that glucose-induced but not K^+-evoked $[Ca^{2+}]_i$ increase was diminished in HNF-1α-deficient mice. In addition, insulin secretory responses to glucose and arginine in the perfused pancreas and perifused islets from these HNF-1α-null mice have also been shown to be dramatically reduced (Pontoglio *et al*., 1998).

In conclusion, the present findings demonstrate that suppression of HNF-1α activity has multiple and severe consequences for the β-cell. HNF-1α function is essential for insulin gene transcription and maintenance of cellular insulin content. Metabolism–secretion coupling is defective because of pronounced reduction of mitochondrial oxidation and ATP generation in response to the nutrient stimuli. These results propose a molecular basis for the impaired insulin secretion characteristic of the diabetic phenotype of MODY3 patients. The present study establishes a cell model for further exploration of the role of HNF-1α in transcription of the specific genes that are involved in mitochondrial oxidation and insulin secretion.

Materials and methods

Establishment of stable cell lines

The INS-1 cell line-derived clones were cultured in RPMI 1640 (11 mM glucose, unless indicated otherwise) supplemented with 10% fetal calf serum and other additions as given previously (Asfari *et al*., 1992). Stable transfection was performed using the calcium phosphate–DNA co-precipitation method followed by glycerol shock (Kingston *et al*., 1990). The establishment of the first-step stable clone INS-r3 expressing the reverse tetracycline-dependent transactivator was described in detail previously (Wang and Iynedjian, 1997). The plasmids used in the second round of stable transfection were constructed by inserting either wild-type HNF-1α or DNHNF-1α cDNA (the latter corresponding to SM6; Nicosia *et al*., 1990; both kindly supplied by Dr R.Cortese, Istituto Di Ricerche Di Biologia Molecolare, Pomezia, Italy) downstream of the tetracycline operator–cytomegalovirus minimal promoter in plasmid PUHD10-3 (Gossen *et al*., 1995). Plasmid pTK-hygro (a kind gift from Dr N.Quintrell) encoding the second selection marker, hygromycin resistance cassette, was co-transfected for selection by culture in the presence of 200 μg/ml of hygromycin. Resistant clones were picked individually with cloning rings and maintained in long-term culture with 150 μg/ml G418 and 100 μg/ml hygromycin. Immunoblotting with antibodies raised against HNF-1α (N or C terminus, kindly provided by Dr R.Cortese) was used for screening clones with high-level expression of HNF-1α or DNHNF-1α protein after 24 h of cell culture with 1000 ng/ml doxycycline.

Immunoblot analysis

Immunoblotting procedures were performed as described previously (Wang and Iynedjian, 1997) using enhanced chemiluminescence (Pierce) for detection. Nuclear extract from HNF-1α#15 or DNHNF-1α#31 cells was fractionated on a 9% SDS–polyacrylamide gel, transferred to nitrocellulose by electroblotting, incubated in a sequential order with a blocking solution, primary antibody (used at the following dilutions: anti-HNF-1α antibodies from Dr R.Cortese, 1:4000; anti-TFIIE-α antibody from Santa Cruz Biotechnology, 1:3000; anti-PDX1 antibody from Dr T.Edlund, 1:5000); and HRP-conjugated secondary antibody (Pierce, 1:10 000). Nuclear extracts from the non-transfected parent INS-1 cells and primary islets were used to demonstrate the endogenous HNF-1α level.

Nuclear extract preparation and electrophoretic mobility-shift assay (EMSA)

Cells in 10-cm dishes were cultured in complete INS-1 medium with or without 500 ng/ml doxycycline for 48 h. Thereafter, nuclear extracts were prepared according to Schreiber *et al*. (1988).

EMSA was performed as previously described by Philippe (1991) using the following double-stranded oligonucleotides: rat L-PK L1 element (Miquerol *et al*., 1994) CTAGCTGGTTATACTTTAACCAGGACTCGATCGACCAATATGAAATTGGTCCTGAG; Rat insulin I FLAT element (Emens *et al*., 1992) GATCTTGTTAATAATCTAATTACCCTAGAACAATTATTAGATTAATGG.

The double-stranded oligonucleotides were end-labelled with [α-^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase I and used as probes. Binding reactions were conducted for 15 min at room temperature with 2 or 8 μg nuclear extract, 10^4 c.p.m. probe, 1 μg BSA, 2 mM DTT, 50 mM KCl, 6.25% glycerol, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 μg poly(dI.dC) and 1 μg denatured salmon sperm DNA in a final volume of 20 μl. In competition experiments, the unlabelled competitor DNA (50-, 100- and 200-fold molar excess) was added simultaneously with the ^{32}P-end-labelled oligonucleotide duplex probe. For supershift experiments, 1 μl of the polyclonal rabbit antiserum was added to the reaction mixture after 7 min, and then continued for a further 8 min. The samples were subjected to electrophoretic separation on a 4% non-denaturing polyacrylamide gel. The gel was then dried, and labelled DNA–protein complexes were located by autoradiography.

Total RNA extraction and Northern blot analysis

Total RNA was extracted from cells cultured in 10-cm dishes by the guanidinium thiocyanate/phenol/chloroform method (Chomczynscki and Sacchi, 1987). Total RNA (20 μg) was denatured with glyoxal and dimethylsulphoxide and separated on 1% agarose gels as described (McMaster and Carmichael, 1977). Resolved RNA was blotted to nylon

membranes by vacuum transfer (VacuGene™ XL, Pharmacia, Uppsala, Sweden), followed by UV cross-linking. The membranes were prehybridized and then hybridized to ^{32}P-labelled random primer cDNA probes according to standard protocols (Sambrook *et al*., 1989). cDNA fragments used as probes for L-PK, GLUT-2, glucokinase, insulin, PDX1, Beta-2 and HNF-1α mRNA detection were obtained from corresponding expression vectors kindly given by Drs A.Kahn, B.Thorens, P.B.Iynedjian, J.Philippe, T.Edlund, M.-J.Tsai and R.Cortese.

Transient transfection and luciferase reporter enzyme assay

Cells from DNHNF-1α#31 or HNF-1α#15 lines at 60–70% confluence were harvested from T75 flasks by trypsinization and seeded into 6-well culture dishes at a density of 1.5×10^6 cells per well. After culturing in complete medium for 18 h, cells in each well were replenished with 1 ml fresh medium 2 h before transfection. Plasmids –410INSluc, Far-FLAT*wt*, Far-FLAT*MF* and Far-FLAT*ME* were kindly supplied by Dr M.S.German (University of California, San Francisco, USA). The transfection mixture containing calcium phosphate–DNA precipitates formed with 10 μg of plasmid, essentially as described by Kingston *et al*. (1990), was placed onto cells. At 20 h after transfection, cells were glycerol-shocked for 2 min and rinsed twice with medium before culturing for 48 h with or without 500 ng/ml doxycycline in complete medium. The cytosolic luciferase was extracted as previously reported (Iynedjian *et al*., 1996). Separate cell extracts were obtained from duplicate wells to monitor the reproducibility of the transfection efficiency (replicates generally varied by <10% in luciferase activity). The luciferase assay was performed by adding 30 μl of a reaction mixture, containing 20 mM Tricine pH 7.8, 4 mM $MgCl_2$, 0.1 mM EDTA, 33 mM dithiothreitol, 530 μM ATP, 270 μM CoA and 300 μM firefly luciferin (Iynedjian *et al*., 1996), into 30 μl of cell extract with an input of 20 μg of protein. The sample was mixed and light emission measured immediately in a luminometer (TD-20/20, Turner Designs).

Measurements of cellular insulin content and insulin secretion

Insulin content was determined after extraction with acid ethanol as described previously (Asfari *et al*., 1992). Insulin secretion was measured over a period of 30 min, in Krebs–Ringer–bicarbonate–HEPES buffer (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, 0.1% BSA) containing indicated stimulators. Insulin was detected by radioimmunoassay using rat insulin as standard and an antibody for rat insulin determination purchased from LINCO (St Louis, MO, USA).

Measurement of glycolysis, mitochondrial oxidation and intracellular ATP

Glucose utilization was measured and calculated as reported previously (Wang and Iynedjian, 1997). In brief, the production of ^{3}H-labelled water from cells was measured during 30 min incubation with KRBH containing the indicated concentration of glucose and D-5-[^{3}H]glucose at a constant specific activity of 0.313 nCi/nmol.

For measurement of mitochondrial oxidation (Sekine *et al*., 1994), cells in 35-mm dishes treated with polyornithine were cultured in medium containing 2.5 mM glucose in the presence or absence of 500 ng/ml doxycycline for 48 h. Cells were then transferred into a thermostatic glass chamber, and preincubated for 30 min in KRBH. Oxidation was initiated by replacing the buffer with 1 ml of KRBH containing 0.5 μCi/chamber of 12.8 mM D-[^{14}C(U)]glucose or 20 mM L-[1-^{14}C]leucine (DuPont-NEN, Boston, MA, USA). After 1 h incubation at 37°C in sealed chambers, 0.5 ml of 0.1 M HCl was added onto the cell layers to stop the reaction and 1 ml benzethonium hydroxide (Fluka, Buchs, Switzerland) was injected into the bottom of the chamber to bind the CO_2 liberated by the cells. Following an overnight incubation at room temperature, $^{14}CO_2$ production was measured in benzethonium extracted with 5 ml ethanol and counted in an LS6500 liquid scintillation counter (Beckman, Palo Alto, CA, USA).

For the assay of ATP, cells in 6-well dishes were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 48 h. Cells were then stimulated for 8 min in KRBH and scraped into 0.5 ml of ice-cold 10% trichloroacetic acid. The extract was sonicated on ice and centrifuged for 6 min. The resulting supernatant was neutralized with 2 M K_2CO_3. After appropriate dilution in a buffer containing 20 mM HEPES pH 7.75 and 3 mM $MgCl_2$, samples or ATP standards in a volume of 20 μl were added to 20 μl luciferin–luciferase reagent (Boehringer Mannheim), and luminescence was measured immediately in the same luminometer as described above in the luciferase assay. Cellular DNA content measured in each experiment in parallel wells was used to normalize the ATP production.

Cell membrane potential and $[Ca^{2+}]_i$ measurements

Cells in 10-cm dishes were cultured in medium containing 2.5 mM glucose in the presence or absence of 500 ng/ml doxycycline. After a 46 h culture period, cells were trypsinized and the cell suspension was further maintained for 2 h under spinner culture conditions at 37°C in the same medium. For cell membrane potential measurements, 2×10^6 cells were pelleted, resuspended in 2 ml KRBH with 2.5 mM glucose and 100 μM bis-oxonol (Molecular Probes, Eugene, OR, USA) and transferred to a thermostatic cuvette. Cells were then excited at 540 nm and emission was recorded at 580 nm (Asfari *et al*., 1992). For $[Ca^{2+}]_i$ measurements, 1 μM Fura-2 AM (Teflabs, Austin, TX, USA) was added to the spinner culture for the last 30 min. An aliquot of 2×10^6 cells was then pelleted, washed in KRBH and resuspended in 2 ml KRBH with 2.5 mM glucose and 0.1 mM EGTA. $[Ca^{2+}]_i$ was monitored as the calibrated ratio of dual wavelength excitation (340/380 nm). All fluorescence measurements were performed at 37°C with gentle stirring in an LS-50B fluorimeter (Perkin-Elmer, Bucks, UK).

Acknowledgements

We are grateful to Ms D.Harry, Ms C.Bartley, Ms G.Chaffard and Mr J.Nicolet for expert technical assistance. We thank Dr R.Cortese, Istituto Di Ricerche Di Biologia Molecolare, Pomezia, Italy, for kindly supplying HNF-1α cDNAs and antibodies. We are indebted to Drs T.Edlund (PDX-1 cDNA and antibody), B.Thorens (GLUT-2 cDNA and antibody), M.S.German (–410INSluc cDNA and three insulin minienhancer constructs), M.-J.Tsai (Beta-2 cDNA), P.B.Iynedjian (glucokinase cDNA and INS-r3 cells), J.Philippe (insulin-I cDNA), H.Bujard (PUHD10-3 plasmid), N.Quintrell (pTKhygro plasmid), M.A.Glucksmann (Pro-291insC cDNA) and A.Kahn (L-PK cDNA). We are also grateful to Dr B.Ritz-Laser for helpful advice on the gel shift assays. This work was supported by the Swiss National Science Foundation (No. 32-49755.96).

References

Antoine,B., Lefrançois-Martinez,A.-M., Le Guillou,G., Leturque,A., Vandewalle,A. and Kahn,A. (1997) Role of the GLUT 2 glucose transporter in the response of the L-type pyruvate kinase gene to glucose in liver-derived cells. *J. Biol. Chem.*, **272**, 17937–17943.

Asfari,M., Janjic,D., Meda,P., Li,G., Halban,P.A. and Wollheim,C.B. (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology*, **130**, 167–178.

Byrne,M.M. *et al*. (1994) Insulin secretory abnormality in subjects with hyperglycemia due to glucokinase mutations. *J. Clin. Invest*., **93**, 1120–1130.

Byrne,M.M. *et al*. (1996) Altered insulin secretory response to glucose in diabetic and nondiabetic subjects with mutations in the diabetes susceptibility gene MODY3 on chromosome 12. *Diabetes*, **45**, 1503–1510.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*., **162**, 156–159.

De Simone,V., De Magistris,L., Lazzaro,D., Gerstner,J., Monaci,P., Nicosia,A. and Cortese,R. (1991) LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. *EMBO J*., **10**, 1435–1443.

Dumonteil,E. and Philippe,J. (1996) Insulin gene: organization, expression and regulation. *Diabete Metab.*, **22**, 164–173.

Emens,L.A., Landers,D.W. and Moss,L.G. (1992) Hepatocyte nuclear factor 1 alpha is expressed in a hamster insulinoma line and transactivates the rat insulin I gene. *Proc. Natl Acad. Sci. USA*, **89**, 7300–7304.

Frain,M., Swart,G., Monaci,P., Nicosia,A., Stämpfli,S., Frank,R. and Cortese,R. (1989) The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. *Cell*, **59**, 145–157.

Froguel,P. *et al*. (1993) Familial hyperglycemia due to mutations in glucokinase: definition of a subtype of diabetes mellitus. *N. Engl. J. Med.*, **328**, 697–702.

Fromont-Racine,M. *et al*. (1990) Effect of 5′ flanking sequence deletions on expression of the human insulin gene in transgenic mice. *Mol. Endocrinol*., **4**, 669–677.

German,M.S., Wang,J., Chadwick,R.B. and Rutter,W.J. (1992a) Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix–loop–helix protein: building a functional insulin minienhancer complex. *Genes Dev.*, **6**, 2165–2176.

German,M.S., Moss,L.G., Wang,J. and Rutter,W.J. (1992b) The insulin and islet amyloid polypeptide genes contain similar cell-specific promoter

elements that bind identical β-cell nuclear complexes. *Mol. Cell. Biol*., **12**, 1777–1788.
Glucksmann,M.A. *et al*. (1997) Novel mutations and a mutational hotspot in the MODY3 gene. *Diabetes*, **46**, 1081–1086.
Gossen,M., Freundlieb,S., Bender,G., Müller,G., Hillen,W. and Bujard,H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science*, **268**, 1766–1769.
Gragnoli,C., Lindner,T., Cockburn,B.N., Kaisaki,P., Gragnoli,F., Marozzi,G. and Bell,G.I. (1997) Maturity-onset diabetes of the young due to a mutation in the hepatocyte nuclear factor-4α binding site in the promoter of the hepatocyte nuclear factor-1α gene. *Diabetes*, **46**, 1648–1651.
Guillam,M.T., Hummler,E., Schaerer,E., Yeh,J.I., Birnbaum,M.J., Beermann,F., Schmidt,A., Deriaz,N. and Thorens,B. (1997) Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2. *Nature Genet*., **17**, 327–330.
Iynedjian,P.B., Marie,S., Wang,H., Gjinovci,A. and Nazaryan,K. (1996) Liver-specific enhancer of the glucokinase gene. *J. Biol. Chem*., **271**, 29113–29120.
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.
Kaisaki,P.J. *et al*. (1997) Mutations in the hepatocyte nuclear factor-1alpha gene in MODY and early-onset NIDDM: evidence for a mutational hotspot in exon 4. *Diabetes*, **46**, 528–535.
Kennedy,E.D., Maechler,P. and Wollheim,C.B. (1998) Effects of depletion of mitochondrial DNA in metabolism secretion coupling in INS-1 cells. *Diabetes*, **47**, 374–380.
Kennedy,H.J., Viollet,B., Rafiq,I., Kahn,A. and Rutter,G.A. (1997) Upstream stimulatory factors (USF2) activity is required for glucose stimulation of L-pyruvate kinase promoter activity in single living islet β-cells. *J. Biol. Chem*., **272**, 20636–20640.
Kingston,R.E., Chen,C.A. and Okayama,H. (1990) Transfection of DNA into eukaryotic cells. In Ausubel,F.M., Brent,R., Kingston,R.E., Morre,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology.* John Wiley & Sons, USA, pp. 9.1.1–9.1.9.
Kobayashi,T., Nakanishi,K., Nakase,H., Kajio,H., Okubo,M., Murase,T. and Kosaka,K. (1997) *In situ* characterization of islets in diabetes with a mitochondrial DNA mutation at nucleotide position 3243. *Diabetes*, **46**, 1567–1571.
Lee,Y.-H., Sauer,B. and Gonzalez,F.J. (1998) Laron dwarfism and non-insulin-dependent diabetes mellitus in the *Hnf-1*α knock out mouse. *Mol. Cell. Biol*., **18**, 3059–3068.
Lehto,M. *et al*. (1997) Characterization of the MODY3 phenotype: early-onset diabetes caused by an insulin secretion defect. *J. Clin. Invest*., **99**, 582–591.
Lichtsteiner,S. and Schibler,U. (1989) A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. *Cell*, **57**, 1179–1187.
Lindner,T., Yamagata,K., Ogata,M., Tomonaga,O., Kuroki,H., Kasahara,T., Iwamoto,Y. and Bell,G.I. (1997) Mutation in hepatocyte nuclear factor-1β gene (TCF2) associated with MODY. *Nature Genet*., **17**, 384–385.
Maassen,J.A. and Kadowaki,T. (1996) Maternally inherited diabetes and deafness: a new diabetes subtype. *Diabetologia*, **39**, 375–382.
MacDonald,M.J. (1995) Is liver type pyruvate kinase present in the normal pancreatic islet? *Diabetologia*, **38**, 125.
Maechler,P., Kennedy,E.D., Pozzan,T. and Wollheim,C.B. (1997) Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic β-cells. *EMBO J*., **16**, 3833–3841.
Marie,S., Diaz-Guerra,M.-J., Miquerol,L., Kahn,A. and Iynedjian,P.B. (1993) The pyruvate kinase gene as a model for studies of glucose-dependent regulation of gene expression in the endocrine pancreatic β-cell type. *J. Biol. Chem*., **268**, 23881–23890.
Matschinsky,F.M. (1996) Banting lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes*, **45**, 223–241.
McMaster,G.K. and Carmichael,C.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl Acad. Sci. USA*, **74**, 4835–4838.
Miquerol,L., Lopez,S., Cartier,N., Tulliez,M., Raymondjean,M. and Kahn,A. (1994) Expression of the L-type pyruvate kinase gene and the hepatocyte nuclear factor 4 transcription factor in exocrine and endocrine pancreas. *J. Biol. Chem*., **269**, 8944–8955.
Naya,F.J., Huang,H.-P, Qui,Y., Mutoh,H., DeMayo,F.J., Leiter,A.B. and Tsai,M.-J. (1997) Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/Neuro D-deficient mice. *Genes Dev.*, **11**, 2323–2334.
Newgard,C.B. and McGarry,J.D. (1995) Metabolic coupling factors in pancreatic β-cell signal transduction. *Annu. Rev. Biochem*., **64**, 689–719.
Nicosia,A., Monaci,P., Tomei,L., De Francesco,R., Nuzzo,M., Stunnenberg,H. and Cortese,R. (1990) A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA binding structure of LFB1. *Cell*, **6**, 1225–1236.
Nishigori,H., Yamada,S., Kohama,T., Tomura,H., Sho,K., Horikawa,Y., Bell,G.I., Takeuchi,T. and Takeda,J. (1998) Frameshift mutation, A263fsinsGG, in the hepatocyte nuclear factor-1β gene associated with diabetes and renal dysfunction. *Diabetes*, **47**, 1354–1355.
Philippe,J. (1991) Insulin regulation of the glucagon gene is mediated by an insulin-responsive DNA element. *Proc. Natl Acad. Sci. USA*, **88**, 7224–7227.
Pontoglio,M., Barra,J., Hadchouel,M., Doyen,A., Kress,C., Bach,J.P., Babinet,C. and Yaniv,M. (1996) Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell*, **84**, 575–585.
Pontoglio,M. *et al*. (1998) Defective insulin secretion in hepatocyte factor 1α-deficient mice. *J. Clin. Invest*., **101**, 2215–2222.
Prentki,M. (1996) New insights into pancreatic β-cell metabolic signaling in insulin secretion. *Eur. J. Endocrinol*., **134**, 272–286.
Rey-Campos,J., Chouard,T., Yaniv,M. and Cereghini,S. (1991) vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1. *EMBO J*., **10**, 1445–1457.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In Sambrook,J., Fritsch,E.F. and Maniatis,T. (eds), *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 7.3–7.84.
Sander,M. and German,M.S. (1997) The β cell transcription factors and development of the pancreas. *J. Mol. Med*., **75**, 327–340.
Schreiber,E., Matthias,P., Muller,M.M. and Schaffner,W. (1988) Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping bandshift assay (PCBA). *EMBO J*., **7**, 4221–4229.
Sekine,N. *et al*. (1994) Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic β-cells. *J. Biol. Chem*., **269**, 4895–4902.
Shelton,K.D., Franklin,A.J., Khoor,A., Beechem,J. and Magnuson,M.A. (1992) Multiple elements in the upstream glucokinase promoter contribute to transcription in insulinoma cells. *Mol. Cell. Biol*., **12**, 4578–4589.
Soejima,A., Inoue,K., Takai,D., Kaneko,M., Ishihara,H., Oka,Y. and Hayashi,J.I. (1996) Mitochondrial DNA is required for regulation of glucose-stimulated insulin secretion in a mouse pancreatic β-cell line: MIN6. *J. Biol. Chem*., **271**, 26194–26199.
Stoffers,D.A., Ferrer,J., Clarke,W.L. and Habener,J.F. (1997) Early-onset type-II diabetes mellitus (MODY4) linked to IPF-1. *Nature Genet*., **17**, 138–139.
Wallace,D.C., Shoffner,J.M., Trounce,I., Brown,M.D., Ballinger,S.W., Corral-Debrinski,M., Horton,T., Jun,A.S. and Lott,M.T. (1995) Mitochondrial DNA mutations in human degenerative diseases and aging. *Biochim. Biophys. Acta*, **1271**, 141–151.
Wang,H. and Iynedjian,P.B. (1997) Modulation of glucose responsiveness of insulinoma β-cells by graded overexpression of glucokinase. *Proc. Natl Acad. Sci. USA*, **99**, 4372–4377.
Wollheim,C.B., Lang,J. and Regazzi,R. (1996) The exocytotic process of insulin secretion and its regulation by Ca^{2+} and G-proteins. *Diabetes Rev.*, **4**, 276–297.
Yamagata,K. *et al*. (1996a) Mutations in the hepatocyte nuclear factor-4α gene in maturity-onset diabetes of the young (MODY 1). *Nature*, **384**, 458–460.
Yamagata,K. *et al*. (1996b) Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). *Nature*, **384**, 455–458.
Yamagata,K. *et al*. (1998) Mutation P291fsinsC in the transcription factor hepatocyte nuclear factor-1α is dominant negative. *Diabetes*, **47**, 1231–1235.
Yanuka-Kashles,O., Cohen,H., Cihail,T., Aran,A., Benvenisty,N. and Reshef,L. (1994) Transcription regulation of the phosphoenolpyruvate carboxykinase gene by cooperation between hepatic nuclear factors. *Mol. Cell. Biol*., **14**, 7124–7133.

Received May 29, 1998; revised August 18, 1998; accepted September 21, 1998