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Transcription Factor Hepatocyte Nuclear Factor 6 Regulates Pancreatic Endocrine Cell Differentiation and Controls Expression of the Proendocrine Gene *ngn3*

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Hepatocyte nuclear factor 6 (HNF-6) is the prototype of a new class of cut homeodomain transcription factors. During mouse development, HNF-6 is expressed in the epithelial cells that are precursors of the exocrine and endocrine pancreatic cells. We have investigated the role of HNF-6 in pancreas differentiation by inactivating its gene in the mouse. In *hnf6*$^{-/-}$ embryos, the exocrine pancreas appeared to be normal but endocrine cell differentiation was impaired. The expression of neurogenin 3 (Ngn-3), a transcription factor that is essential for determination of endocrine cell precursors, was almost abolished. Consistent with this, we demonstrated that HNF-6 binds to and stimulates the *ngn3* gene promoter. At birth, only a few endocrine cells were found and the islets of Langerhans were missing. Later, the number of endocrine cells increased and islets appeared. However, the architecture of the islets was perturbed, and their β cells were deficient in glucose transporter 2 expression. Adult *hnf6*$^{-/-}$ mice were diabetic. Taken together, our data demonstrate that HNF-6 controls pancreatic endocrine differentiation at the precursor stage and identify HNF-6 as the first positive regulator of the proendocrine gene *ngn3* in the pancreas. They also suggest that *HNF-6* is a candidate gene for diabetes mellitus in humans.

The pancreas contains exocrine cells that produce digestive enzymes, ducts through which these enzymes transit to the gut, and endocrine cells, organized in islets of Langerhans, that produce insulin, glucagon, somatostatin, and pancreatic polypeptide (PP). The pancreas arises from the primitive gut epithelium as a dorsal bud and a ventral bud, which later fuse to form a single organ (reviewed in reference 29). The pancreatic epithelium, surrounded by mesenchyme, then proliferates and branches into multiple evaginations. During the initial stage of pancreas development (embryonic day 9.5 [e9.5] to approximately e14.5 in the mouse), the pancreatic epithelium consists mainly of cells that are the precursors of the acinar, ductal, and endocrine cells (29). Starting at e9.5, early glucagon-expressing cells are found in the epithelium. Around e12, glucagon cells are found both within the epithelium and in small clusters that are distinct from the epithelium. The fate of these clusters is unknown. Starting around e14.5 to e15, a wave of differentiation is associated with an increase in the synthesis of digestive enzymes and hormones, ultimately resulting in the formation of the acini, ducts, and islets of Langerhans around e18 to e19. The insulin-expressing cells (β cells) are then in the core of the islets, and the cells expressing glucagon (α cells), somatostatin (δ cells), and PP are located at their periphery (reviewed in references 29 and 32).

A number of transcription factors are involved in endocrine pancreas development (reviewed in references 8 and 25). They belong to the class of basic helix-loop-helix factors (Ngn-3, NeuroD/Beta2, and Hes-1) or are homeoproteins of the LIM (Isl-1), paired-box (Pax-4 and Pax-6), Antennapedia (Hb9 and Pdx-1; also known as IPF-1, IDX-1, STF-1, IUF-1, and GSF), or NK-2 (Nkx2.2) class. Targeted disruption of the genes coding for these transcription factors indicated that they are required for pancreatic morphogenesis and/or for the differentiation of one or several pancreatic endocrine cell types (1, 2, 10, 13, 17, 18, 23, 24, 26, 31, 33–36). The temporal and cellular expression profiles of these factors and the results of gene disruption experiments led to the proposal that pancreatic endocrine cell differentiation relies on the activation of a cascade of transcription factors (8, 16). Moreover, it was proposed recently that pancreatic endocrine cell differentiation is controlled by a lateral specification mechanism involving the Notch signaling pathway, in which Ngn-3 is the earliest marker of endocrine cell precursors (3, 10, 17).

We isolated previously a cDNA coding for the transcription factor hepatocyte nuclear factor 6 (HNF-6) (22). HNF-6 belongs to the new ONECUT class of cut homeodomain proteins, whose members contain a single cut domain and a divergent homeodomain (21, 22). In the mouse embryo, *hnf6* is expressed in several tissues, including the epithelial cells of the pancreas, starting at the onset of its development (20, 30). During formation of the acini, ducts, and islets, the expression of *hnf6* becomes restricted to the acini and ductal cells (20, 30). Transient transfection experiments have identified target genes for HNF-6. These include *hnf3*β, which is coexpressed with *hnf6* in the developing pancreatic epithelium (20, 30). Since the expression of Pdx-1, a factor whose absence leads to

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pancreatic agenesis (18), is controlled by HNF-3β (36) and since HNF-3β expression is stimulated by HNF-6, it has been proposed that HNF-6 is a key component of the pancreatic transcription factor cascade (11). To investigate the role of HNF-6 in pancreatic cell differentiation, we inactivated its gene by homologous recombination in the mouse.

MATERIALS AND METHODS

Construction of targeting vector and generation of knockout mice. The targeting construct was made by subcloning *hnf6* gene fragments from a strain 129 mouse genomic library in the pPNT vector. R1 embryonic stem (ES) cells (a gift from A. Nagy) derived from blastocysts of mouse strain 129 were electroporated with the linearized construct and were selected with G418 and ganciclovir. Independent ES clones containing an inactivated *hnf6* allele were aggregated with Swiss strain morula-stage embryos as described previously (6), and the embryos were transferred into pseudopregnant Swiss foster mothers. Two chimeric males from independent clones were test bred with Swiss mice for germ line transmission. Heterozygous offspring was intercrossed to generate $hnf6^{-/-}$ progeny. The progeny derived from each of these two males displayed the same phenotype. The phenotype of the $hnf6^{+/-}$ mice was normal. These mice were therefore used as controls together with wild-type littermates.

Immunohistochemistry. Dissected pancreas, fixed in Bouin's solution or in 4% paraformaldehyde in phosphate-buffered saline, was embedded in paraffin, sectioned at 5 μm, and immunostained. Primary antibodies were mouse antiinsulin (Novo BioLabs clone HUI-018), mouse antiglucagon (Novo BioLabs clone GLU-001), rabbit anti-islet amyloid polypeptide, rabbit anti-glucose transporter 2 (Glut-2) (Chemicon AB1342 or gift from B. Thorens), rabbit anti-Pdx-1, mouse antisomatostatin (Novo BioLabs clone SOM-018), rabbit anti-PP (Lilly), rabbit anti-Pax-6, mouse anti-Isl-1, and rabbit anti-Nkx6.1. Primary antibodies were detected by immunoperoxidase using biotinylated sheep anti-rabbit or anti-mouse immunoglobulin G (Boehringer Mannheim/Roche), a streptavidin-peroxidase conjugate (Boehringer Mannheim/Roche), and DAB+ (Dako). For immunofluorescence, primary antibodies were detected with secondary antibodies coupled to Texas red or Cy-2 (Jackson Immunochemicals).

In situ hybridization. Nonradioactive in situ cohybridization with RNA antisense probes labeled with digoxigenin-UTP or fluorescein-UTP was performed as described in reference 9. The Ngn-3 probe is 0.75 kb long and encompasses the entire Ngn-3 coding sequence (7). The HNF-6 probe spans nucleotides 1214 to 1607 of the mouse HNF-6 cDNA (nucleotide numbering as in GenBank accession no. U95945).

Multiplex RT-PCR analysis. Multiplex reverse transcription-PCR (RT-PCR), which allows coamplification of several cDNAs from total RNA, was performed as described elsewhere (15). Amplification products were quantified using a PhosphorImager, and relative mRNA concentrations were calculated as ratios to the coamplified internal standard. Primer sequences were 5′-TGGCGCCTCAT CCCTTGGATG-3′ and 5′-CAGTCACCCACTTCTGCTTCG-3′ (Ngn-3), 5′-C TGGTTCCCTGAGGGTTTCAA-3′ and 5′-GGAACTTCTTGGTCTCCAGG T-3′ (Notch-1), 5′-CAACATGGGCCGCTGTCCTC-3′ and 5′-CACATCTGCT TGGCAGTTGATC-3′ (Notch-2), 5′-GCAGCTGTGAACAACGTGGAG-3′ and 5′-AACCGCACAATGTCCTGGTGC-3′ (Notch-3), 5′-TCAACACGACA CCGGACAAACC-3′ and 5′-GGTACTTCCCCAACACGCTCG-3′ (Hes-1), 5′-ACCCTTCACCAATGACTCCTATG-3′ and 5′-ATGATGACTGCAGCAAAT CGC-3′ (TATA binding protein [TBP]), and 5′-GACCTGCAGAGCTCCAAT CAAC-3′ and 5′-CACGACCCTCAGTACCAAAGGG-3′ (glucose-6-phosphate dehydrogenase [G6PDH]), 5′-CAGCACCTCACGCCCACCTC-3′ and 5′-CTT CCCATGTTCTTGCTCTTTCC-3′ (HNF-6).

Cloning of the mouse *ngn3* gene promoter. A 7.0-kb-long genomic *Xba*I-*Xho*I fragment was cloned in pBluescript (10), and a 5.1-kb region from 4.95 kb upstream to 0.15 kb downstream of the ATG initiator codon was sequenced using Li-Cor 4000L and Beckman CEQ 2000 automated DNA sequencers.

Transfections. Rat-1 fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Cells (3×10^5) grown in serum-free DMEM on 60-mm-diameter dishes were cotransfected by lipofection using DOTAP and 3 μg of a reporter vector containing the *ngn3* gene promoter cloned upstream of the firefly luciferase gene, 1.5 μg of the pECE-HNF6α expression vector, and 500 ng of pRL138 plasmid coding for *Renilla* luciferase as internal control. After 5 h, the medium was replaced by DMEM plus 10% fetal calf serum and further incubated for 40 h before measuring luciferase activities with a Dual-Luciferase kit (Promega). Luciferase activities were measured with a TD-20/20 Luminometer (Promega) and expressed as the ratio of reporter activity (firefly luciferase) to internal control activity (*Renilla* luciferase). pECE-HNF6α and pRL138 have been described elsewhere (21, 22). The *ngn3* promoter reporter vector p3957ngn3-luc contains a 3,957-bp-long *Bstu*I-*Bstu*I genomic fragment (from −4573 to −617 relative to the ATG initiator codon) cloned in the *Sma*I site of pGL3 basic (Promega).

Electrophoretic mobility shift assays (EMSA). Recombinant HNF-6 was in vitro transcribed and translated using a wheat germ extract (TNT expression system; Promega). Five microliters of the reaction mix was incubated on ice for 20 min in a final volume of 20 μl containing 10 mM HEPES (pH 7.6), 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EGTA, 50 mM KCl, 10% (vol/vol) glycerol, 3 μg of poly(dI-dC), and the ^{32}P-labeled probe (30,000 cpm). The samples were loaded on a 6% acrylamide gel (acrylamide/bisacrylamide ratio of 29:1) in 0.25× Tris-borate-EDTA buffer and electrophoresed at 200 V. The sense-strand sequences of the double-stranded oligonucleotide probes, derived from the mouse *ngn3* gene, were 5′-CTTCCCGATAGCATCCATAGTGGGGCGGGG-3′ (proximal site) and 5′-GCTCAGTGCCAAATCCATGTGTCAGCTTCT-3′ (distal site) (the HNF-6 binding consensus is underlined).

Glucose, insulin, and glucagon measurements. Blood glucose (tail vein) was measured using an Elite glucometer (Bayer). Insulin and glucagon levels were measured by radioimmunoassay on 25 μl of plasma using the sensitive rat insulin radioimmunoassay and glucagon radioimmunoassay pancreas-specific kits (Linco).

RESULTS

Targeted disruption of the *hnf6* gene and generation of HNF-6 knockout mice. We inactivated the *hnf6* gene in the mouse by homologous recombination (Fig. 1). A neomycin resistance gene (*neo*) cassette was used to replace the proximal promoter region and the first exon. This exon codes for a region of the protein that is essential for DNA binding and transcriptional activity (21). The strategy for disrupting the *hnf6* gene is shown in Fig. 1A. The recombinant ES clones were identified by Southern blotting using probes located 5′ and 3′ from the recombination site (Fig. 1B). The resulting transgenic mice were genotyped by PCR as in Fig. 1C. Lack of *hnf6* expression in $hnf6^{-/-}$ pancreas was confirmed by RT-PCR (Fig. 1D). $hnf6^{+/-}$ mice were phenotypically normal. Cross-breeding of these mice produced $hnf6^{-/-}$ offspring at the frequency of 22% (83/376). The $hnf6^{-/-}$ mice were growth retarded at birth and showed a reduced growth rate (Fig. 1E). Seventy-five percent of them died between postnatal day 1 (P1) and P10, probably as a result of liver failure. The surviving mice reached adulthood.

Defective endocrine cell differentiation in HNF-6 knockout mice. To analyze the pancreas of $hnf6^{-/-}$ mice, we first performed histological and immunohistochemical examinations on embryos at various stages of development. Exocrine tissue was histologically normal. In keeping with this, the expression of the exocrine marker p48 (19) was normal at e14.5 (not shown). In contrast, endocrine development was severely inhibited in all embryos. Indeed, the number of glucagon-expressing cells was reduced by 85% in $hnf6^{-/-}$ embryos compared to control embryos at e12.5 and e15.5 (Fig. 2C to F). This reduction involved the glucagon-expressing cells found interspersed in the epithelium. However, glucagon expression was normal at e10.75 (Fig. 2A and B), and the number of clusters of early glucagon cells was normal at e12.5 and e15.5 (arrows in Fig. 2C to F). No insulin was found at e15.5 in $hnf6^{-/-}$ embryos, in contrast to control animals, in which insulin-expressing cells were found scattered throughout the epithelium (Fig. 2I and J). At P4, $hnf6^{-/-}$ animals showed a markedly reduced number of α cells (Fig. 2G and H), β cells (Fig. 2K and L), δ cells (Fig. 2M and N), and PP cells (Fig. 2O and P). These four cell types were scattered within, and in the vicinity of, pancreatic ducts, instead of being clustered in typical islets as in control littermates.

In $hnf6^{-/-}$ mice, the endocrine cells became organized in islets only 2 to 3 weeks after birth. The architecture of the islets was abnormal, as they showed no mantle of α cells around the core of β cells at 5 weeks (Fig. 3A and B) and at 10 weeks many α cells were found throughout the islets, instead of being located at their periphery (not shown). The differentiation state of islet cells in 5-week-old $hnf6^{-/-}$ mice was investigated by monitoring the coexpression of hormonal and metabolic markers. Glut-2, which in wild-type mice is expressed in β cells, was undetectable by immunofluorescence in most of the $hnf6^{-/-}$

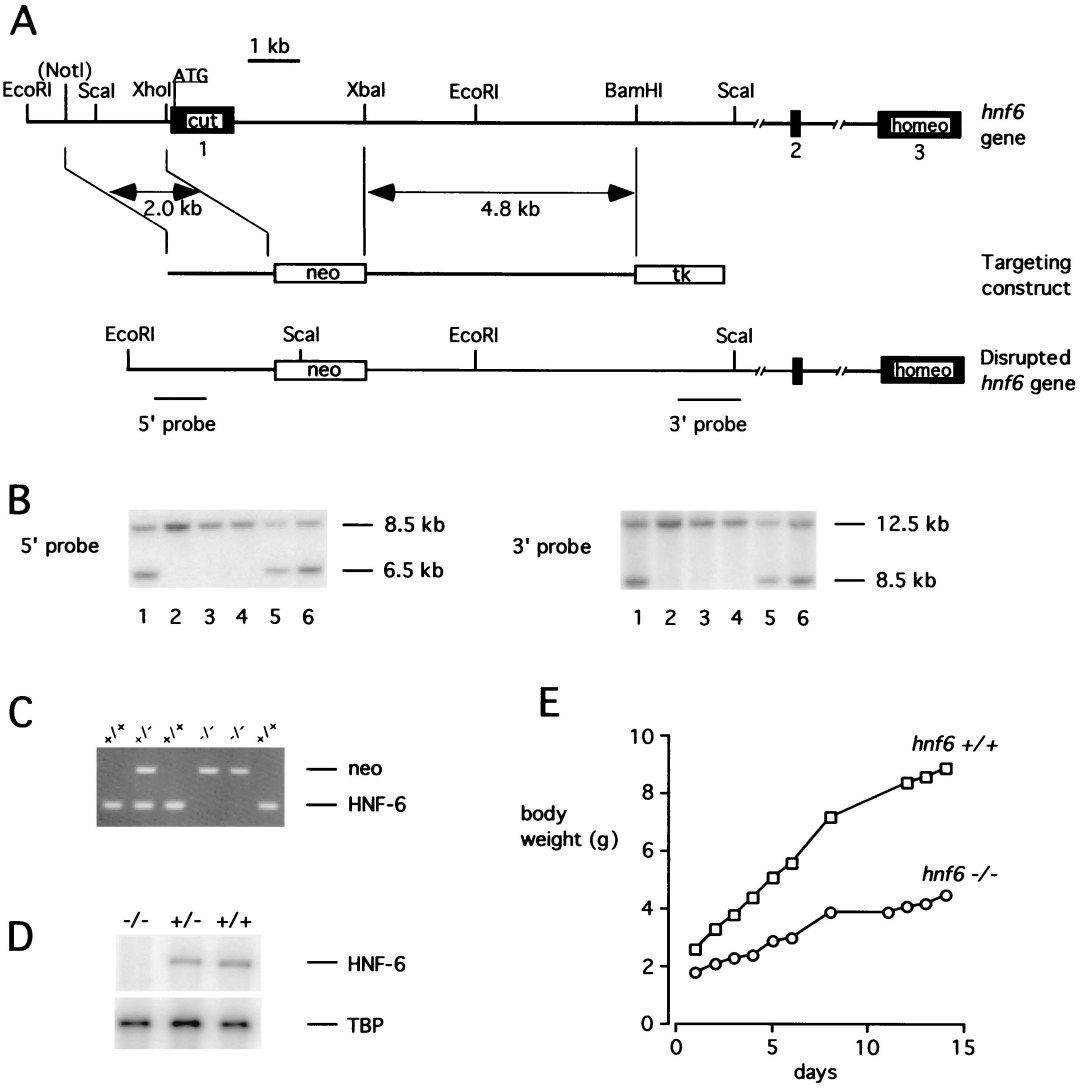

FIG. 1. Targeted disruption of the *hnf6* gene and generation of *hnf6*$^{-/-}$ mice. (A) Scheme of the *hnf6* gene, targeting construct, and product of homologous recombination. The three exons are shown as black boxes. Cut and homeo refer to the cut domain and homeodomain; neo and tk refer to the neomycin resistance and thymidine kinase genes. The *Not*I site derived from the polylinker of a genomic phage clone. (B) Southern blot analysis of DNA isolated from six ES cell clones resistant to G418 and ganciclovir. Correct homologous recombination at the 5′ end was confirmed by hybridization of *Eco*RI-digested DNA with the 5′ probe. This probe detected an 8.5-kb wild-type and a 6.5-kb recombinant fragment. Correct homologous recombination at the 3′ end was confirmed by hybridization of *Sca*I-digested DNA with the 3′ probe, which detected a 12.5-kb wild-type and an 8.5-kb recombinant fragment. (C) PCR genotyping of tail DNA. Primer pairs amplifying sequences *neo* (5′-CTGTGCTCGACGTTGTCACTG-3′ and 5′-GATCCCCTCAGAAGAACTCGT-3′) and of exon 1 of the *hnf6* gene (5′-CAGCACCTCACGCCCACCTC-3′ and 5′-CAGCCACTTCCACATCCTCCG-3′) were added simultaneously in the PCR. (D) Multiplex RT-PCR analysis of total RNA from e14.5 pancreas with primers located in exons 1 and 3 of the *hnf6* gene and with sequences of TBP as internal control. *hnf6* mRNA was undetectable in *hnf6*$^{-/-}$ pancreas, and the ratio of HNF-6 to TBP amplification products in *hnf6*$^{+/-}$ mice was 52% of that in *hnf6*$^{+/+}$ mice. (E) Weight gain curve of a representative *hnf6*$^{-/-}$ mouse shows a reduced growth rate compared to a wild-type littermate.

mice. In the *hnf6*$^{-/-}$ mice in which some Glut-2 could be visualized, its expression was markedly reduced (Fig. 3C and D). Unlike in control mice, IAPP (amylin) was absent from several insulin-positive cells (Fig. 3E and F).

We conclude that in *hnf6*$^{-/-}$ mice, (i) endocrine pancreas development is severely inhibited; (ii) the appearance of the islets of Langerhans is delayed; (iii) when the islets form, they display a perturbed architecture and contain cells that have not reached full maturity.

Analysis of differentiation markers in *hnf6*$^{-/-}$ mice. To explore how endocrine pancreas development is perturbed in *hnf6*$^{-/-}$ embryos, we analyzed by immunohistochemistry the expression of pancreatic transcription factors at various stages of development. Pdx-1 and Nkx6.1 are markers of the early pancreatic epithelium. Their expression indicates that endodermal cells have been specified to a pancreatic fate (27). Isl-1 and Pax-6 are expressed in postmitotic cells that have started to differentiate into either one of the four endocrine cell types (1, 8, 31, 34).

The expression of *pdx1* and of *nkx6.1* was normal at e10.75, e12.5, and e15.5 (Fig. 4A to J). Cells expressing *isl1* and *pax6* were found in normal numbers at e10.75 (Fig. 4K, L, Q, and R) but in much lower numbers at e12.5 and e15.5 in *hnf6*$^{-/-}$ embryos than in control embryos (Fig. 4M to P and S to V). In line with the normal expression of glucagon in the early glucagon cell clusters (Fig. 2D and F), the expression of *isl1* and

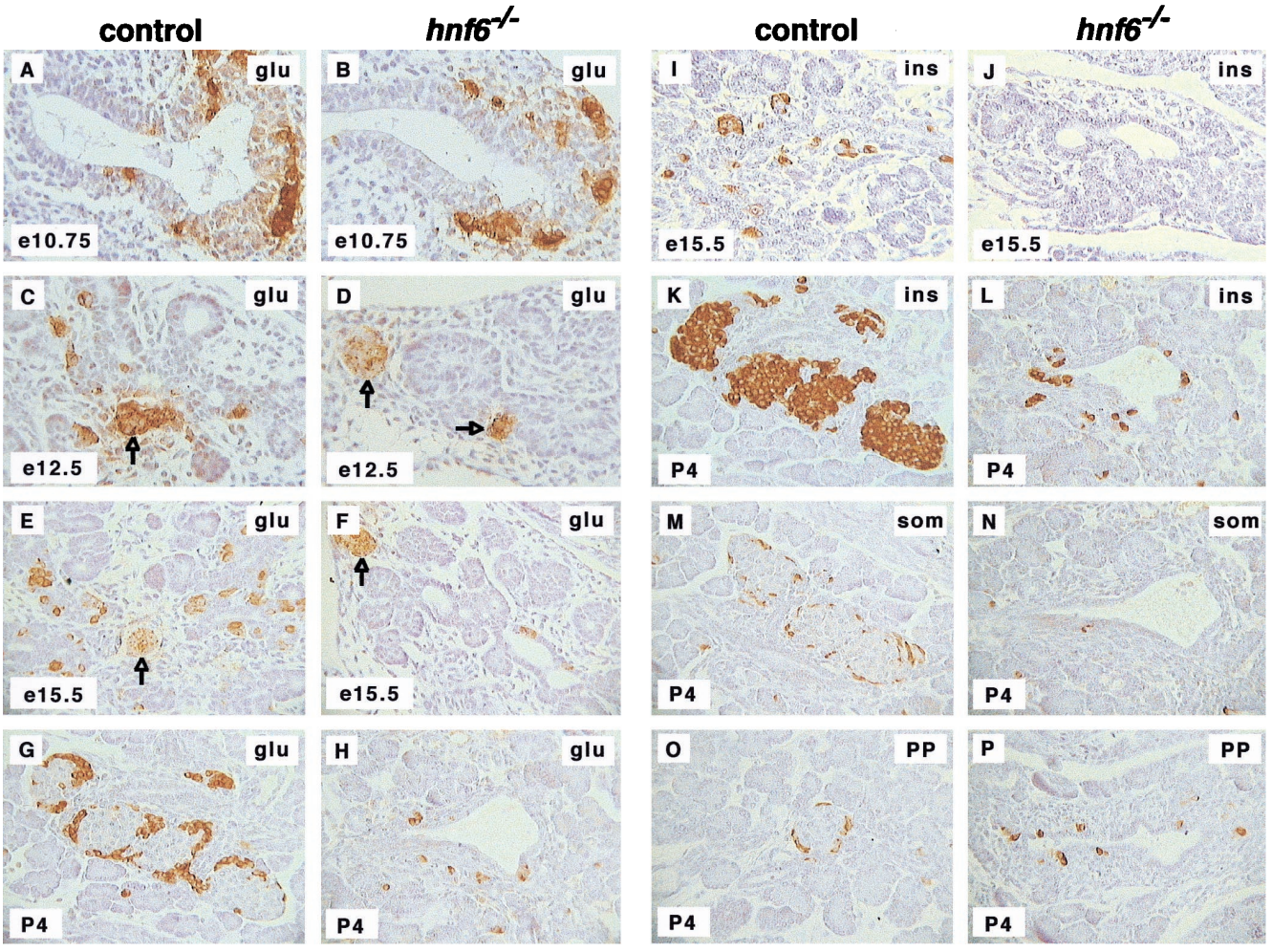

FIG. 2. Defective endocrine cell differentiation and islet morphogenesis in *hnf6*$^{-/-}$ mice. In *hnf6*$^{-/-}$ mice, the number of glucagon (glu)-expressing cells was normal at e10.75 (A and B) but was reduced at e12.5, e15.5, and P4 (C to H). However, the number of clusters of early glucagon-expressing cells was normal at e12.5 and e15.5 (arrows in panels C to F). In *hnf6*$^{-/-}$ mice, there was a reduced number of insulin (ins)-expressing cells at e15.5 and P4 (I to L), of somatostatin (som)-expressing cells at P4 (M and N), and of PP-expressing cells at P4 (O and P). At P4, hormone-producing cells were found near pancreatic ducts in *hnf6*$^{-/-}$ mice, instead of being organized in islets as in control littermates (G, H, and K to P). Original magnifications: ×400 (A to D, I, and J) and ×312.5 (E to H and K to P).

pax6 was detected at normal levels in these cell clusters (arrows in Fig. 4M, N, S, T, and V).

Starting around e15, the pancreas of *hnf6*$^{-/-}$ embryos showed cystic structures. These were delineated by cells that had characteristics of the early pancreatic epithelium, as they all expressed *pdx1* and *nkx6.1* (Fig. 4F and J). Within the epithelium lining the cysts, a few cells expressed the endocrine markers *isl1* and *pax6* (Fig. 4P and V). After birth, the cysts developed as enlarged ducts or as large cavities (up to 2 cm). Their epithelium expressed *pdx1* (Fig. 3H), contrary to normal ductal cells (Fig. 3G). Insulin- or glucagon-expressing cells were then detected within, or in close association with, the epithelium lining the cysts (Fig. 3B, D, and F) or the pancreatic ducts (Fig. 3I and J). Insulin-negative, Glut-2-positive cells were also seen in the epithelium lining the cysts at 5 weeks (data not shown), which is interesting since *glut2* is expressed not only in mature β cells but also in the undifferentiated pancreatic epithelium (28).

We conclude from these experiments that in the absence of HNF-6, the pancreatic epithelium is specified. However, this epithelium fails to give rise, between e10.75 and e12.5, to the expected pool of *isl1*- and *pax6*-expressing cells. Instead, this epithelium generates cystic structures delineated by *pdx1*- and *nkx6.1*-expressing cells.

Reduced expression of the proendocrine gene *ngn3* in *hnf6*$^{-/-}$ embryos. A lateral specification mechanism is involved in pancreatic endocrine cell differentiation (3). In this mechanism, the proendocrine gene *ngn3*, which is the earliest marker of endocrine cell precursors, plays a dual role. First, Ngn-3 is essential for endocrine differentiation (3, 10). Second, it is proposed that Ngn-3 stimulates the synthesis of Notch ligands, which activate the Notch pathway in neighboring Notch-expressing cells. This increases the expression of *hes1*, which decreases that of *ngn3* and consequently inhibits endocrine differentiation.

To determine how HNF-6 controls endocrine differentiation, we measured the expression of genes involved in the pancreatic Notch signaling pathway. We microdissected out the pancreas of e14.5 and e17.5 embryos and measured mRNA contents by RT-PCR (Fig. 5A). Our results showed that *notch-1*, *-2*, and *-3* as well as *hes1* were expressed at similar levels in control and *hnf6*$^{-/-}$ embryos. In contrast, the mRNA coded by *ngn3* was nearly undetectable in *hnf6*$^{-/-}$ embryos. Consistent with this, in situ hybridization experiments showed

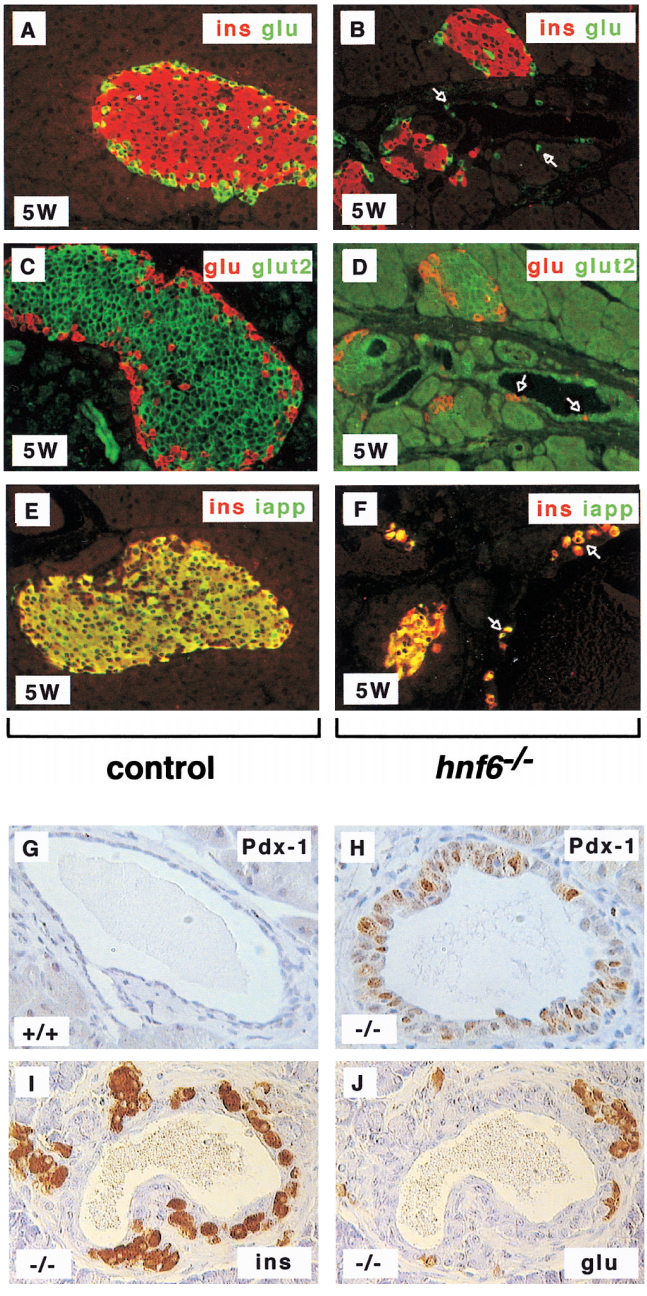

FIG. 3. Formation of abnormal islets of Langerhans in *hnf6*$^{-/-}$ mice. (A and B) At 5 weeks (5W), islets of Langerhans were detected in *hnf6*$^{-/-}$ mice, but their architecture was perturbed and no mantle of glucagon (glu)-expressing cells (green) was seen around the insulin (ins)-expressing cells (red). (C and D) Glut-2 expression was low and could be detected only in some *hnf6*$^{-/-}$ mice (glucagon, red; Glut-2, green). A few glucagon-expressing cells were found in the epithelium lining the cysts (arrows in panel D). (E and F) Insulin (red) and IAPP (green) were coexpressed in control and *hnf6*$^{-/-}$ mice, but insulin-positive, IAPP-negative cells were found in *hnf6*$^{-/-}$ mice. The epithelium of the cysts showed a few cells that express hormones (arrows in panels B, D, and F). (G) Section through a duct in a control animal showed the expected absence of *pdx1* expression. (H) The epithelium of a cyst showed *pdx1* expression in *hnf6*$^{-/-}$ mice. (I and J) Similarly to what is observed during regeneration after pancreatectomy, insulin- or glucagon-expressing cells were detected near ducts in *hnf6*$^{-/-}$ mice. Panels A to F and G to H are from 5- and 10-week-old animals, respectively. Original magnifications: ×200 (A to F), ×640 (G and H), and ×500 (I and J).

that the number of *ngn3*-positive cells was strongly reduced in e14.5 *hnf6*$^{-/-}$ embryos compared to wild-type embryos (Fig. 5B). In the few *ngn3*-positive cells, labeling was weaker than in wild-type embryos. These observations suggested that HNF-6 controls directly or indirectly the expression of the *ngn3* gene. We therefore determined by in situ cohybridization if *ngn3* and *hnf6* are coexpressed in the pancreatic epithelium of normal embryos during development. The results showed that *hnf6* is expressed throughout the pancreatic epithelium and that the *ngn3*-positive cells coexpress *hnf6* (Fig. 5C and data not shown). To investigate whether HNF-6 can directly control transcription of the *ngn3* gene, we searched for HNF-6 binding sites in the *ngn3* promoter. As no information on this promoter was available, we cloned a fragment of the mouse *ngn3* gene (10) and sequenced 4.95 kb upstream of the coding region. We identified a TATA box consensus and found two nucleotide sequences that match (21) the HNF-6 DNA-binding consensus 5′-(A/T/G)(A/T)(A/G)TC(A/C)ATN(A/T/G)-3′. These are located 453 bp (5′-GCATCCATAG-3′) and 3,187 bp (5′-AAA TCCATGT-3′) upstream of the TATA box. In EMSA, each of these sites bound in vitro-synthesized HNF-6 (Fig. 5D). The difference in the intensity of the retarded complexes generated with the two probes indicated that the distal site binds HNF-6 with higher affinity than the proximal one. To test if HNF-6 can stimulate transcription from the *ngn3* gene promoter, transient transfection experiments were then performed with a 3,957-bp-long *ngn3* gene fragment containing the two HNF-6 sites cloned upstream of the luciferase gene. The results showed that this *ngn3* gene fragment displays promoter activity and that cotransfection of an HNF-6 expression vector stimulates this activity fourfold (Fig. 5E).

We conclude from this set of experiments that *hnf6* and *ngn3* are coexpressed in the pancreatic epithelium, that HNF-6 can stimulate *ngn3* gene expression, and that inactivation of the *hnf6* gene drastically reduces expression of the *ngn3* gene in the endocrine cell precursors.

Perturbed glucose homeostasis in *hnf6*$^{-/-}$ mice. The reduction in the number of endocrine cells and the absence of islets of Langerhans at birth prompted us to determine how glycemia is controlled in the postnatal period. Blood glucose, glucagon, and insulin levels were measured in 4-day-old mice. The mice were starved for 4 h to standardize for the feeding status. Figure 6A shows that blood glucose levels were significantly lower in *hnf6*$^{-/-}$ mice than in their wild-type littermates. In *hnf6*$^{-/-}$ mice the insulin levels were below the threshold sensitivity of the assay (<0.05 ng/ml; $n = 4$), in contrast to the wild-type mice, for which a value of 0.46 ± 0.04 ng/ml ($n = 3$) was found. Glucagon levels were reduced by approximately 20% in the *hnf6*$^{-/-}$ mice compared to wild-type mice (Fig. 6B). We interpreted the data as follows. Low insulinemia in *hnf6*$^{-/-}$ mice is consistent with the fasting state of the animals and with the low number of β cells in the pancreas (Fig. 2K and L). Low glucagonemia is consistent with the low number of pancreatic α cells (Fig. 2G and H). The apparent discrepancy in *hnf6*$^{-/-}$ mice between a strong reduction in pancreatic α cell number and a mildly reduced blood glucagon level can be explained by the fasting state, which stimulates glucagon secretion. In these mice, however, glucagon secretion is not sufficient to ensure normal glucagonemia and normoglycemia. We conclude that glucose homeostasis is perturbed in newborn *hnf6*$^{-/-}$ mice, at least in part as a consequence of inappropriate insulinemia and glucagonemia.

Five- to ten-week-old *hnf6*$^{-/-}$ mice had islets of Langerhans, but the morphology of the islets was abnormal (Fig. 3). To further investigate glucose homeostasis in adult *hnf6*$^{-/-}$ mice, we performed glucose tolerance tests on 10-week-old animals.

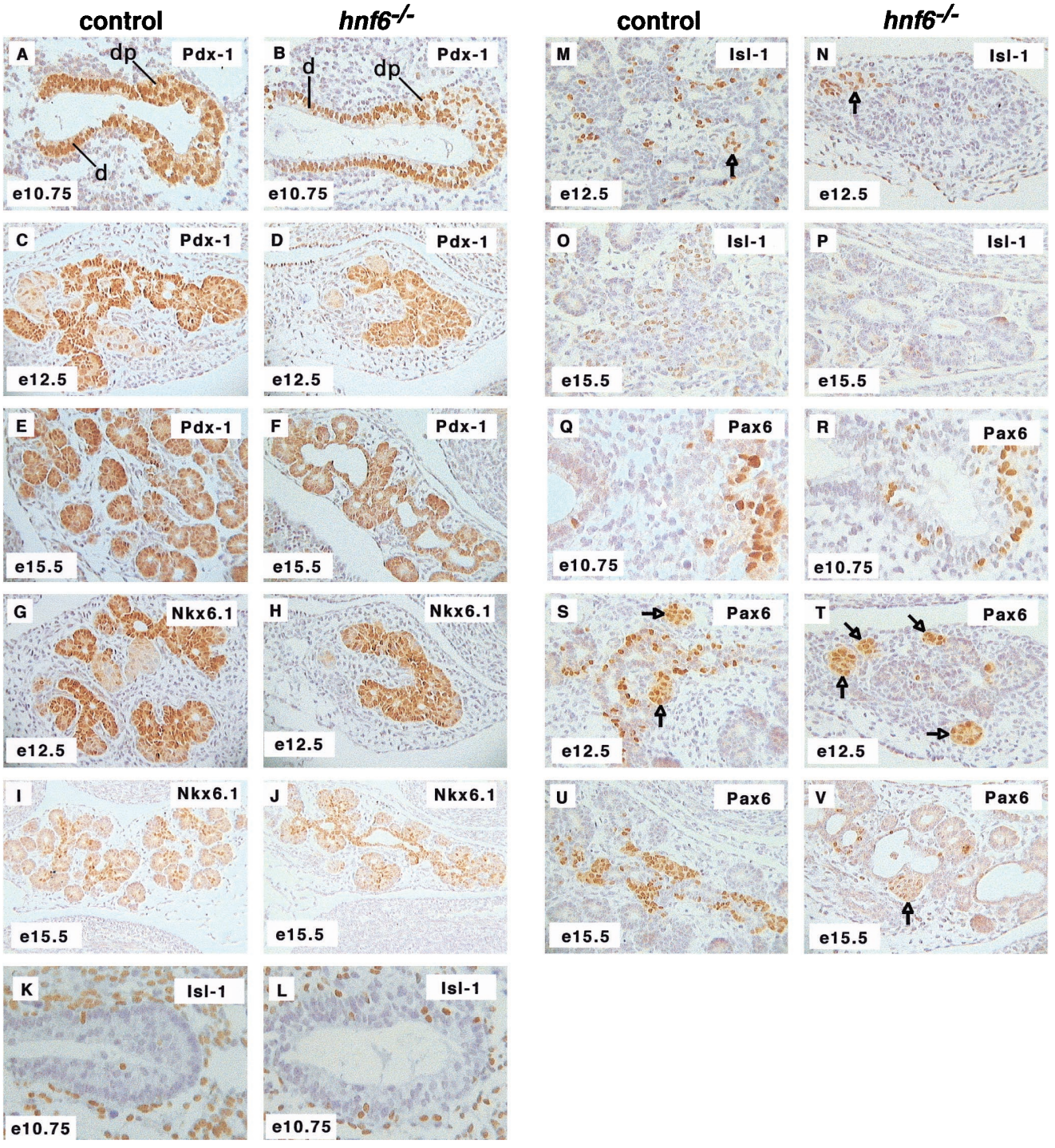

FIG. 4. Expression of differentiation markers in the pancreas of *hnf6*$^{-/-}$ embryos. Expression of the early pancreatic epithelium markers Pdx-1 (A to F) and Nkx6.1 (G to J) was normal at e10.75, e12.5, and e15.5. Cells expressing the endocrine markers *isl1* and *pax6* were found in normal numbers at e10.75 (K, L, Q, and R) but in markedly reduced numbers at e12.5 and e15.5 (M to P and S to V). The expression of *isl1* and of *pax6* was normal in the clusters of early glucagon-expressing cells (arrows in panels M, N, S, T, and V). At e15.5, the pancreatic epithelium of *hnf6*$^{-/-}$ embryos displayed cystic structures delineated by *pdx1*- and *nkx6.1*-expressing cells (F and J). d, duodenum; dp, dorsal pancreas. Original magnifications: ×312.5 (B, C, D, G, H, U, and V), ×200 (I and J), ×640 (K, L, Q, and R), ×400 (A, E, F, M to P, S, and T).

hnf6$^{+/-}$ mice showed normal fasting glycemia and normal blood glucose response curves (data not shown). In contrast, *hnf6*$^{-/-}$ mice exhibited elevated fasting glycemia and were glucose intolerant (Fig. 6C). The perturbed glucose response curve was, at least in part, a consequence of insufficient glucose-induced insulin response, as shown by the low levels of insulin 1 h after glucose injection (Fig. 6D). In addition, glucagonemia, measured after an overnight fasting period, was slightly lower than in the wild-type mice. We conclude that lack of HNF-6 results in diabetes.

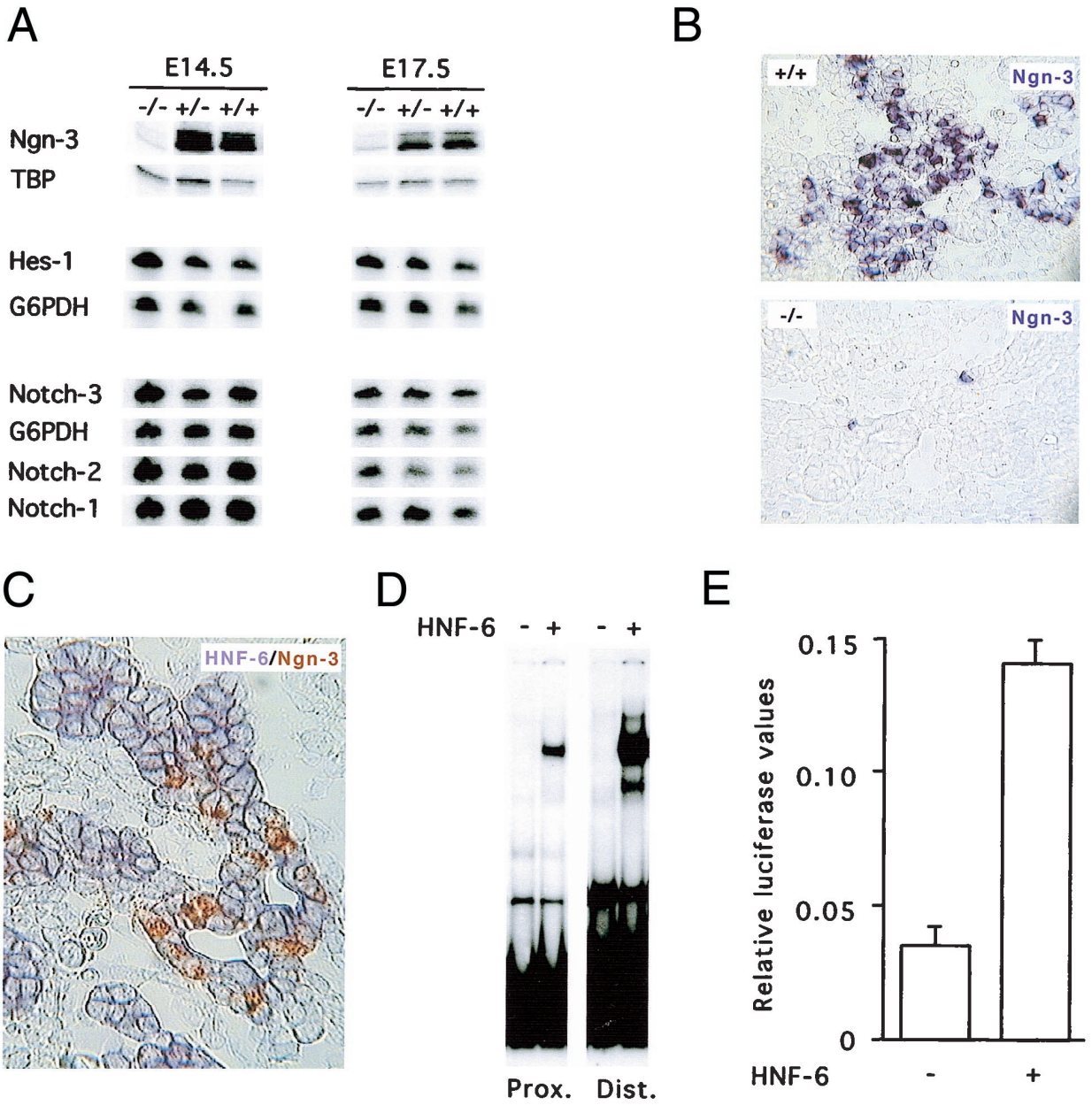

FIG. 5. HNF-6 controls expression of the *ngn3* gene. (A) Multiplex semiquantitative RT-PCR experiments were performed on total RNA extracted from microdissected pancreas from $hnf6^{+/+}$, $hnf6^{+/-}$, and $hnf6^{-/-}$ embryos at e14.5 and e17.5. The expression of *ngn3* was downregulated in $hnf6^{-/-}$ embryos, and that of *notch-1*, *-2*, and *-3* and of *hes1* was unaffected, compared to wild-type and heterozygous embryos. G6PDH mRNA or TBP mRNA was coamplified as internal control. (B) In situ hybridization on sections of e14.5 embryos showed that a digoxigenin-labeled *ngn3* probe detected fewer *ngn3*-positive cells in the pancreas of $hnf6^{-/-}$ embryos than in wild-type embryos. (C) In situ cohybridization with fluorescein-labeled *ngn3* (brown staining) and digoxigenin-labeled *hnf6* (blue staining) probes on a section of a e14.5 wild-type pancreas showed that *ngn3*-positive cells coexpress *hnf6*. (D) EMSA show that in vitro-translated HNF-6 binds to two sites of the *ngn3* gene promoter. A retarded band was observed when either the proximal (Prox.) or the distal (Dist.) site was used as a probe in the presence of HNF-6-programmed wheat germ extracts but not with unprogrammed extracts. (E) HNF-6 stimulates the *ngn3* gene promoter. Rat-1 cells were transiently cotransfected with a firefly luciferase reporter plasmid containing 3,957 bp of *ngn3* promoter sequence and an internal control plasmid coding for *Renilla* luciferase, in the presence or absence of HNF-6 expression vector, as indicated (mean ± standard error of the mean, $n = 4$).

DISCUSSION

In the present work we addressed the question of the role of the ONECUT transcription factor HNF-6 in pancreatic cell differentiation. The early, specified, pancreatic epithelium contains precursors of the exocrine and endocrine cells (29), and it expresses *pdx1*, *nkx6.1*, and *hnf6*. Our results show that HNF-6 is dispensable for reaching this specified state. Indeed, expression of *pdx1* and *nkx6.1* was normal at the initiation of pancreas development (e10.75) in the $hnf6^{-/-}$ embryos. Further differentiation of the pancreatic epithelium into exocrine pancreatic cells was unaffected in $hnf6^{-/-}$ embryos. In contrast, the number of the four endocrine cell types was reduced and the islets of Langerhans were absent at birth, indicating that HNF-6 is involved in endocrine cell differentiation.

Ngn-3 was recently characterized as a proendocrine factor, since its expression is required for endocrine determination (3) and its absence in $ngn3^{-/-}$ mice results in lack of pancreatic endocrine cells (10). In wild-type embryos, *ngn3* expression

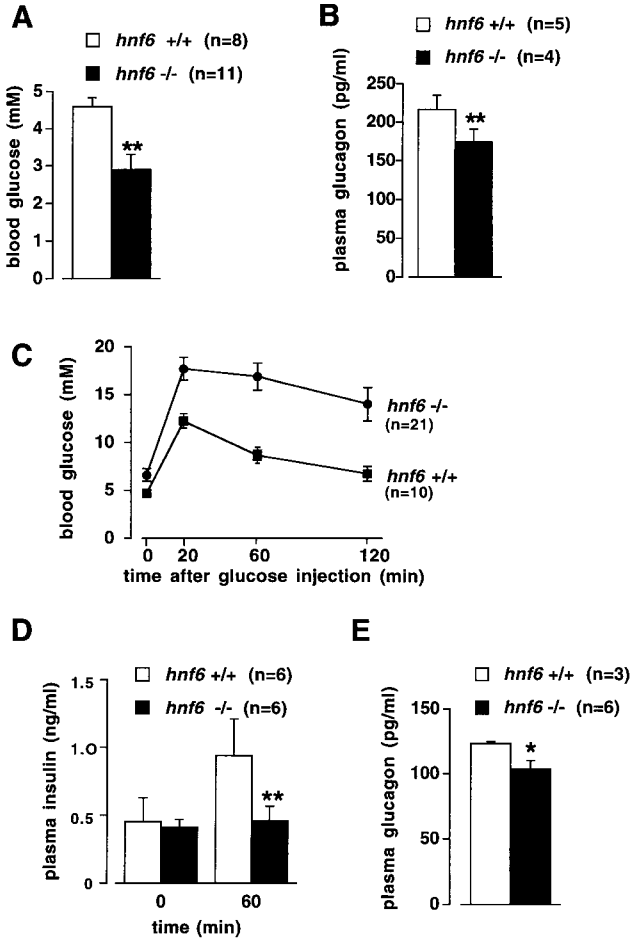

FIG. 6. Perturbed glucose homeostasis in *hnf6*$^{-/-}$ mice. (A and B) Four-day-old mice were starved for 4 h, and blood glucose and plasma glucagon levels were measured. The *hnf6*$^{-/-}$ mice showed hypoglycemia and slightly reduced glucagonemia compared to wild-type mice. (C and D) Ten-week-old mice were fasted overnight and injected intraperitoneally with glucose (0.2 g/ml; 2 g/kg of body weight). Blood glucose levels were measured at the times indicated and showed that the *hnf6*$^{-/-}$ mice were glucose intolerant (C). Glucose intolerance in *hnf6*$^{-/-}$ mice was associated with absence of insulin response. Plasma insulin levels were measured before and 60 min after the injection of glucose for the tolerance tests (D). (E) Plasma glucagon levels were measured in 10-week-old mice after overnight fasting. The data showed slightly reduced glucagonemia in *hnf6*$^{-/-}$ mice compared to wild-type animals. *, $P < 0.05$; **, $P < 0.01$.

starts around e9.5, peaks around e13.5 to e15.5, and disappears postnatally. It is proposed (3, 17) that expression of *ngn3* induces both endocrine differentiation and the synthesis of Notch ligands. Binding of Notch ligands to receptors of neighboring cells would stimulate in these cells the synthesis of Hes-1, a factor that represses *ngn3* expression. In *hnf6*$^{-/-}$ embryos, the concentration of Ngn-3 mRNA was very low at e14.5 to e17.5. This was not a consequence of increased *hes1* gene expression, since the levels of Hes-1 mRNA were normal. Instead, our observations suggested that HNF-6 stimulates *ngn3* expression and that absence of HNF-6 results in reduced *ngn3* gene activation. Indeed, we showed that HNF-6 can bind to and activate the *ngn3* promoter. Given the known requirement of Ngn-3 for endocrine differentiation, it is not surprising that the *ngn3* deficiency seen in *hnf6*$^{-/-}$ embryos is associated with impaired endocrine differentiation. To explain the pancreatic endocrine phenotype of the *hnf6*$^{-/-}$ embryos, we propose that the absence of HNF-6 leads, in most cells, to a reduction of *ngn3* gene expression below the threshold required to induce endocrine differentiation. In a few cells, *ngn3* expression would be above the threshold, consistent with the known cell-to-cell variation in transcriptional response of a particular gene (5). In these cells, endocrine differentiation would be initiated and allowed to proceed along the next steps characterized by expression of differentiation markers such as Pax-6 and Isl-1. The low number of cells that express these markers at e12.5 and e15.5 would therefore reflect the reduction in the number of precursor cells that have entered the endocrine differentiation pathway.

From our in situ hybridization experiments, we conclude that all cells of the pancreatic epithelium at e14.5 express *hnf6* but only a fraction express *ngn3*. This raises the question as to how *ngn3* gene activation by HNF-6 is restricted to a subpopulation of HNF-6-expressing cells. The uniform distribution of *hnf6* expression throughout the epithelium may suggest that HNF-6 acts as an accessory protein for a cell-type-restricted transcription factor with which it would cooperate to activate the *ngn3* gene promoter. Alternatively, the activity of HNF-6 could be modulated by cell-type-specific mechanisms.

According to the lateral specification model (see above), *hes1* is activated by Notch and represses endocrine differentiation (3, 17). However, the broad expression of *hes1* in the pancreatic epithelium, including in cells that are not in contact with differentiating endocrine cells, suggested that the role of *hes1* is not restricted to lateral specification (17). Our data on *hnf6*$^{-/-}$ mice further support this interpretation. Indeed, according to the lateral specification model, one would expect reduced expression of *ngn3* to be associated with reduced expression of *hes1*. This was not the case in the *hnf6*$^{-/-}$ mice. Our data therefore clearly point to the existence of Ngn-3-independent mechanisms of *hes1* gene activation.

After birth, the number of endocrine cells increased in the pancreas of *hnf6*$^{-/-}$ mice. These cells were observed near enlarged ducts and near cysts. They were most likely at the origin of the islets formed postnatally in the *hnf6*$^{-/-}$ mice. Endocrine cells located near ducts have been observed in rats during pancreas regeneration after partial pancreatectomy (4). The presence of endocrine cells within the cystic epithelium of *hnf6*$^{-/-}$ mice suggests that it retained some differentiation potential. However, most of the cells lining the cysts expressed *pdx1* and *glut2*, but not insulin, suggesting that these cells had failed to complete differentiation. No expression of *ngn3* was detected by RT-PCR or by in situ hybridization in the pancreas of *hnf6*$^{-/-}$ mice after birth (data not shown). Other factors of the ONECUT class have been identified in humans (14), and their expression in the pancreas should be analyzed to determine if they may participate in the control of pancreatic development.

HNF-6 stimulates *hnf3*β (20, 30) and HNF-3β stimulates *pdx1* in the pancreas (36). This suggested that HNF-6 could control indirectly *pdx1* gene expression. Our data would dismiss this hypothesis since expression of *pdx1*, as shown here, and of *hnf3*β (data not shown) was unaffected in *hnf6*$^{-/-}$ embryos. However, we cannot exclude that a HNF-6→HNF-3β→Pdx-1 cascade functions in wild-type embryos and that, in the absence of HNF-6, activation of the *hnf3*β gene is compensated for by other factors.

Glucose homeostasis was perturbed in *hnf6*$^{-/-}$ mice. At birth, their insulinemia was very low, consistent with the low number of pancreatic β cells. Contrary to what has been observed in knockout mice that have few or no β cells (10, 18, 24, 31, 33), the newborn *hnf6*$^{-/-}$ mice were hypoglycemic both in the fasted (Fig. 6) and in the fed (data not shown) state. Blood glucose levels depend on the balance between intestinal glu-

cose transport, glucose consumption, and hepatic gluconeogenesis. Understanding the mechanisms by which newborn *hnf6*$^{-/-}$ mice control glucose homeostasis therefore requires characterization of these parameters. The fact that fasted newborn *hnf6*$^{-/-}$ mice did survive rules out hypoglycemia as a cause of the high mortality rate (75%) seen between P1 and P10. It is more likely that these mice die from liver failure. Indeed, work in progress in our laboratory suggests that *hnf6*$^{-/-}$ mice have a variable liver phenotype characterized by abnormal biliary differentiation and liver necrosis.

The pancreatic phenotype in newborn animals is transient. Five- to ten-week-old mice had islets of Langerhans, but they became diabetic. They had normal levels of pancreatic glucokinase mRNA (data not shown). However, full differentiation was not reached since islet morphology was abnormal and since IAPP was not expressed in several β cells. Moreover, the glucose transporter Glut-2 was barely detectable in the β cells of *hnf6*$^{-/-}$ mice. This may, at least in part, explain their diabetic phenotype. Indeed, Glut-2-deficient mice have diabetes because of a decreased glucose-induced insulin response (12), as seen here in the *hnf6*$^{-/-}$ mice. We conclude from our work that *HNF6* is a candidate gene for diabetes mellitus in humans.

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