Distinct Gene Expression Programs Function in Progenitor and Mature Islet Cells*

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Homeodomain transcription factor Nkx2.2 is required for the final differentiation of the β-cells in the pancreas and for the production of insulin. Nkx2.2 is expressed in islet cell precursors during pancreatic development and persists in a subset of mature islet cells including all β-cells. To understand the mechanisms regulating the expression of Nkx2.2 in these different cell populations, we outlined the structure of the mouse nkx2.2 gene and identified regions that direct cell type-specific expression. The nkx2.2 gene has two noncoding alternative first exons (exons 1a and 1b). In transgenic mice, sequences upstream from exon 1a directed expression predominantly in mature islet cells. Within this exon 1a promoter, cooperative interactions between HNF3 and basic helix-loop-helix factors neurogenin-3 or NeuroD1 binding to adjacent sites played key roles in its islet cell-specific expression. In contrast, sequences upstream from exon 1b restricted expression specifically to islet cell precursors. These studies reveal distinct mechanisms for directing the expression of a key differentiation factor in precursors versus mature islet cells.

The development and differentiation of organs such as the pancreas involve sequential modifications in gene expression controlled by a cascade of transcription factors. Recently, several mouse strains with mutations in genes encoding transcription factors that are expressed in the pancreatic β-cells have been found to have severe abnormalities in pancreatic development (1–12). Mice homozygous for a null mutation of the pancreatic β-cell neogenesis, in the broad initial pancreatic precursor population, in a subset of the neurogenin-3-expressing islet progenitor cells, and in differentiated islet cells. In addition, Nkx2.2 is expressed at least three distinct stages in islet cell differentiation: in the initial pancreatic precursor population, in a subset of the neurogenin-3-expressing islet progenitor cells, and in differentiated islet cells. Therefore, Nkx2.2 is expressed predominantly in differentiated endocrine cells including α-, β-, and PP cells, but not δ-cells. Thus, Nkx2.2 expression is limited to the differentiated endocrine cells in different cell populations are unknown.

To understand the mechanisms that regulate the expression of Nkx2.2, we outline here the structure of the mouse nkx2.2 gene and identify the regions that direct cell type-specific expression. The nkx2.2 gene has three alternative first exons (exons 1a, 1b, and 1c). We found that the 5′-flanking region of exon 1a drives the expression of Nkx2.2 predominantly in differentiated islet cells and is activated by cooperative interactions between HNF3β and either neurogenin-3 or the related bHLH factor NeuroD1. On the other hand, the 5′-flanking region of exon 1b directs expression predominantly to islet precursor cells. These data reveal distinct mechanisms regulating Nkx2.2 expression in progenitor cells and in mature islet cells and support a model in which HNF3β and neurogenin-3 lie upstream from Nkx2.2 in the hierarchy of β-cell differentiation factors.

Materials and Methods

Cloning of the Mouse nkx2.2 and Human NKX2B Gene Promoters—Plasmids containing mouse nkx2.2 genomic DNA were kindly provided by L. Sussel (University of Colorado, Denver (12)). The PI artificial chromosome clone containing the human NKX2B gene and the plasmid

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containing human *NKX2B* exons 1c and 2 were kindly provided by G. Bell and H. Furuta (University of Chicago) (16). From the PI artificial chromosome clone, the fragment containing exons 1a and 1b was isolated by Southern blot analysis using a fragment of the mouse *nkx2.2* gene containing exon 1a and 5′/H11032- flanking sequences. The mouse and human upstream regions were sequenced and are available from GenBank.

**Oligonucleotide-capping Rapid Amplification of 5′-cDNA Ends (5′-RACE)**—Total RNA was isolated from the mouse neural tube at embryonic day 11.5, pancreas at day 2.5, and isolated adult islets of Langerhans, and from the mouse *HIK252*-cell tumor line HIK252. The 5′-end of the mouse *nkx2.2* cDNA from each cell was identified by the oligonucleotide-capping RACE method using the GeneRacer Kit according to the manufacturer’s instructions (Invitrogen). Briefly, 2 μg of total RNA was dephosphorylated, decapped, and ligated to GeneRacer RNA oligonucleotides. Then reverse transcription was carried out using an oligo(dT) primer. Using this cDNA pool as a template, we carried out 30 cycles of PCR using the GeneRacer 5′-primer and HW323 (5′-CACTTGGCTCAATTGCCTG GCPTCC-3′) as primers. For nested PCR, we used the GeneRacer 5′-nested primer and HW324 (5′-CAACCCAGAATAAGTGGCGTACTGCG-3′) as primers and performed 25 cycles of PCR. The PCR products were subcloned into pCR4-TOPO (Invitrogen) and sequenced.

**Reporter Gene Constructs**—To generate reporter plasmids, fragments of the 5′-region of the mouse *nkx2.2* gene obtained by restriction digestion or PCR were ligated upstream from the luciferase gene in the pFOXLuc1 plasmid or upstream from the thymidine kinase minimal promoter in the pFOXLuc1TK (17). Mutagenesis of the reporter gene constructs was performed using the QuikChange mutagenesis kit according to the manufacturer’s instructions (Stratagene). All constructs were confirmed by sequencing.

**Cell Culture and Transient Transfections**—The βTC3 cell line and the α-cell tumor line αTC1.6 were grown in Dulbecco’s modified Eagle’s medium supplemented with 2.5% fetal bovine serum and 15% horse serum. NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. The rat floor plate-derived line Z13 (a generous gift from T. Jessell, Columbia University, New York) was grown in OptiMEM with 10% fetal bovine serum. For transient mammalian cell transfections, cells were plated in six-well tissue culture plates 24 h before transfection. For standard reporter gene analyses, 1.8 μg of each luciferase reporter plasmid and 0.2 μg of the CMV-βGal plasmid were cotransfected into cells using the TRANSFAST cationic lipid reagent (Promega) according to the manufacturer’s instructions. For assessing the effect of each transcription factor on the Nkx2.2 promoter, we cotransfected 10 ng of the expression vector with

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**Fig. 1. Structure of the *nkx2.2* gene.**

A, map of the mouse *nkx2.2* gene. The coding sequence is shown in black. B, the three identified splice variants of the *nkx2.2* mRNA. In C–E, comparisons of the proximal sequences of the human *NKX2B* and mouse *nkx2.2* 1a (C), 1b (D), and 1c (E) promoters are shown. Conserved bases are marked by asterisks. The major transcription start sites identified by oligonucleotide-capping RACE methods are shown as boldface underlines, and potential transcription factor binding sites are indicated in boldface letters.
Fig. 2. Identification of an islet cell-specific enhancer element in the nKx2.2 promoter. A, the upper panel shows a map of the mouse nKx2.2 gene. Reporter plasmids were constructed with the nKx2.2 gene fragments shown, inserted upstream from the luciferase gene in the pFOXLuc1 plasmid using appropriate restriction enzyme sites. Luciferase reporter plasmids were cotransfected with a CMV promoter-driven β-galactosidase expression plasmid into a β-cell-derived line, βTC3 (hatched bars); α-cell derived line, δTC1.6 (open bars); a neural tube derived line, Z13 (striped bars); and a fibroblast line, NIH3T3 (filled bars). Relative luciferase activities were calculated with the activity of cells transfected with the promoterless parent vector pFOXLuc1 set at 1. B, varying lengths of the 5′-flanking region of the mouse nKx2.2 1a promoter were inserted upstream from the luciferase gene in pFOXLuc1 as shown. These luciferase reporter plasmids were then cotransfected with a CMV promoter-driven β-galactosidase expression plasmid into the cell lines βTC3 (hatched bars), δTC1.6 (open bars), and NIH3T3 (filled bars). Activity in αTC cells was not determined (N.D.) for the −634 and −539 bp promoters. Relative luciferase activities were calculated with the activity of cells transfected with the promoterless parent vector pFOXLuc1 set at 1. All data are shown as the mean ± S.E. The abbreviations for restriction enzyme sites used in this figure are: B, BamHI; K, KpnI; N, Nhel; X, XhoI; P, PstI; S, SpeI; A, ApaI; Not, NotI.

1.8 μg of the reporter gene vector. Controls for transcription factor experiments always contained equal amounts of the empty CMV expression vector (pBAT12). 48 h after transfection, cells were harvested, and luciferase and β-galactosidase assays were performed as described previously (17). All transfection experiments were performed in triplicate on at least three separate occasions. Luciferase activity was corrected for transfection efficiency by dividing by the β-galactosidase activity.

Construction of Plasmids—The HNF3β deletion mutant constructs were generated by PCR starting with the CMV-HNF3β plasmid (a generous gift from M. Stoffel, Albert Einstein College of Medicine, New York (18)) as a template and subcloned into either the pB Tat1 T7 in vitro transcription vector (17), the pB Tat2 CMV expression vector, or the pPG11 glutathione S-transferase (GST) fusion vector. The truncated neurogenin-3 constructs were generated by PCR and subcloned into the pCITE4a T7 in vitro transcription vector (Amersham Biosciences), the pBAT12 CMV expression vector, or the pPIG11 parental vector (17), the pBAT12 CMV expression vector, or the pPG11 glutathione S-transferase (GST) fusion vector. The truncated neurogenin-3 constructs were generated by PCR and subcloned into the pCITE4a T7 in vitro transcription vector (Amersham Biosciences), the pBAT12 CMV expression vector, or the pPG11 parental vector (17). pPIG11 and neurogenin-3 expression plasmids were trans-
the Nkx2.2 1b construct we characterized 15 independent lines, four gave detectable expression in the same pattern. For the Nkx2.2 1c construct, we examined eight lines, and none gave detectable expression.

The embryonic, neonatal, and adult tissues were harvested from the established mouse lines. The pancreases from adult mice were harvested after heart perfusion with 4% paraformaldehyde. Harvested tissues were prefixed for 30 min at 4°C in 4% paraformaldehyde. Tissues were incubated overnight with 400 μg/ml X-gal substrate at room temperature. Gross embryos and dissected pancreases were visualized using a Leica dissecting microscope and imaged with a Spot RT digital camera and Openlab software. The tissues were fixed again in 4% paraformaldehyde, paraffin embedded, and sectioned at 5 μm.

Immunohistochemical Analyses—Immunohistochemical and immunofluorescence analyses were performed on paraffin sections as described previously (8). The primary antibodies were used at the following dilutions: guinea pig anti-insulin (Linco), 1:5,000; guinea pig anti-glucagon (Linco), 1:10,000; rabbit anti-neurogenin-3 (14), 1:5,000; guinea pig anti-PDX-1 (14), 1:5,000; rabbit anti-HNF3β (23) (gift of T. Jessell), 1:1,000; mouse monoclonal anti-Nkx2.2 (23) (Developmental Studies Hybridoma Bank, University of Iowa), 1:10.

For immunohistochemistry, biotinylated anti-rabbit, anti-guinea pig or anti-mouse antibodies were used at a 1:200 dilution (Vector) and were detected with the ABC Elite immunoperoxidase system (Vector). The secondary antibodies used for immunofluorescence were as follows: FITC-conjugated anti-rabbit, anti-mouse or anti-guinea pig diluted 1:100 (Jackson Laboratory); Cy3-conjugated anti-rabbit diluted 1:800 (Jackson Laboratory). Fluorescence and brightfield images were visualized with a Zeiss axioskop II and imaged with a Hamamatsu ORCA100 digital camera and Openlab software.
Structure of the Mouse nkx2.2 Gene—As an initial step in assessing its regulation, we identified the transcription initiation sites in the mouse nkx2.2 gene using 5'-RACE. Using primers complementary to the 5'-end of the known nkx2.2 cDNA sequence (12), 5'-RACE was performed with cDNA from fetal mouse pancreas at embryonic day 11.5, neural tube at day 10.5, adult pancreatic islets, the /H9252 TC3 cell line, and the fibroblast line NIH3T3. Sequencing of the PCR products revealed three major transcription start sites (Fig. 1A), which produce three different splice products (Fig. 1B). Two novel exons, 1a and 1b, each are spliced upstream from exon 1c. Exon 1b is located ~8 kb upstream from exon 1c and the translation initiation site, and exon 1a is located ~0.7 kb farther upstream. Although the 5'-RACE results are not quantitative, the adult islet and βTC3 RNA produced predominantly exon 1a-containing products, whereas the pancreatic bud and neural tube RNA produced predominantly exon 1b products, suggesting that transcription initiating from these exons is regulated in a tissue-specific manner. Products starting with exon 1c were found at low abundance in βTC3 cells and all four tissues but not in NIH3T3 cells.

To identify sequences that might control transcription of the nkx2.2 gene, we sequenced the regions flanking each major transcription start site in both the mouse and human genes (Fig. 1, C–E). As shown in Fig. 1, promoter 1a contains no TATA box although it has a conserved GC-rich region that is frequently observed in non-TATA box promoters (24). Promoters 1b and 1c each have a TATA box sequence ~30 bp upstream from the THYMIDINE KINASE minimal promoter driving luciferase and expression plasmids expressing the transcription factor cDNAs indicated under the control of the CMV promoter were cotransfected into NIH3T3 cells. Relative luciferase activities were calculated with the activity of cells transfected with the expression vector without cDNA insert (~) set at 1. All data are shown as the mean ± S.E.
of promoters 1a and both 1c are highly conserved between mouse and human and contain multiple potential binding sites for bHLH proteins (E boxes), homeodomain proteins, and nkx2 class homeodomain proteins (25). Promoter 1a also contains a conserved consensus binding site for HNF3 (26). The proximal region of promoter 1b is less well conserved and contains two conserved E boxes but no other identifiable pancreatic transcription factor binding sites.

**Promoter Function in Vitro**—To test for the ability to drive transcription in cell lines, we constructed a series of plasmids with upstream fragments of the mouse nkx2.2 gene linked to the firefly luciferase gene. As shown in Fig. 2A, the relative activities of the three promoters were compared in the β-cell-derived line βTC3, the α-cell-derived line αTC1.6, the rat fetal floor plate cell-derived line Z13, and the fibroblast line NIH3T3. These cell lines were chosen because Nkx2.2 protein was detected by Western blot analysis in βTC3 cells, αTC1.6 cells, and Z13 cells, but not in NIH3T3 cells (data not shown).

In agreement with the 5′-RACE results, the nkx2.2 1a promoter drove luciferase expression only in islet cell lines, whereas the nkx2.2 1b promoter functioned in Z13 cells. The nkx2.2 1c promoter showed minimal activity in all cell lines.

Although the longest construct, containing sequences from promoter 1a through 1c, produced less absolute activity than the shorter constructs, all transfections were performed with the same mass of DNA, so that the molar concentration for this large plasmid was 2–3-fold lower than for the shorter promoters. Because, in addition, the transfection efficiencies of such large plasmids may be decreased, relative activity of this very large construct can best be judged by comparing the βTC3 cells and NIH3T3 cells infected with the same construct. Using that comparison, the activity of the longest construct was not significantly different from the construct with the isolated 1a promoter in βTC3 cells.

Focusing on the Nkx2.2 1a promoter, we mapped sequences within the proximal 2,800 bp important for expression in islet cell lines. As shown in Fig. 2B, a series of truncations of the promoter demonstrated that removal of the sequence between −247 and −121 bp completely disrupted the activity of the promoter in βTC3 cells. Within this region, we identified seven potentially important elements based on their similarity to known transcription factor binding sites. Mutations were introduced into each of these sites in the context of the −247 bp reporter gene construct and tested in βTC3 cells (Fig. 3, A and B).

Mutations introduced into the homeodomain binding site (H0), or the two 5′-E boxes (E1 and E2) singly or together had modest effects on promoter activity. In contrast, mutation of either the HNF3 (recently renamed FoxA) binding site (H3) or the adjacent E box (E4) blocked promoter activity almost completely. The fact that both mutations can independently abolish promoter activity suggests that these two elements may work synergistically.

Next, we generated reporter gene constructs containing three tandem repeats of the H3/E4 region inserted upstream from the minimal promoter. As shown in Fig. 3C, this small mini-enhancer is capable of activating transcription in a cell type-specific and orientation-independent manner. Together with the mutation data, these results demonstrate that the H3 and E4 elements are both necessary and sufficient for nkx2.2 1a promoter activity in the transfected cell lines.

**Transcription Factors Binding to the nkx2.2 E1a Promoter**—To identify factors that bind to H3 and E4, we performed EMSAs using double-stranded oligodeoxynucleotides corresponding to H3 and E4 as probes. The H3 site conforms to an HNF3 binding consensus (26). The three member of the HNF3 family of winged helix transcription factors play key roles in development and gene expression in endoderm-derived tissues (27), and HNF3β (FoxA2) is a key regulator of the pancreatic/duodenal homeobox gene pdx-1 (28–30). As shown in Fig. 4A, in vitro translated HNF3β can bind to H3. A complex of similar mobility was detected in nuclear extracts from αTC1.6 cells and βTC3 cells, but not in NIH3T3 cell, and this complex was recognized by antiserum to HNF3β but not to HNF3α or HNF3γ.

E boxes contain the consensus sequence CANNTG, bind to dimers of the bHLH class of transcription factors, and mediate cell-specific gene expression. Among the bHLH proteins expressed in the pancreas, neurogenin-3 and NeuroD1 play critical roles in islet development and gene expression (2, 7, 14, 31–33). As shown in Fig. 4B, in vitro translated neurogenin-3 and NeuroD1 can bind to E4 when dimerized with the ubiquitous bHLH protein E47. A complex of similar mobility detected in nuclear extracts from αTC1.6 cells and βTC3 cells, but not NIH3T3 cells, was recognized by antisera to NeuroD1 and E47.

**HNF3β and Neurogenin-3 Synergistically Activate the nkx2.2 1a Promoter**—To test the ability of the bHLH proteins and HNF3β to activate the islet-specific H3/E4 element in non-islet cells, we expressed various bHLH factors in NIH3T3 cells along with a luciferase construct driven by either the −247 bp nkx2.2
A promoter (Fig. 5A) or by three copies of the H3/E4 element from the 1a promoter upstream from a minimal THYMIDINE KINASE promoter (Fig. 5D). As shown in Fig. 5A, HNF3β/E47 alone activated the nkx2.2 1a promoter modestly; and coexpression of HNF3β/E47 did not provide any further activation. On the other hand, the addition of NeuroD1 or neurogenin-3 to E47 and HNF3β synergistically activated the promoter. Activity of the three factors transfected together was significantly greater than the combined activities of the individual transcription factors. To keep all transfections comparable, no attempt was made to optimize relative synergistic activity by varying plasmid concentrations.

In contrast, the related non-pancreatic bHLH factors MyoD and Tal1 did not synergize with HNF3β, although MyoD significantly activated the promoter and mini-enhancer construct in the absence of HNF3β. Synergistic activation of the nkx2.2 1a promoter requires the DNA binding domains of HNF3β and neurogenin-3 (Fig 5B), and intact H3 and E4 sites (Fig 5C). These results demonstrate that neurogenin-3 and HNF3β synergistically activate the nkx2.2 1a promoter when bound to the H3 and E4 sites.

To map the regions of the HNF3β and neurogenin-3 proteins outside of the DNA binding domains that are necessary for this synergy, we generated eukaryotic expression vector constructs expressing truncated neurogenin-3 and HNF3β proteins and tested their ability to synergize on the nkx2.2 minimal promoter. Interestingly, no single domain outside the DNA binding domains was absolutely required by neurogenin-3 or HNF3β for synergy (Fig. 6). Instead, neurogenin-3 requires either the carboxyl- or the amino-terminal end of the molecule, both of which contain a transcription activation domain. Similarly, HNF3β requires any one of its three activation domains (34).

In contrast to the 1a promoter, activity of the nkx2.2 1b promoter was not enhanced by neurogenin-3, in islet or non-islet cell lines.

HNF3β Physically Interacts with Neurogenin-3—To determine whether synergy between HNF3β and neurogenin-3 involves a physical interaction, we tested for a direct interaction between the two proteins. A FLAG-tagged HNF3β expression plasmid was transfected along with a neurogenin-3 expression plasmid (Fig. 7). HNF3β and neurogenin-3 interact directly through their DNA binding domains. A, neurogenin-3 and the FLAG peptide or HNF3β fused to the FLAG peptide were expressed in NIH3T3 cells. Nuclear extracts were isolated, immunoprecipitated (IP) with a FLAG antibody, separated by SDS-PAGE, and immunoblotted (IB) with the FLAG antibody (upper panel) or a neurogenin-3 (Ngn3) antibody (lower panel). Panels B–G show pulldown assays of HNF3β/neurogenin-3 interactions. The various truncated fragments of HNF3β and neurogenin-3 shown in B and C were translated in vitro with [35S]methionine and compared for translation efficiency by separating on SDS-polyacrylamide gel and comparing 35S incorporation (shown in D and F). In vitro translated [35S]methionine-labeled HNF3β and neurogenin-3 proteins were then incubated with GST or GST-fused neurogenin-3 (amino acids 76–213) (E) or HNF3β (amino acids 141–262) (G) and glutathione-Sepharose. After washing, Sepharose-bound proteins were separated by SDS-PAGE, and binding was gauged by retained 35S label (shown in E and G).

2 S. Smith, H. Watada, and M. S. German, unpublished data.

3 H. Watada and M. S. German, unpublished data.
plasmid into NIH3T3 cells and nuclear extracts were isolated. Immunoprecipitation was performed with a FLAG antibody, followed by Western blotting with a neurogenin-3 antibody. As shown in Fig. 7A, neurogenin-3 coprecipitated with FLAG-tagged HNF3β but not with the FLAG peptide alone. These results demonstrate that HNF3β can directly interact with neurogenin-3 in vivo.

To map the regions of HNF3β and neurogenin-3 which are involved in this interaction, we used a pulldown analysis with GST fused to neurogenin-3 amino acids 76–213. As shown in Fig. 7E the neurogenin-3-GST fusion protein bound in vitro translated HNF3β proteins containing the winged helix domain. It did not bind, however, to HNF3β proteins lacking the winged helix domain, demonstrating that neurogenin-3 interacts with the winged helix domain of HNF3β. Similar pulldown assays revealed that HNF3β bound to the bHLH domain of neurogenin-3 (Fig. 7G). In contrast, the bHLH domains of E47, MyoD, and NeuroD1 did not interact with HNF3β with similar affinity, although a weaker interaction with NeuroD1 was detectable. These results demonstrate a specific physical interaction between the neurogenin-3 bHLH domain and the HNF3β winged helix domain.

Promoter Function in Vivo—To determine when and where the individual nksx2.2 promoters function during fetal development and islet cell differentiation, we generated transgenic mice with each of the three promoters driving the bacterial lacZ gene encoding β-galactosidase. As shown in Fig. 8, the −1754 bp nksx2.2 1a promoter produced obvious β-galactosidase activity in the fetal pancreatic bud as early as embryonic day 10.5, along with expression in the developing neural tube. By day 15.5, strong β-galactosidase activity was observed in central epithelial cells of the developing pancreas and scattered cells in the gut. At 1 and 3 weeks after birth, β-galactosidase activity was restricted to islets. Three independent transgenic lines showed identical expression patterns.

Although Nksx2.2 is expressed in all or most of the early epithelial cells of the pancreatic bud (12), immunohistochemical analyses showed that at embryonic days 10.5 (Fig. 9, A–C) and 12.5 (data not shown) the 1a promoter drove β-galactosidase expression predominantly in differentiated hormone-expressing cells and not in the more abundant PDX-1 expressing progenitor cells. At this stage, most of the differentiated endocrine cells in the pancreatic buds express glucagon, but these early glucagon-expressing cells are distinct from the glucagon-expressing α-cells found in the mature islets after birth (35). At embryonic day 15.5, the 1a promoter drove β-galactosidase expression in all insulin-positive cells and a subset of glucagon-positive cells (Fig. 9, D and E). Although most of the Nksx2.2-positive cells expressed β-galactosidase at day 15.5, some did not (data not shown), suggesting that the expression of Nksx2.2 in those cells may be regulated by other promoters. All β-galactosidase-expressing cells expressed HNF3β (Fig. 9G).

A small subset of β-galactosidase-expressing cells also expressed neurogenin-3, although most neurogenin-3-expressing cells did not express β-galactosidase (Fig. 9F; and see also Fig.
transgenic mouse is shown after X-gal staining. The bar indicates 1 mm.

If neurogenin-3 initiates β-galactosidase expression, we assume that it would take some period of time for β-galactosidase protein accumulation to reach detectable levels, by which time neurogenin-3 expression, which is brief, would already be declining, resulting in only a few cells that express detectable levels of both neurogenin-3 and β-galactosidase. Therefore, this expression pattern is consistent with the initiation of β-galactosidase expression in neurogenin-3-expressing cells. In adult animals, the expression of β-galactosidase was detected in islets in insulin-expressing cells, but little or no β-galactosidase activity could be detected in glucagon- or somatostatin-expressing cells (Fig. 9, H–J, and data not shown).

To test whether the minimal nks2.2 1a promoter is sufficient to drive correct expression, we also generated mice carrying a transgene with the −247 bp nks2.2 1a promoter driving the lacZ gene. Among six transgenic mouse lines carrying the transgene, two transgenic lines expressed β-galactosidase. Although the level of β-galactosidase activity was lower than in the −1754 bp promoter transgenic lines, the −247 bp promoter produced the same expression pattern (data not shown).

The nks2.2 1b promoter gave a very different expression pattern in vivo. We generated transgenic mice carrying a transgene with −665 bp of the nks2.2 1b promoter driving the lacZ gene. Among 15 independent transgenic mouse lines carrying the transgene, 4 lines expressed detectable levels of β-galactosidase in the same pattern. As shown in Fig. 10A, β-galactosidase activity was faintly detectable in a small region of the developing spinal cord at embryonic day 12.5. Although not apparent grossly, very faint β-galactosidase activity was detectable in the dissected pancreas.

By embryonic day 15.5, much stronger β-galactosidase activity appeared in central regions of the pancreas of the nks2.2 1b promoter transgenic embryos (Fig. 10B); but by birth and in the adult, β-galactosidase activity was undetectable in the pancreas. In the embryonic day 15.5 pancreas, β-galactosidase expression was restricted to neurogenin-3-positive cells and was not detected in mature islet cells (Fig. 11, D–F). Careful examination reveals that most, but not all, neurogenin-3-expressing cells had some detectable β-galactosidase activity. There was little if any β-galactosidase expression in cells that did not stain for neurogenin-3. These results revealed that the nks2.2 1b promoter was active in the cells of the islet precursor population during the major phase of islet cell neogenesis.

Finally, we also generated transgenic mice with a construct carrying 3.6 kb of the nks2.2 exon 1c promoter ligated upstream from the β-galactosidase gene. We established eight independent lines, but β-galactosidase expression was not detectable in any of these lines (data not shown).

**DISCUSSION**

The transit of cells from a multipotent precursor state to a specific differentiated fate involves progressive changes in their gene expression program. In the developing pancreas, most of the cells that form the initial buds have the potential to differentiate along several different paths. When a subset of these cells commits to the pathway to endocrine cells, they activate a set of islet transcription factor genes that include neurogenin-3 and pax4 (14, 31, 36). These genes, however, are unique to the islet precursor cells and are switched off as the cells differentiate further into mature endocrine cells. In the differentiated, post-mitotic islet cells, a different set of islet transcription factor genes is activated, including NeuroD1, pax6, and isl1 (8, 14, 37).

Nks2.2 is expressed in the early pancreatic progenitor cells, the neurogenin-3-expressing islet precursor cells, and the differentiated islet cells (12, 14). The data presented here indicate that distinct mechanisms direct the expression of Nks2.2 in these three cell populations. The transgenic animal studies demonstrate that sequences 5′ of exon 1a direct expression in a
few neurogenin-3-expressing islet precursor cells, but primarily in mature islet cells (α-, β-, and PP cells), whereas sequences 5' of exon 1b direct expression to neurogenin-3-expressing islet precursor cells. These distinct promoters in turn are regulated by different sets of transcription factors.

The sequence of the exon 1b promoter is less well conserved between mouse and human than the sequences upstream from exons 1a or 1c, but two ideal bHLH binding sites (E boxes) are conserved. Despite the presence of these binding sites and the observation that activity of the exon 1b promoter closely parallels the expression of neurogenin-3 in vivo, several other lines of evidence suggest that neurogenin-3 may not directly control the 1b promoter. First, in the transgenic fetuses it should take some time for the 1b promoter to be activated and for β-galactosidase protein to accumulate after neurogenin-3 appears and then for β-galactosidase activity to decay after neurogenin-3 is gone (38); but the restriction of detectable β-galactosidase activity to cells expressing neurogenin-3 provides no evidence for such a lag. Second, in cell lines, the E boxes are not required for full activity of the 1b promoter3; and coexpression of neurogenin-3 and/or NeuroD1 (28) terminating regulation of several pancreatic genes, including the pancreatic/duodenal homeobox gene (pdx1) (28–30) and the neurogenin-3 gene itself (39). Unfortunately, the role of HNF3β in pancreatic development and gene expression cannot be determined by studying HNF3β homozygous null mutant mice because they die early in embryogenesis well before formation of the pancreas (40, 41); although a β-cell-specific disruption of the HNF3β has been obtained, the expression of Nkx2.2 was not studied in these animals (42).

It should be noted that the expression patterns of β-galactosidase in the transgenic fetuses using the nkhx2.2 1a and 1b promoters do not recapitulate the broad expression of nkhx2.2 protein seen prior to embryonic day 13 in the pancreatic buds of normal mice. This shortcoming could result from the absence of key sequences that lie outside the regions of the nkhx2.2 gene used for these transgenic animals. Furthermore, the use of individual, isolated promoters, although necessary to identify their distinct functions, could also limit expression if sequence elements from two or more of the promoters cooperate in driving expression in some cell types. For example, sequences in the 1a promoter could affect transcription from the 1b promoter and broaden its activity to additional cell types.

Finally, it is interesting to speculate on the role of the Nkx2.2 binding sites found in the Nkx2.2 1a and 1c promoters. These sites fit the ideal Nkx2.2 binding consensus (25) and are completely conserved between mouse and human. It seems possible that, once Nkx2.2 expression has been initiated from the 1b promoter, Nkx2.2 itself may feedback through the 1a and 1c promoters to maintain its expression in mature islet cells, in cooperation with other factors. In this model, a cascade of signals in the form of transcription factors initiates Nkx2.2 expression, but a network of interdependent signals maintains Nkx2.2 expression and the differentiated phenotype of the mature islet cells.

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