THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 19, Issue of May 9, pp. 17130–17140, 2003
Printed in U.S.A.

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

Received for publication, December 26, 2002, and in revised form, February 24, 2003
Published, JBC Papers in Press, February 25, 2003, DOI 10.1074/jbc.M213196200

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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 homeodomain transcription factor Nkx2.2 develop severe hyperglycemia and die shortly after birth (12). The mutant embryos lack insulin-producing β-cells and have fewer α-cells and PP cells. Remarkably, in these mutants there remains a large population of islet cells that do not produce any of the four endocrine hormones. These cells express some β-cell markers, such as islet amyloid polypeptide and PDX-1, but lack other definitive β-cell markers including GLUT2, glucokinase, and the β-cell-specific homeodomain factor Nkx6.1. These mice demonstrate that Nkx2.2 is necessary for the final differentiation of β-cells.

The onset of Nkx2.2 expression in mouse endoderm is coincident with the onset of dorsal pancreatic bud evagination at embryonic day 9.5. Most or all of the epithelial cells of the pancreas express Nkx2.2 from the onset of bud formation until embryonic day 12.5; thereafter, Nkx2.2 expression becomes more restricted. During the peak period for β-cell neogenesis, from embryonic day 13.5 to 18.5 (13), Nkx2.2 is expressed in a subset of incompletely differentiated endocrine precursor cells that coexpress the bHLH[1] proendocrine transcription factor neurogenin-3 (12, 14). Unlike neurogenin-3, which is expressed exclusively in precursor cells, Nkx2.2 is expressed also in differentiated endocrine cells. In the mature pancreas, Nkx2.2 expression is limited to the differentiated endocrine cells including α-, β-, and PP cells, but not δ-cells. Therefore, Nkx2.2 is expressed at least three distinct stages in islet cell differentiation: 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 in the developing ventral neural tube and mature neurons in the central nervous system (15). However, the mechanisms that control Nkx2.2 expression in these different 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

* This work was supported in part by National Institutes of Health Grants DK553401 and DK21344. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY44657 (mouse) and AY44658 (human).

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; CMV, cytomegalovirus; EMSA(s), electrophoretic mobility shift assays; GST, glutathione *S*-transferase; LUC, luciferase; 5′-RACE, rapid amplification of 5′-cDNA ends; TK, thymidine kinase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

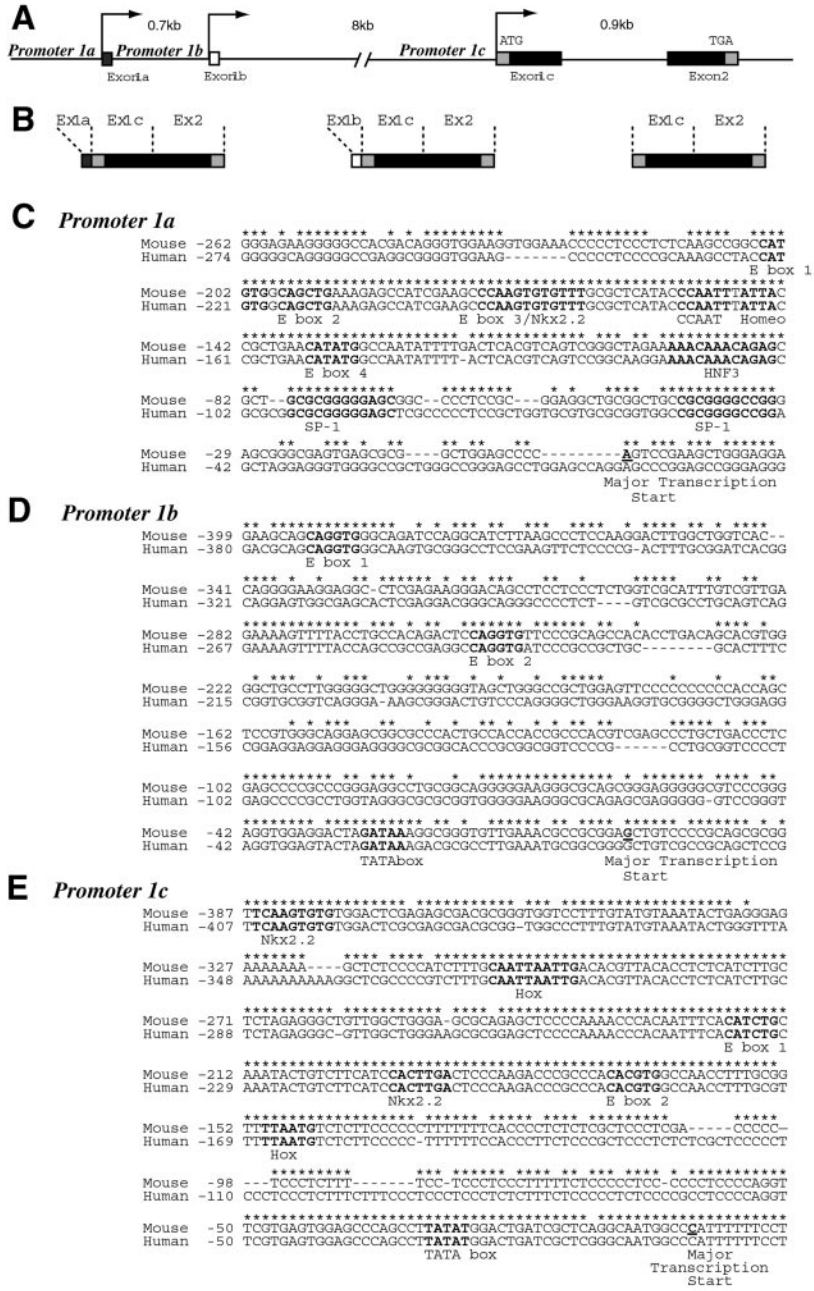

FIG. 1. **Structure of the *nkx2.2* gene.** *A*, map of the mouse *nkx2.2* gene. The coding sequence is shown in *black*. *B*, the three identified splice variants of the *nkx2.2* mRNA. In *C–E*, comparisons of the proximal sequences of the human *NKX2B* and mouse *nkx2.2* 1a (*C*), 1b (*D*), and 1c (*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*.

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′-flanking sequences. The mouse and human upstream regions were sequenced and are available from GenBank.

Oligoucleotide-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 β-cell tumor line βTC3. 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′-CACTTGGTCAATTCGTGG CTCTCC-3′) as primers. For nested PCR, we used the GeneRacer 5′-nested primer and HW324 (5′-CACGCAGAAATGTAGGCTGTGACTGG-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 cell-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 CMVβ-Gal 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

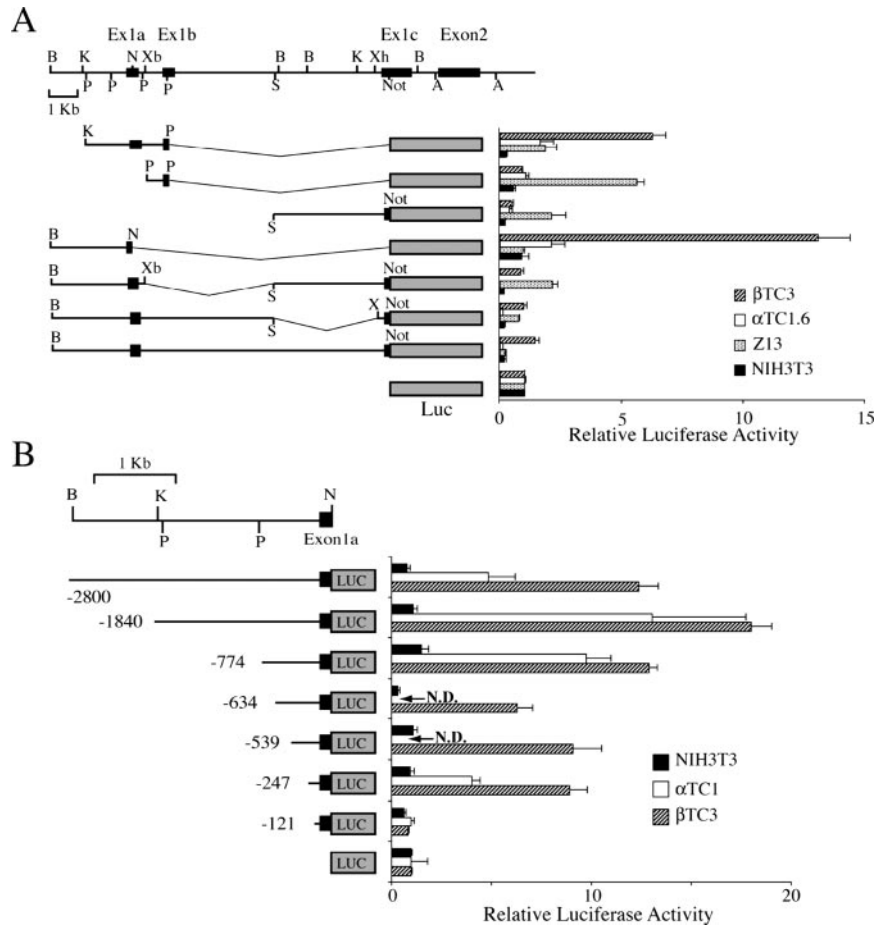

FIG. 2. **Identification of an islet cell-specific enhancer element in the nkx2.2 promoter.** *A*, the *upper panel* shows a map of the mouse *nkx2.2* gene. Reporter plasmids were constructed with the *nkx2.2* gene fragments shown, inserted upstream from the luciferase gene in the pFOXLuc1 plasmid using 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, αTC1.6 (*open bars*); a neural tube derived line, Z13 (*stippled 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*), αTC1.6 (*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*, *Bam*HI; *K*, *Kpn*I; *N*, *Nhe*I; *Xb*, *Xba*I; *Xh*, *Xho*I; *P*, *Pst*I; *S*, *Spe*I; *A*, *Apa*I; *Not*, *Not*I.

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 pBAT11 T7 *in vitro* transcription vector (17), the pBAT12 CMV expression vector, or the pPIG11 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), pBAT12, or pPIG11. HNF3β, E47, NeuroD1, and neurogenin-3 proteins were produced *in vitro* using the TNT Quick Coupled Lysate System® (Promega) and the *in vitro* expression vectors as templates.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from αTC1.6 cells, βTC3 cells, and NIH3T3 cells were prepared following the procedure described by Sadowski and Gilman (19). Single-stranded oligonucleotides corresponding to the sequences in the mouse Nkx2.2 promoter were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled oligonucleotide was column purified and annealed to an excess of complementary strand. EMSA buffers and electrophoresis conditions were as described previously (17). One μl of *in vitro* reaction mixture or 2 μg of nuclear extract was used for each 10-μl binding reaction. For antibody experiments, 1 μl of antiserum was added to each binding reaction, and the mix was incubated for 15 min at room temperature before PAGE. The antisera directed against HNF3α, β, and γ were generous gifts from R. H. Costa (University of Illinois at Chicago (20)). The following oligonucleotides along with their complementary strands were used as binding probes: H31, CGGGCTAGAAAAACAAACAGAGCGCTGCGC; E4, GATCCATTGGCCATATGTTCAGCGGTAATAAATTGA.

Immunoprecipitation Analyses—The FLAG-fused HNF3β expression plasmids were generated using the pcDNA3FLAG plasmid (a generous gift from S. Tomita, University of California, San Francisco (21)). The FLAG-fused HNF3β and neurogenin-3 expression plasmids were transfected into NIH3T3 cells, nuclear extracts were isolated, and 50 μg of each nuclear extract was used for immunoprecipitation. The immunoprecipitation procedures were performed using FLAG-tagged protein immunoprecipitation kit (Sigma) according to the manufacturer's instructions.

In Vitro Protein-Protein Interaction Assay—GST fusion proteins were produced in *Escherichia coli* BL21 competent cells via the pPIG plasmid system (22). *In vitro* translated and [^{35}S]methionine-labeled proteins were prepared using the TNT-coupled reticulocyte lysate system (Promega). 25 μl of ^{35}S-labeled protein was mixed with 10 μg of GST fusion protein bound to glutathione-agarose beads in a total volume of 600 μl of interaction buffer (40 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40). Samples were then incubated for 1 h at 4 °C with gentle rocking, and the beads were washed three times with interaction buffer. The bound protein was eluted with 2 × SDS sample buffer, fractionated on SDS-polyacrylamide gels, and visualized by autoradiography.

Generation of Transgenic Mice and Detection of β-Galactosidase—We isolated 1.8 kb and 0.2 kb of the *nkx2.2* exon 1a promoter (from −1754 to +85 bp relative to the 1a transcription start site and from −247 to +85 bp), 0.7 kb of the exon 1b promoter (from −665 to +109 bp relative to the 1b transcription start site), and 3.6 kb of the exon 1c promoter (from approximately −3600 to +106 bp relative to the 1c transcription start site) using suitable restriction enzymes or PCR. After cloning into the pβgal-Enhancer vector (Clontech, CA), we obtained pβgal.Nkx2.2E1a-1800, pβgal.Nkx2.2E1a-200, pβgal.Nkx2.2E1b-700, and pβgal.Nkx2.2E1c-3600. Each plasmid was purified using Endo Free Plasmid kit (Qiagen), linearized, and microinjected (1.5 ng/μl) into oocyte pronuclei from C3FeB6 mice. The injected embryos were transferred to pseudopregnant BDF1 female mice. After checking the genotype with PCR primers for the *lacZ* sequence, we established multiple mouse lines with each construct by crossing each founder with C57B6 mice. For the Nkx2.2 1a −1754 bp construct we characterized five independent lines, three gave detectable expression of β-galactosidase in identical patterns. For the Nkx2.2 1a −247 bp construct we characterized six independent lines, two gave detectable expression in the same patterns. For

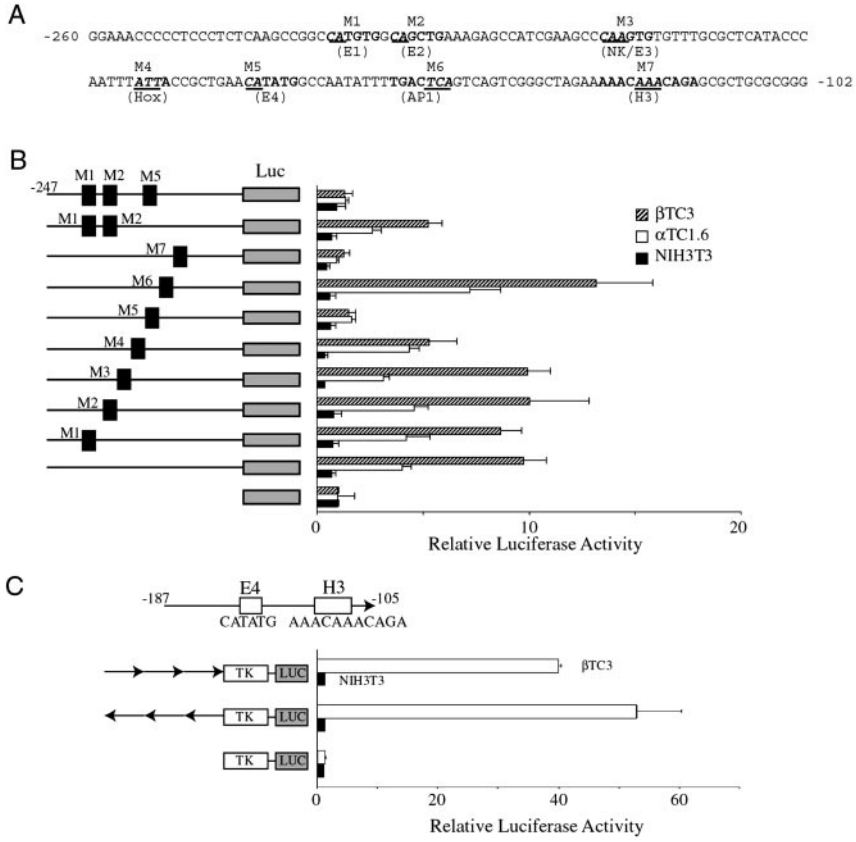

FIG. 3. **E box and HNF3 binding sites are necessary and sufficient for nkx2.2 1a promoter activity.** *A*, the sequence of the domain mapped in Fig. 2 is shown. Potential binding sites for pancreatic transcription factors are shown in *boldface*. The locations of mutations introduced into the promoter are *underlined*. *B*, reporter plasmids were constructed with the indicated mutations in the minimal *nkx2.2* 1a promoter upstream from the luciferase gene and then were cotransfected with a CMV promoter-driven β-galactosidase expression plasmid into βTC3 (*hatched bars*), αTC1.6 (*open bars*), or NIH3T3 (*filled bars*) cells. *C*, three tandem repeats of the *nkx2.2* promoter sequences from −187 to −105 bp were ligated bidirectionally upstream from the THYMIDINE KINASE minimal promoter-driving luciferase gene and then were cotransfected with a CMV promoter-driven β-galactosidase expression plasmid into βTC3 (*open bars*) or NIH3T3 (*filled bars*) cells. Relative luciferase activities were calculated with the activity of cells transfected with the promoterless pFOXLuc1 plasmid set at 1. All data are shown as the mean ± S.E.

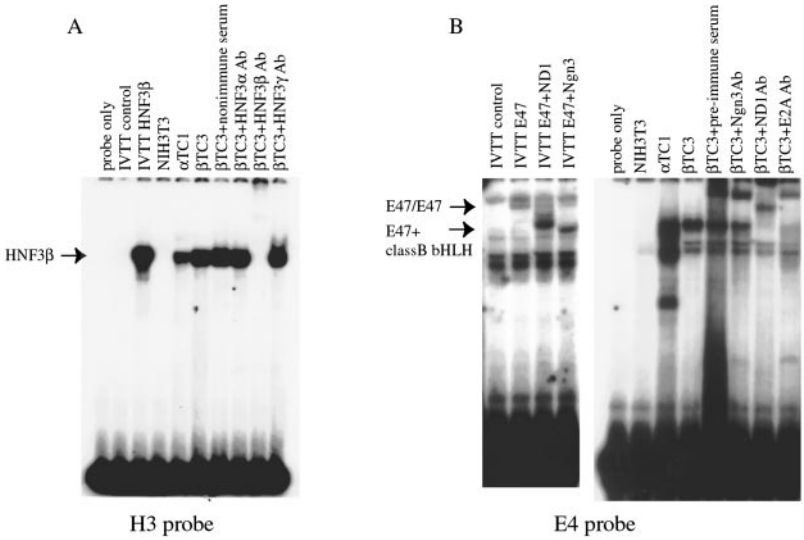

FIG. 4. **HNF3β and proendocrine bHLH proteins bind to the *nkx2.2* 1a promoter.** *A*, EMSAs using *in vitro* translated HNF3β, or nuclear extracts from NIH3T3, αTC1.6, and βTC3 cells are shown. ^{32}P-Labeled oligonucleotides encoding the H3 enhancer element (sequences are shown under "Materials and Methods") were incubated with 1 μl of *in vitro* translation product or 2 μg of nuclear extract. In the *four right lanes*, an additional 1 μl of non-immune serum or the indicated immune serum was added to the binding mix. *B*, EMSAs using *in vitro* translated E47, NeuroD1 (*ND1*), or neurogenin-3 (*Ngn3*), or nuclear extracts from NIH3T3, αTC1.6, or βTC3 cells are shown. ^{32}P-Labeled oligonucleotides encoding the E4 enhancer element were incubated with 1 μl of each *in vitro* translated protein or 2 μg of nuclear extracts. In the indicated *lanes*, an additional 1 μl of non-immune serum or the indicated immune serum was added to the binding mix. Binding mixes were subjected to electrophoresis on a 5% polyacrylamide gel.

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.

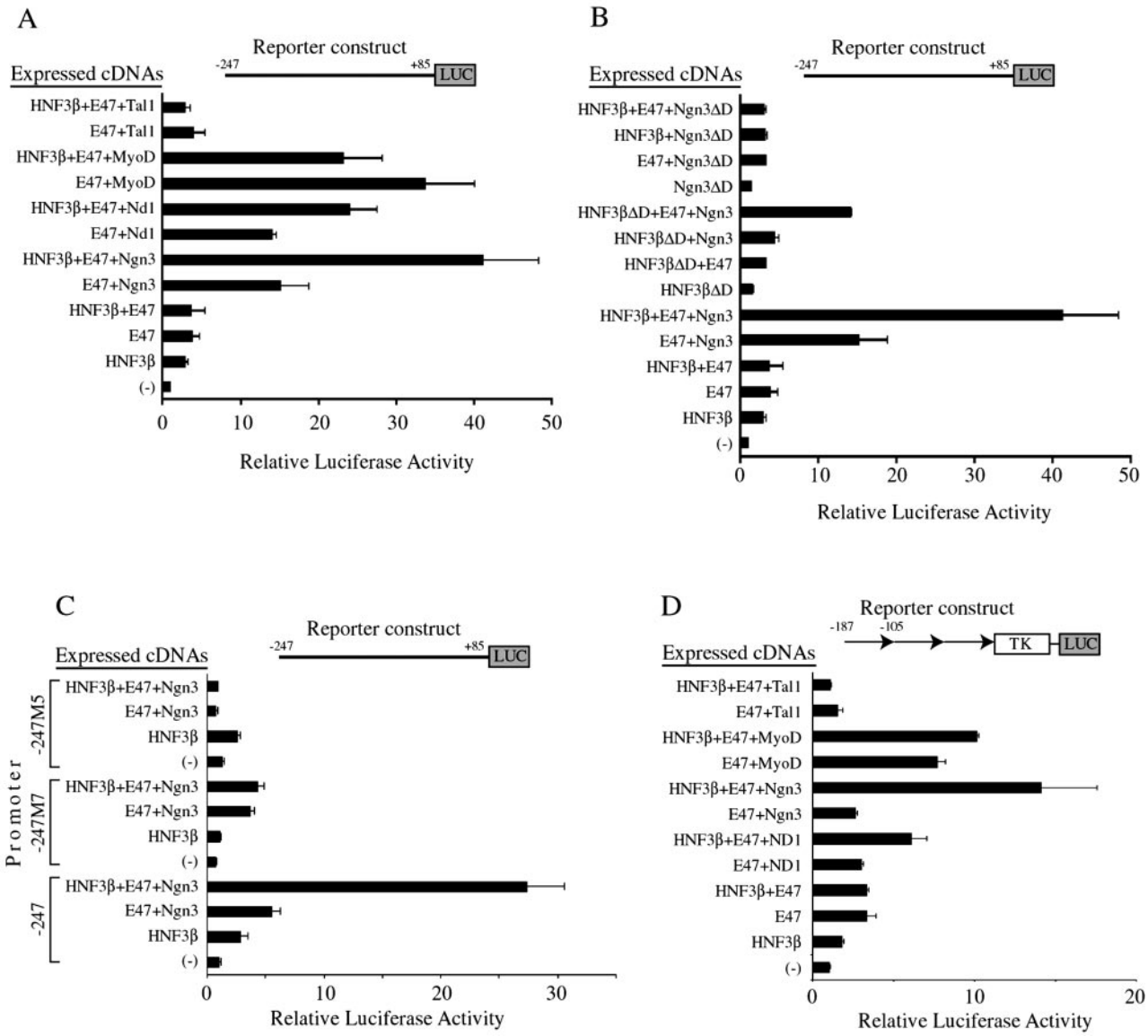

FIG. 5. **HNF3β and neurogenin-3 cooperatively activate the nkx2.2 1a promoter.** *A* and *B*, a reporter plasmid containing the *nkx2.2* 1a proximal promoter driving luciferase and expression plasmids expressing the transcription factor cDNAs indicated under the control of the CMV promoter were cotransfected into NIH3T3 cells. Ngn3ΔD indicates the neurogenin-3 cDNA with the DNA binding domain deleted (Ngn3Δ76–131 is shown in Fig. 7*C*). HNF3βΔD indicates the HNF3β cDNA with the DNA binding domain deleted (HNF3βΔ46–257 shown in Fig. 7*B*). *C*, reporter plasmids containing the *nkx2.2* 1a proximal promoter with or without the mutations indicated driving luciferase and expression plasmids expressing the transcription factor cDNAs indicated under the control of the CMV promoter were cotransfected into NIH3T3 cells. *D*, reporter plasmids containing three tandem copies of the *nkx2.2* mini-enhancer element (from −187 to −105 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.

RESULTS

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 β-cell tumor line βTC3, and the fibroblast line NIH3T3. Sequencing of the PCR products revealed three major transcription start sites (Fig. 1*A*), which produce three different splice products (Fig. 1*B*). Two novel exons, 1a and 1b, each are spliced upstream from exon1c. 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 exon1c 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 major transcription start site. The proximal sequences

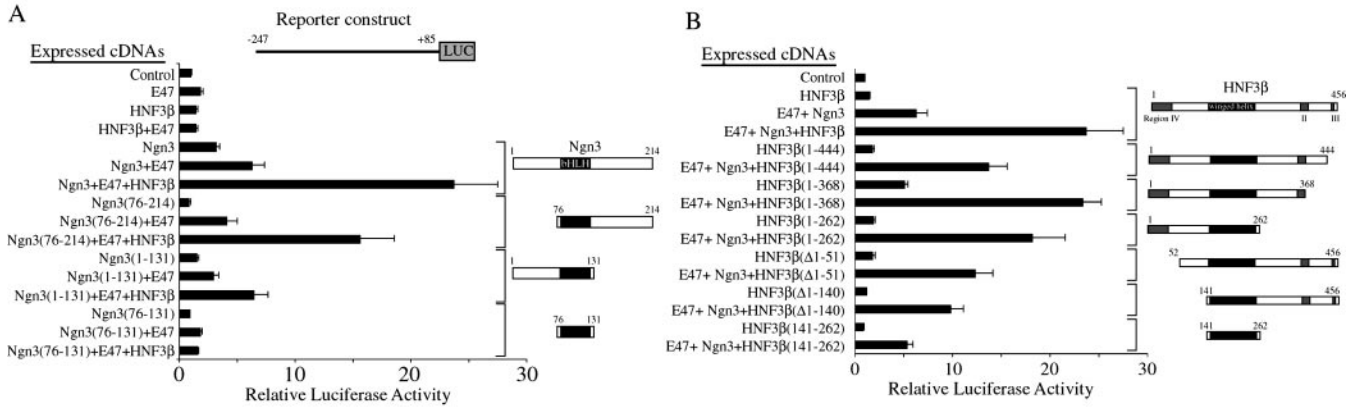

FIG. 6. **Regions of HNF3*β* and neurogenin-3 required for cooperative activation of the *nkx2.2* 1a promoter.** *A*, a reporter plasmid containing the *nkx2.2* 1a proximal promoter (−247 bp) driving luciferase was cotransfected into NIH3T3 cells along with plasmids containing the CMV promoter driving the expression of HNF3*β*, E47, and neurogenin-3 with the deletions indicated. *B*, a reporter plasmid containing the *nkx2.2* 1a proximal promoter (−247 bp) driving luciferase was cotransfected into NIH3T3 cells along with plasmids containing the CMV promoter driving the expression of neurogenin-3, E47, and HNF3*β* with the deletions indicated. Relative luciferase activities were calculated with the activity of cells transfected with the expression vector without cDNA insert (*control*) 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. 2*A*, the relative activities of the three promoters were compared in the *β*-cell-derived line *β*TC3, the *α*-cell-derived line *α*TC1.6, 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 *β*TC3 cells, *α*TC1.6 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 *β*TC3 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 *β*TC3 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. 2*B*, 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 *β*TC3 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 *β*TC3 cells (Fig. 3, *A* and *B*).

Mutations introduced into the homeodomain binding site (Ho), 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. 3*C*, 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 HNF3*β* (FoxA2) is a key regulator of the pancreatic/duodenal homeobox gene *pdx-1* (28–30). As shown in Fig. 4A, *in vitro* translated HNF3*β* can bind to H3. A complex of similar mobility was detected in nuclear extracts from *α*TC1.6 cells and *β*TC3 cells, but not in NIH3T3 cell, and this complex was recognized by antiserum to HNF3*β* but not to HNF3*α* or HNF3*γ*.

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. 4*B*, *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 *α*TC1.6 cells and *β*TC3 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 HNF3*β* 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*

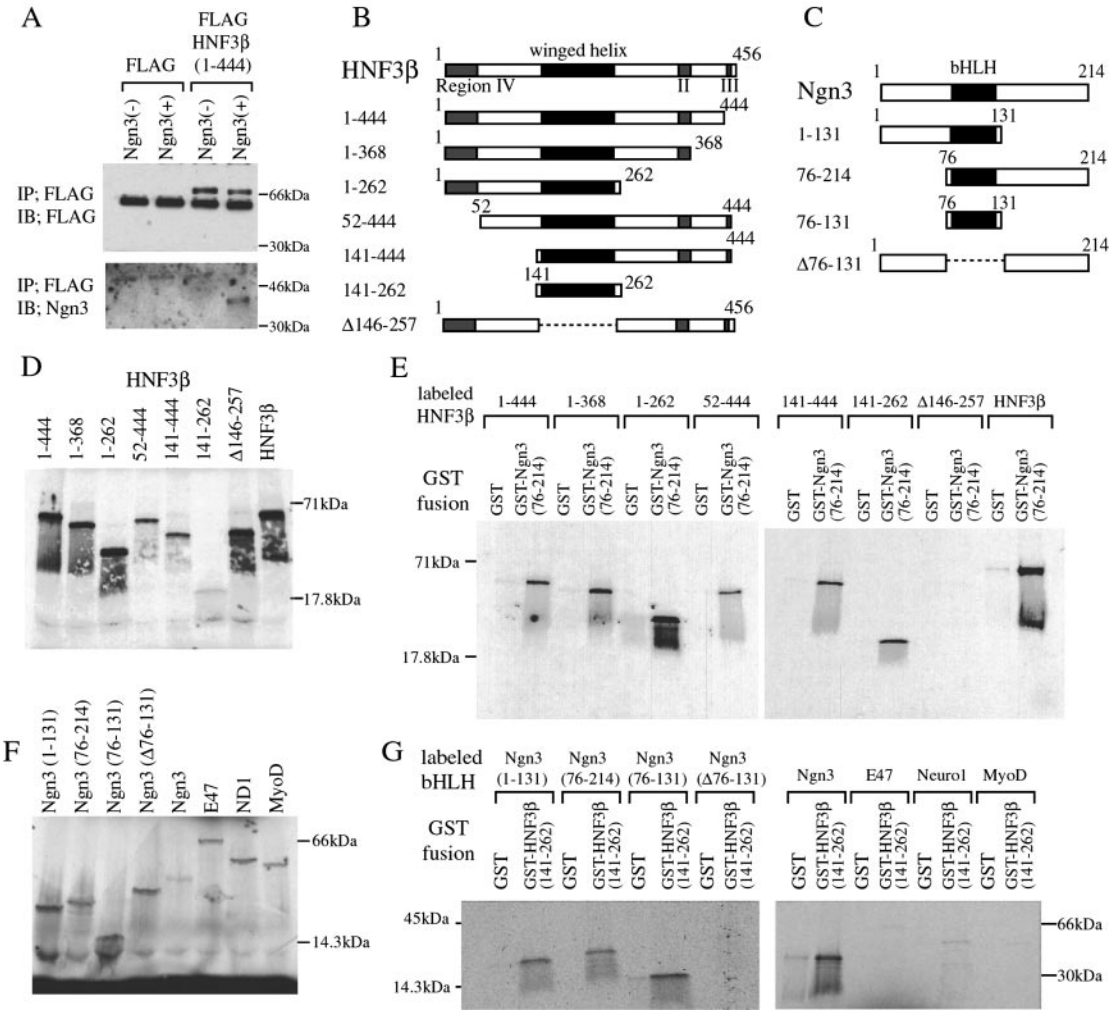

FIG. 7. **HNF3β and neurogenin-3 interact directly through their DNA binding domains.** *A*, neurogenin-3 and the FLAG peptide or HNF3β fused to the FLAG peptide were expressed in NIH3T3 cells. Nuclear extracts were isolated, immunoprecipitated (*IP*) with a FLAG antibody, separated by SDS-PAGE, and 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 [^{35}S]methionine and compared for translation efficiency by separating on SDS-polyacrylamide gel and comparing ^{35}S incorporation (shown in *D* and *F*). *In vitro* translated [^{35}S]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 ^{35}S label (shown in *E* and *G*).

1a promoter (Fig. 5*A*) or by three copies of the H3/E4 element from the 1a promoter upstream from a minimal THYMIDINE KINASE promoter (Fig. 5*D*). As shown in Fig. 5*A*, HNF3β or E47 alone activated the *nkx2.2* 1a promoter modestly; and coexpression of HNF3β with 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 5*B*), and intact H3 and E4 sites (Fig 5*C*). 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* 1a 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.[2] 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.[3]

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

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

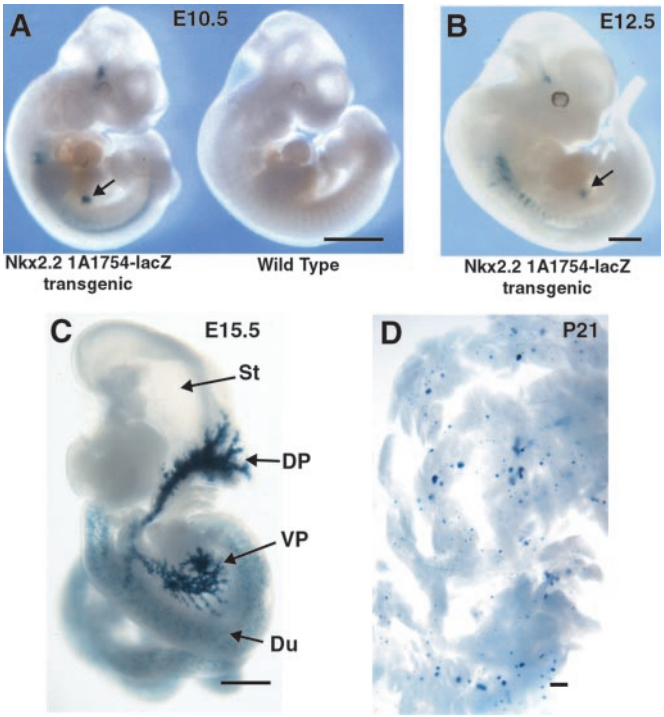

FIG. 8. **Gross expression of the −1754 bp *nkx2.2* 1a promoter *in vivo*.** In *A*, a transgenic embryo at E10.5 with the −1754 bp *nkx2.2* 1a promoter ligated upstream from the bacterial *lacZ* gene is shown on the *left* with a nontransgenic littermate on the *right*. Both embryos were incubated with X-gal, yielding the *blue* product outlining areas of β-galactosidase expression in the pancreas (*arrow*) and neural tissues of the transgenic embryo. In *B*, an Nkx2.2 1A1754-lacZ transgenic embryo at E12.5 is shown after X-gal staining. The *arrow* indicates staining in the pancreas. In *C*, viscera from an Nkx2.2 1A1754-lacZ transgenic embryo at E15.5 is shown after X-gal staining. The *blue* stain can be detected in the central areas of the dorsal and ventral pancreas and in a *speckled pattern* in the gut (distinct from the hazy staining in the lumen of the gut produced by endogenous β-galactosidase activity). In *D*, the pancreas from a 3-week-old Nkx2.2 1A1754-lacZ transgenic mouse is shown after X-gal staining. The *bar* indicates 1 mm. *St*, stomach; *DP*, dorsal pancreas; *VP*, ventral pancreas; *Du*, duodenum.

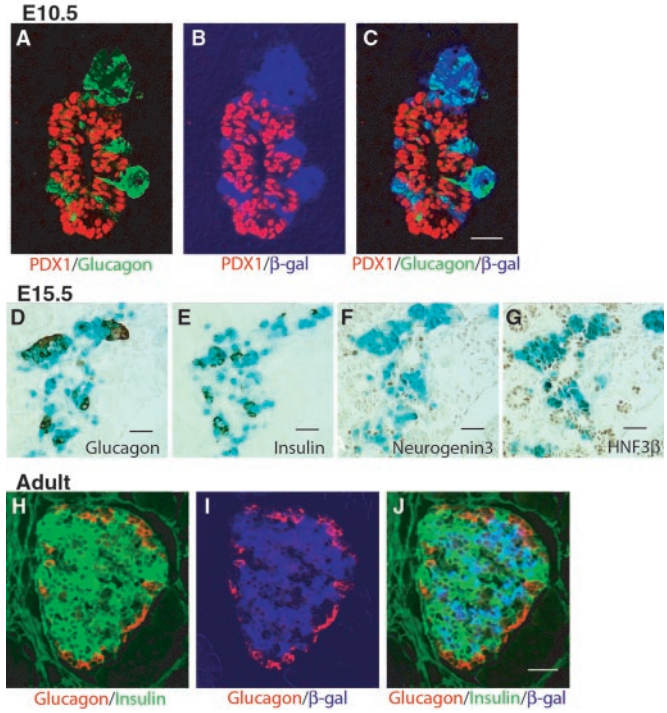

FIG. 9. **Expression pattern of the −1754 bp nkx2.2 1a promoter *in vivo*.** In *A–C*, a single section through the dorsal pancreatic bud at E10.5 from an *nkx2.2* 1a1754-lacZ transgenic embryo is shown. β-Galactosidase activity was detected with X-gal and is shown in *blue*; PDX-1 (*red*) and glucagon (*green*) were detected by immunofluorescence. In *D–G*, serial sections from the pancreas of an Nkx2.2 1a1754-lacZ transgenic embryo at E15.5 are shown. β-Galactosidase activity was detected with X-gal (*blue*); glucagon, insulin, neurogenin-3, and HNF3β were detected by immunohistochemistry with peroxidase labeling (*brown*). In *H–J*, a single section through the pancreas of an adult Nkx2.2 1a1754-lacZ transgenic embryo is shown. β-Galactosidase activity was detected with X-gal and is shown in *blue*; glucagon (*red*) and insulin (*green*) were detected by immunofluorescence. The *bar* indicates 25 μm.

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. 7*A*, 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. 7*E* 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. 7*G*). 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 *nkx2.2* 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 *nkx2.2* 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 Nkx2.2 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 Nkx2.2-positive cells expressed β-galactosidase at day 15.5, some did not (data not shown), suggesting that the expression of Nkx2.2 in those cells may be regulated by other promoters. All β-galactosidase-expressing cells expressed HNF3β (Fig. 9*G*).

A small subset of β-galactosidase-expressing cells also expressed neurogenin-3, although most neurogenin-3-expressing cells did not express β-galactosidase (Fig. 9*F*; and see also Fig.

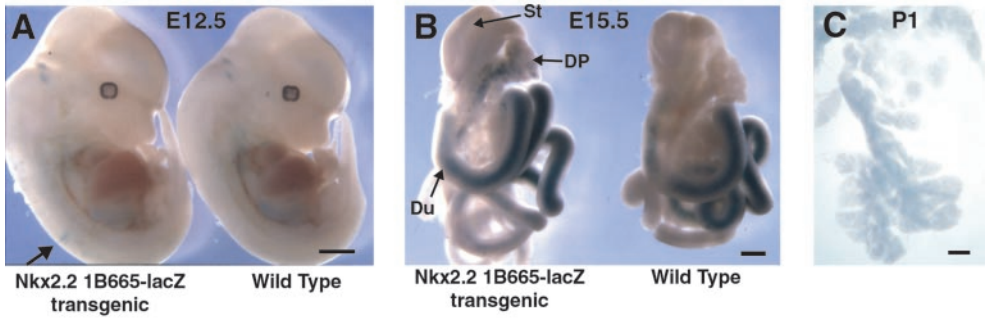

FIG. 10. **Gross expression of the −665 bp nkx2.2 1b promoter *in vivo*.** In *A*, a transgenic embryo at E12.5 with the −665 bp *nkx2.2* 1b promoter ligated upstream from the bacterial *lacZ* gene is shown on the *left* with a nontransgenic littermate on the *right*. Both embryos were incubated with X-gal, yielding the *blue* product outlining areas of β-galactosidase expression in neural tissues (*arrow*) of the transgenic embryo. In *B*, viscera from an *nkx2.2* 1b665-lacZ transgenic embryo at E15.5 on the *left* and a nontransgenic littermate on the *right* are shown after X-gal staining. The *blue* stain can be detected in the central areas of the pancreas. Hazy blue staining in the gut lumen of both embryos is produced by endogenous β-galactosidase activity. *St*, stomach; *DP*, dorsal pancreas; *Du*, duodenum, In *C*, the pancreas from a 1-day-old *nkx2.2* 1b665-lacZ transgenic mouse is shown after X-gal staining. The *bar* indicates 1 mm.

11, *A–C*). 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 *nkx2.2* 1a promoter is sufficient to drive correct expression, we also generated mice carrying a transgene with the −247 bp *nkx2.2* 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 *nkx2.2* 1b promoter gave a very different expression pattern *in vivo*. We generated transgenic mice carrying a transgene with −665 bp of the *nkx2.2* 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 *nkx2.2* 1b promoter transgenic embryos (Fig. 10*B*); 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 *nkx2.2* 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 *nkx2.2* 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).

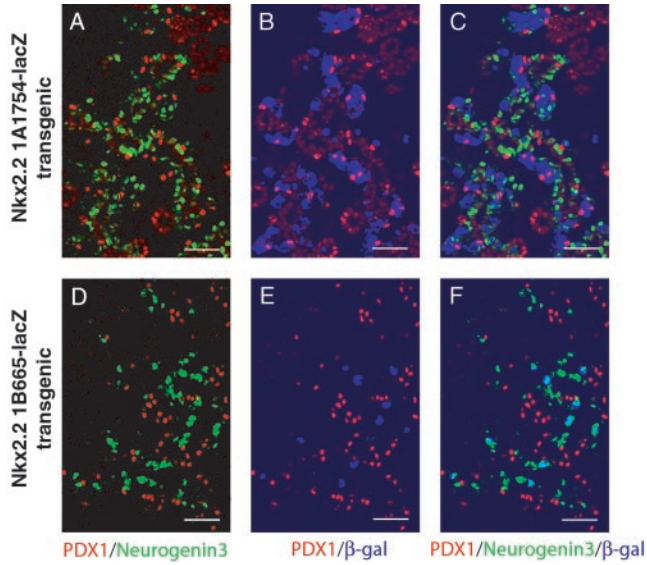

FIG. 11. **Comparison of the activity of the −1754 bp Nkx2.2 1a promoter and the −665 bp nkx2.2 1b promoter in islet progenitor cells *in vivo*.** Single sections through the pancreas at E15.5 are shown from an *nkx2.2* 1a1754-lacZ (*A–E*) and an *nkx2.2* 1b665-lacZ (*F–I*) transgenic embryo. β-Galactosidase activity was detected with X-gal and is shown in *blue*; PDX-1 (*red*) and neurogenin-3 (*green*) were detected by immunofluorescence. The *bar* indicates 25 μm.

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).

Nkx2.2 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 Nkx2.2 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 promoter[3]; and coexpression of neurogenin-3 has no effect on promoter activity,[3] together showing that neurogenin-3 does not directly activate the 1b promoter.

The close match of neurogenin-3 and β-galactosidase expression in the 1b promoter transgenic fetuses suggests instead that the *neurogenin-3* gene promoter and *nkx2.2* 1b promoter may be regulated in parallel in the developing pancreas. Although this is an attractive hypothesis, there are no common sequence elements between the two promoters and no binding sites in the 1b promoter for the endoderm factors that regulate the neurogenin-3 promoter; in addition, the 1b promoter is not inhibited by HES1[3] as is the neurogenin-3 promoter. The tight connection between neurogenin-3 expression and *nkx2.2* 1b promoter activity may depend on factors that have not yet been identified.

On the other hand, our data provide good evidence that bHLH proteins (neurogenin-3 and/or NeuroD1) regulate the *nkx2.2* 1a promoter. In the 1a promoter transgenic fetuses, activity of the promoter overlaps with neurogenin-3 expression, but only in a few cells. Together with the evidence that neurogenin-3 can directly activate the promoter through the E4 site, these data suggest a model in which neurogenin-3 initially activates the 1a promoter. In this model, neurogenin-3 expression would be extinguished in most cells by the time detectable levels of β-galactosidase activity accumulate, and NeuroD1 would maintain *nkx2.2* 1a promoter activity in the mature cells.

Neurogenin-3 does not act alone, however, in activating the *nkx2.2* 1a promoter. Our data show that full activation by neurogenin-3 requires the presence of the forkhead/winged helix factor HNF3β (FoxA2) binding to the adjacent H3 site. This synergy requires DNA binding and transcriptional activation domains on both proteins and is associated with a physical interaction between the two proteins which maps to their DNA binding domains.

Interestingly, HNF3β plays a wide ranging role in endoderm development (27). In this study, we found that HNF3β is expressed broadly in pancreatic endoderm at embyro day 15.5, including ductal cells, exocrine cells, and all Nkx2.2-positive cells. Recently HNF3β has been implicated in the transcriptional 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 *nkx2.2* 1a and 1b promoters do not recapitulate the broad expression of nkx2.2 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 *nkx2.2* 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.

Acknowledgments—We thank members of the German, Hebrok, and Vaisse laboratories for helpful comments and criticism and T. Jessell for providing Z13 cells, S. Tomita for providing the pcDNA3FLAG vector, L. Sussel for the mouse *nkx2.2* genomic DNA, and G. Bell and H. Furuta for the human *NKX2B* DNA.

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J. Biol. Chem. 2003, 278:17130-17140.
doi: 10.1074/jbc.M213196200 originally published online February 25, 2003