Cooperative Transcriptional Regulation of the Essential Pancreatic Islet Gene NeuroD1 (Beta2) by Nkx2.2 and Neurogenin 3**

Keith R. Anderson†**, Ciara A. Torres†, Keely Solomon‡, Thomas C. Becker**, Christopher B. Newgard**, Christopher V. Wright†, James Hagman§§, and Lori Sussel∥∥

From the †Department of Biochemistry and §§Program in Molecular Biology, University of Colorado Health Science Center, Denver, Colorado 80045, the ‡Department of Genetics and Development, Columbia University, New York, New York 10032, the ¶Department of Cell and Development Biology, Vanderbilt University, Nashville, Tennessee 37232, the **Sarah W. Stedman Nutrition and Metabolism Center and Departments of Pharmacology and Cancer Biology and Medicine, Duke University Medical Center, Durham, North Carolina 27704, and the ∥∥Integrated Department of Immunology, National Jewish Health Center, Denver, Colorado 80206

Nkx2.2 and NeuroD1 are two critical regulators of pancreatic β cell development. Nkx2.2 is a homeodomain transcription factor that is essential for islet cell type specification and mature β cell function. NeuroD1 is a basic helix-loop-helix transcription factor that is critical for islet β cell maturation and maintenance. Although both proteins influence β cell development directly downstream of the endocrine progenitor factor, neurogenin3 (Ngn3), a connection between the two proteins in the regulation of β cell fate and function has yet to be established. In this study, we demonstrate that Nkx2.2 transcriptional activity is required to facilitate the activation of NeuroD1 by Ngn3. Furthermore, Nkx2.2 is necessary to maintain high levels of NeuroD1 expression in developing mouse and zebrafish islets and in mature β cells. Interestingly, Nkx2.2 regulates NeuroD1 through two independent promoter elements, one that is bound and activated directly by Nkx2.2 and one that appears to be regulated by Nkx2.2 through an indirect mechanism. Together, these findings suggest that Nkx2.2 coordinatey activates NeuroD1 with Ngn3 within the endocrine progenitor cell and also plays a role in the maintenance of NeuroD1 expression to regulate β cell function in the mature islet. Collectively, these findings further define the conserved regulatory networks involved in islet β cell formation and function.

The pancreas is an intricate organ composed of exocrine tissue that secretes digestive enzymes into pancreatic ducts, and the endocrine islets of Langerhans that produce the metabolic hormones insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. The different pancreatic cell lineages arise during the critical developmental events referred to as the primary and secondary transitions (Ref. 1; reviewed in Ref. 2). The primary transition occurs between embryonic day (e) 8.5 and e11.5** and encompasses the initial patterning and specification of the pancreatic endoderm, which originates from the foregut. The secondary transition is the critical stage between e12.5 and e15.5 when endocrine and exocrine progenitors expand and a large second wave of differentiation is initiated. The secondary transition also marks an increase in the expression and/or relocation of a number of transcription factors that are important in pancreatic development, including Pdx1, Ptf1a, Ngn3, Nkx2.2, NeuroD1, Pax4, Pax6, and Nkx6.1 (reviewed in Ref. 3). A large number of studies of these transcription factors have yielded a timeline of gene expression and determined which cell lineages are regulated by each transcription factor (2–7). Notably, temporal and spatial changes in many of the transcription factor expression profiles can re-program a progenitor or precursor cell to alter, prevent, or initiate endocrine differentiation (3, 8–12). The cumulative findings of these studies have illustrated that the regulation of islet cell differentiation depends on complex relationships between the transcriptional regulatory cascades. These regulatory mechanisms are not simple linear relationships, but instead require feedback loops, cross-talk, and context-dependent interactions to allow the appropriate differentiation program to be fully executed.

One of the key regulators involved in the β cell differentiation process is NeuroD1/β2 (hereafter referred to as NeuroD1). NeuroD1 is a basic helix-loop-helix transcription factor that is essential for pancreatic and neuronal embryonic development and postnatal functions (13–18). In the developing pancreas, NeuroD1 is detected as early as e9.5 in the early glucagon-producing populations (14). By birth, NeuroD1 is predominantly localized to the insulin-producing β cell population, although expression is maintained in a subset of glucagon-producing cells in embryonic pancreata and in perinatal immature islets during the critical developmental events referred to as the primary and secondary transitions (Ref. 1; reviewed in Ref. 2). The primary transition occurs between embryonic day (e) 8.5 and e11.5** and encompasses the initial patterning and specification of the pancreatic endoderm, which originates from the foregut. The secondary transition is the critical stage between e12.5 and e15.5 when endocrine and exocrine progenitors expand and a large second wave of differentiation is initiated. The secondary transition also marks an increase in the expression and/or relocation of a number of transcription factors that are important in pancreatic development, including Pdx1, Ptf1a, Ngn3, Nkx2.2, NeuroD1, Pax4, Pax6, and Nkx6.1 (reviewed in Ref. 3). A large number of studies of these transcription factors have yielded a timeline of gene expression and determined which cell lineages are regulated by each transcription factor (2–7). Notably, temporal and spatial changes in many of the transcription factor expression profiles can re-program a progenitor or precursor cell to alter, prevent, or initiate endocrine differentiation (3, 8–12). The cumulative findings of these studies have illustrated that the regulation of islet cell differentiation depends on complex relationships between the transcriptional regulatory cascades. These regulatory mechanisms are not simple linear relationships, but instead require feedback loops, cross-talk, and context-dependent interactions to allow the appropriate differentiation program to be fully executed.

One of the key regulators involved in the β cell differentiation process is NeuroD1/β2 (hereafter referred to as NeuroD1). NeuroD1 is a basic helix-loop-helix transcription factor that is essential for pancreatic and neuronal embryonic development and postnatal functions (13–18). In the developing pancreas, NeuroD1 is detected as early as e9.5 in the early glucagon-producing populations (14). By birth, NeuroD1 is predominantly localized to the insulin-producing β cell population, although expression is maintained in a subset of glucagon-producing cells in embryonic pancreata and in perinatal immature islets during the critical developmental events referred to as the primary and secondary transitions (Ref. 1; reviewed in Ref. 2). The primary transition occurs between embryonic day (e) 8.5 and e11.5** and encompasses the initial patterning and specification of the pancreatic endoderm, which originates from the foregut. The secondary transition is the critical stage between e12.5 and e15.5 when endocrine and exocrine progenitors expand and a large second wave of differentiation is initiated. The secondary transition also marks an increase in the expression and/or relocation of a number of transcription factors that are important in pancreatic development, including Pdx1, Ptf1a, Ngn3, Nkx2.2, NeuroD1, Pax4, Pax6, and Nkx6.1 (reviewed in Ref. 3). A large number of studies of these transcription factors have yielded a timeline of gene expression and determined which cell lineages are regulated by each transcription factor (2–7). Notably, temporal and spatial changes in many of the transcription factor expression profiles can re-program a progenitor or precursor cell to alter, prevent, or initiate endocrine differentiation (3, 8–12). The cumulative findings of these studies have illustrated that the regulation of islet cell differentiation depends on complex relationships between the transcriptional regulatory cascades. These regulatory mechanisms are not simple linear relationships, but instead require feedback loops, cross-talk, and context-dependent interactions to allow the appropriate differentiation program to be fully executed.
(14, 19). Despite the early expression of NeuroD1 in the developing pancreas, NeuroD1 null mice do not exhibit phenotypes until late gestation. At this time the null mice display reduced insulin production and significantly decreased β cell mass due to apoptosis (14). Interestingly, on certain genetic backgrounds, NeuroD1 null mice can survive postnatally with only mild hyperglycemia (13). These findings suggest that NeuroD1 plays a predominant role in the maintenance of functional β cells after they have formed, although it has been suggested that the absence of an earlier phenotype may be due to redundancy with other NeuroD family members (20).

The importance of NeuroD1 in regulating β cell function and, in particular, its critical role in mediating glucose-regulated insulin gene transcription has led to investigations of its regulation. Huang et al. (21) identified a minimal 2.2-kb NeuroD1 promoter that recapitulated NeuroD1 expression in the pancreas during embryonic development and in the adult. This study also determined that Ngn3, another essential pancreatic basic helix-loop-helix protein, was able to activate NeuroD1 through multiple E boxes present within the minimal NeuroD1 promoter (21). Ngn3 is necessary for the production of all endocrine lineages and is expressed in the islet progenitor. Once islet cell types are specified, Ngn3 expression is down-regulated (9). Gasa et al. have postulated that NeuroD1 may take over the transcriptional regulation of previous Ngn3 downstream targets (15). The 2.2-kb minimal promoter element is also sufficient for mediating glucose-responsive NeuroD1 induction by Foxo1 (22). Additional studies have indicated that NeuroD1 is regulated post-transcriptionally in response to glucose levels (reviewed in Ref. 23).

Similar to NeuroD1, the homeodomain containing transcription factor Nkx2.2 is involved in both early central nervous system development and endocrine pancreas specification (24–26). Nkx2.2 null mice die shortly after birth with severe hyperglycemia. Nkx2.2 null mice lack all β cells, most α cells, and a subset of PP cells, which are replaced by the ghrelin-producing population (10, 26). In vitro and in vivo data suggest that Nkx2.2 mediates these early islet cell fate decisions by functioning both as a repressor and activator of transcription depending on the developmental context (27–31). To date, however, only a small number of transcriptional targets of Nkx2.2 have been identified, including Ins2 and MafA (27, 30). Nkx2.2 consensus sites are also present in several regions on the NeuroD1 promoter, and it has been speculated that Nkx2.2 could control its own transcription through a feedback loop (29, 32).

Nkx2.2 and NeuroD1 are both critical players in islet cell development. Their respective null phenotypes suggest that Nkx2.2 functions primarily upstream of NeuroD1 to specify islet cell fate, whereas NeuroD1 plays a more critical role in the survival and maintenance of β cell function once the mature β cells have formed. However, there is evidence suggesting that the relationship between the two factors is more complex. Epistasis analysis has identified a genetic interaction between Nkx2.2 and NeuroD1 in regulating the formation of glucagon-producing cells, which is consistent with the early expression of both factors in the pancreatic epithelium and in the early α cell populations (33). In addition, evidence suggests that NeuroD1 functions upstream of Nkx2.2; adenovirus-mediated expression of NeuroD1 in pancreatic ductal cell lines was able to induce the expression of Nkx2.2 (15). Furthermore, ectopic expression of NeuroD1 was able to induce expression of Nkx2.2 in a human pancreatic δ cell line (19). However, Nkx2.2 expression is not significantly affected in neonatal NeuroD1 null mice, whereas NeuroD1 is significantly down-regulated in the Nkx2.2 null pancreata (33).

To clarify the respective roles of Nkx2.2 and NeuroD1 in the regulation of islet development and β cell function, we examined whether Nkx2.2 functions upstream of NeuroD1 to regulate its transcription. We determined that NeuroD1 transcriptional expression is down-regulated in the pancreata of Nkx2.2 null embryos after e12.5. NeuroD1 is also reduced in the mouse βTC6 cell line in response to RNA interference knockdown of Nkx2.2 expression. Interestingly, Nkx2.2 regulates NeuroD1 through two distinct promoter regions. Nkx2.2 can bind and activate NeuroD1 expression through an Nkx2.2 binding site located 837 bp upstream of the transcriptional start site. Nkx2.2 is also able to activate a 686-bp promoter region that includes the Ngn3 binding sites, however the direct binding of Nkx2.2 to this regulated region was not detected. Furthermore, we demonstrate that Nkx2.2 and Ngn3 can cooperate to activate a minimal NeuroD1 promoter element and the endogenous NeuroD1 gene. These data suggest that, in addition to the complex functional roles of Nkx2.2 and NeuroD1 in regulating islet development and function, Nkx2.2 is also a key component of the regulatory pathway that modulates NeuroD1 expression in the islet.

**EXPERIMENTAL PROCEDURES**

**Mice—**Nkx2.2null/+ and NeuroD1:lacZ/+ heterozygous mice were previously generated by homologous recombination (18, 26). Both mouse strains were maintained on a Swiss Black (Taconic) background. Genotyping of mice and embryos was performed by PCR analysis as previously described (26, 33). Mice were housed and treated according to Columbia University and University of Colorado Denver Health Science Center (UCDHS) Institute of Animal Care and Use Committee approval protocols.

**Reverse Transcription-PCR and Quantitative Real-time PCR—**Total RNA was harvested from NIH3T3, PANC1, αTC1, and βTC6 cell lines and e12.5 to e18.5 whole pancreata using the RNeasy Micro or Mini Kit (Qiagen). For e12.5 to e14.5 embryos, three to five pancreata were pooled per experiment; for e15.5 to e16.5 embryos, two to three pancreata were pooled per experiment; and for e17.5 to e18.5, one to two pancreata were pooled per experiment. Experimental n was ≥3 for each cell line, and n was equal to 3 for each embryonic age. For each cell line and embryonic age group 0.5–1 μg of mRNA was converted to cDNA using the Superscript III Kit (Invitrogen). Quantitative real-time PCR was performed using pre-designed and custom TaqMan primer/probes (Applied Biosystems), and all probes were 6-carboxyfluorescein (FAM®) fluorescently labeled with a 3’ minor groove binder nonfluorescent quencher. All sequences are listed in supplemental Table S1. NeuroD1 mRNA expression for each sample was normalized to the ubiquitous metabolic control gene, cyclophilin B, expression. All quantita-
Nkx2.2 Regulates NeuroD1

tive PCR single-plex reactions were performed on an ABI Prism 7000 Sequence Detection System. Statistical significance was determined by using Student’s t test comparing wild type and Nkx2.2 null expression per age group (* represents a p value < 0.05).

In Situ Hybridization—RNA in situ hybridization was performed as previously described (10) on e15.5 whole embryo frozen 8-μm sections that were fixed overnight with 4% paraformaldehyde. The NeuroD1 antisense Riboprobe was transcribed with T7 polymerase from pCS2:MTmNeuroD1 (provided by Dr. J. Lee, Geron) linearized with EcoRI. In situ hybridizations were performed on wild-type and Nkx2.2 null littermate embryos and followed with rabbit anti-amylose (1:1000, Sigma-Aldrich) immunohistochemistry to mark the exocrine tissue within the pancreas. Images were acquired on a Leica CTR 5000 with 20× magnification.

Immunofluorescence—Immunofluorescence was performed on e14.5 and e16.5 whole embryo frozen 8-μm sections that were fixed overnight with 4% paraformaldehyde. Antibodies used consisted of guinea pig anti-β-galactosidase (1:1000, T. Finger), mouse anti-insulin (1:1000, Sigma), mouse anti-glucagon (1:1000, Sigma), rabbit anti-glucagon (1:200, Phoenix), rabbit anti-somatostatin (1:400, Phoenix), rabbit anti-PP (1:200, Zymed Laboratories Inc.), and rabbit anti-Ngn3 (1:500, Developmental Studies Hybridoma Bank (DSHB)). Secondary antibodies (Jackson ImmunoResearch) were against individual species, all raised in donkey, labeled with either Cy2 or Cy5, and used at 1:300. 4’,6-Diamidino-2-phenylindole (Invitrogen) was used at 1:1000 and incubated for 30 min. Confocal images were taken on a Zeiss META LSM 510 confocal microscope.

Zebrafish—Zebrafish and embryos were raised, maintained, and staged according to standard procedures (34). The AB* (Streisinger Laboratory, University of Oregon, Eugene, OR) line was used in natural matings to obtain embryos. Embryos, 48 hours postfertilization, were maintained in embryo medium containing 0.003% phenylthiourea to inhibit pigmentation. Morpholino oligonucleotides were purchased from Gene Tools and injected into one- or two-cell stage embryos at concentrations of 15–20 ng/embryo as previously described (35). nks2.2a morpholino sequence is as follows: 5’-TGAGGTATCTTA-CATGAGTATTG-3’. Gene Tools standard control morpholino was used for control experiments. The zebrafish nks2.2a plasmid was provided by Dr. B. Appel (UCDHSC). Zebrafish neuroD1 plasmid was provided by Dr. S. Leach (Johns Hopkins). Antisense probes were synthesized with T3 RNA polymerase after BamHI linearization (nks2.2a) and NotI linearization (neuroD). Whole mount in situ hybridization was performed as described previously (36). Yolk was manually removed after in situ hybridization, and embryos were cleared in 80% glycerol/20% phosphate-buffered saline for imaging.

Luciferase Reporter Assays—The NeuroD1 –2.2-kb minimal promoter was fused to the firefly luciferase open reading frame in the pGL3 Basic vector (Promega, Madison, WI) as previously described (21). All deletion constructs were designed with the following restriction digests, gel purified, blunt-ended, and religated as follows: for NDΔ1, the –686/-240 region was excised with PstI and NdeI; for NDΔ2, the –2190/-686 region was excised with SacI and PstI; for NDΔ3 a proximal 128-bp region was excised with Ndel and MluI maintaining the most proximal 113 bp upstream of the transcriptional start site, including the endogenous TATA box; for NDΔ4, the –2190/-240 region was excised with SacI and NdeI; and for NDΔ5, a proximal 573-bp region was excised with PstI and MluI maintaining the most proximal 113 bp upstream of the transcriptional start site, including the endogenous TATA box. NDΔfull, ndk1mut and NDΔ5-ndk1mut have the Site 1 consensus core sequence deleted through PCR mutagenesis (QuikChange mutagenesis kit, Stratagene). Panc1 or βTC6 cells in 12-well plates were co-transfected (FuGENE 6, Roche Applied Science) with 500 ng of pGL3B promoter constructs, 250 ng of effector plasmid (pcDNA3-Nkx2.2, pcDNA3-Nks2.2BDmut, pcDNA3-Ngn3, or both Nkx2.2 and Ngn3 simultaneously), and 50 ng of the internal control pRL-TK-Renilla (Promega) at the time of seeding. Each transfection condition was tested in triplicate (experimental n = 4). Transfected cells were harvested and passaged following the analysis with the Dual-luciferase Reporter Assay (Promega) and plate luminometer, Monolight (BD Biosciences). Firefly luciferase readings were normalized to Renilla luciferase values. For data including regulation by effector genes, normalized luciferase values were further normalized to the promoter construct alone in order to determine fold change differences.

Western Blot—Nuclear protein lysates from mock or transfected Panc1 cells were prepared using the Nuclear Extract Kit (Active Motif). Twenty micrograms of each sample was loaded in each lane of a 10% Bis-Tris polyacrylamide gel (Invitrogen). Proteins were transferred to a polyvinylidene difluorid membrane, and the membrane was blocked in 5% milk for 30 min, incubated with anti-NeuroD1 (1:1000, DSHB) or anti-histone deacetylase 1 (1:500, Santa Cruz Biotechnology) overnight at 4 °C, washed, incubated with anti-mouse-horseradish peroxidase (1:10,000, Zymed Laboratories Inc.) for 1 h at room temperature, washed again, and developed with Western Lightning (GE Biosciences).

Adenovirus Design and Transduction—A small interfering RNA (siRNA) sequence corresponding to the 5’-untranslated region of the mouse Nkx2.2 gene (GCCACGAAATTGAC-CAGGTGA) was used to prepare the siRNA adenovirus (Ad-siNkx2.2) by previously developed methods (37–39). A scrambled control siRNA adenovirus (Ad-siRNAcontrol) (40) and AdCMV-GFP virus (41) were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown). Purified virus stocks were titered and used to treat βTC6 cells, which express endogenous Nkx2.2, NeuroD1, and insulin. 630 plaque-forming units/cell of Ad-siNkx2.2 were used as negative controls in the knockdown experiments (data not shown).
**RESULTS**

**NeuroD1 Gene Expression Is Decreased in Nkx2.2 Null Mice**

Pancreata—Previous studies have suggested that NeuroD1 gene expression is down-regulated in the absence of Nkx2.2 (28, 33). To determine when during pancreatic development NeuroD1 mRNA levels were decreased in the Nkx2.2 null pancreata, we performed semiquantitative real-time PCR on dissected pancreata at daily time points between e12.5 and birth. A reduction of NeuroD1 expression was first evident at e13.5, during the secondary transition, and remained decreased throughout embryogenesis (Fig. 1A). In the Nkx2.2 null mice, all β cells and most α cells are replaced by the ghrelin cell population. Because the reduction in NeuroD1 corresponds with the major wave of islet cell differentiation, we wished to determine whether the reduction of NeuroD1 expression was simply due to the loss of the α and β cell populations that occurs in the Nkx2.2 null embryos. We performed mRNA in situ hybridization analyses to visualize the overall localization of NeuroD1 mRNA at e15.5. Surprisingly, NeuroD1 appeared to be expressed in similar expression domains in the wild-type and Nkx2.2 mutant pancreata, but with a reduced overall expression level in the mutant pancreas (Fig. 1, B and C). Analysis of β-galactosidase expression from the NeuroD1: LacZ knockin allele (18) also suggested that there was a decrease in NeuroD1 expression per cell, rather than a loss in the total number of NeuroD1-expressing cells (Fig. 1, M and Q).

Because the Nkx2.2 null embryos lack β cells and most α cells, the two cell populations reported to express NeuroD1, we wished to determine the endocrine cell types that express NeuroD1 in Nkx2.2 mutant pancreata compared with wild...
Nkx2.2 Regulates NeuroD1

A

Fold Change

Wildtype
Nkx2.2 +/-

Insulin: Bgal: DAPI
Glucagon: Bgal: DAPI
Ghrelin: Bgal: DAPI

Nkx2.2 +/-

Nkx2.2 +/-

Nkx2.2 +/-

Ghrelin: Bgal: Gluc
SS: Bgal: DAPI
PP: Bgal: DAPI
Bgal

Increased Gain
with the ghrelin-producing population, albeit at much lower levels. In e14.5 wild-type mice, β-galactosidase staining could be detected in each of the hormone-producing cell populations, with the exception of somatostatin-producing δ cells (Fig. 1, D–P), similar to the findings of Itkin-Ansari et al. (19). Interestingly, many of the insulin, glucagon, and PP cells at e16.5 did not co-express β-galactosidase, which may reflect the maturation state of the cell. In the Nkx2.2 null mice, as expected from the maintained widespread expression, NeuroD1 is predominantly co-expressed with the ghrelin-producing population, albeit at much lower levels than observed in wild-type islet cells (Fig. 1, F, I, J, and N). β-Galactosidase staining could also be detected at low levels in the few remaining glucagon- and PP-producing cells (Fig. 1, N and P). Similar to wild type, β-galactosidase staining could not be detected in the somatostatin-producing cells. Therefore, the reduction of NeuroD1 in the Nkx2.2 mutant pancreas appears to be a general reduction of β-galactosidase levels in the remaining islet cell populations that normally produce NeuroD1.

**NeuroD1 Regulation by Nkx2.2 Is Conserved in Zebrafish**—To assess whether the Nkx2.2-dependent regulation of NeuroD1 is functionally relevant, we determined whether this regulation is conserved across species. In zebrafish, it has been determined that a knockdown of the Nkx2.2 zebrafish homolog Nkx2.2a by translation- or splice-blocking morpholinos can recapitulate the phenotype of the Nkx2.2 null mouse; the morphant fish display decreased α and β cells, a corresponding increase in ghrelin-producing cells, and no change in the number of δ cells (46). We used the splice-blocking Nkx2.2a morpholino to demonstrate that down-regulation of Nkx2.2a in zebrafish also results in a decrease of NeuroD1 expression compared with wild type pancreata or pancreata from embryos injected with a scrambled morpholino (Fig. 2, C–E). Furthermore, similar to our observation in the Nkx2.2 null mice, expression of NeuroD1 remains widespread throughout the islet despite the changes in islet cell fates, and NeuroD1 mRNA expression is reduced in all cells (Fig. 2E).

**NeuroD1 Is Regulated by Nkx2.2 in β Cells**—In Nkx2.2 null mice it is not possible to assess NeuroD1 regulation in β cells, which are completely absent at all stages of pancreatic development. To determine whether Nkx2.2 also regulates NeuroD1 expression in functional β cells, we utilized a recombinant adenovirus containing an siRNA specific for Nkx2.2 (Ad-siNkx2.2(283)) to suppress the expression of Nkx2.2 in βTC6 cells, which endogenously express Nkx2.2 and NeuroD1. Treatment of the βTC6 cells with Ad-siNkx2.2(283) resulted in suppression of Nkx2.2 mRNA levels by ~65% by 48 h post-transfection.

**Nkx2.2 Regulates NeuroD1**

FIGURE 2. Nkx2.2 regulation of NeuroD1 expression is conserved in zebrafish. In situ hybridization of wild-type 48-hours postfertilization zebrafish embryos for Nkx2.2a (A, B, and B’). In situ hybridization of 48-hours postfertilization zebrafish embryonic pancreas for NeuroD1 (C–E) showing a decrease in NeuroD1 expression in Nkx2.2 splice morphant embryos (E) and either wild-type (C) or control morphant embryos (D).
duction (Fig. 3A) and resulted in a corresponding significant decrease in endogenous NeuroD1 transcripts by 50% (Fig. 3B). These results further suggest that Nkx2.2 regulates NeuroD1 transcription in the islet cell populations where both Nkx2.2 and NeuroD1 are expressed.

**Nkx2.2 Regulates NeuroD1**

**Nkx2.2 Is Sufficient to Activate the Minimal NeuroD1 Promoter**—A minimal 2.2-kb NeuroD1 promoter fragment has been shown to provide appropriate tissue expression *in vivo* during mouse embryogenesis and in the adult (21). To determine whether Nkx2.2 could transcriptionally regulate the min-
imal NeuroD1 promoter, we transfected the full-length minimal NeuroD1 promoter fused to a firefly luciferase (pGL3, Invitrogen) with or without Nkx2.2 into the Panc1 cell line. Panc1 cells are human pancreatic ductal carcinoma cells that do not endogenously express Nkx2.2, NeuroD1, or the pancreatic endocrine hormones to significant levels (supplemental Fig. S2). Ngn3, a known regulator of NeuroD1, was included as a positive control for these studies (21). Interestingly, Nkx2.2 activated the NeuroD1 promoter to similar or higher levels than Ngn3 (Figs. 3C and 4A). In addition, we observed additive activation of the NeuroD1 promoter when Nkx2.2 was co-transfected with Ngn3 (Figs. 3C and 4A). Similarly, endogenous NeuroD1 mRNA expression was activated when Nkx2.2 was transfected or virally transduced into Panc1 or NIH3T3 cell lines (Fig. 3D and supplemental Fig. S4). Furthermore, co-expression of Nkx2.2 and Ngn3 resulted in synergistic activation of endogenous NeuroD1 transcript (Fig. 3D), suggesting that Nkx2.2 and Ngn3 function together to initiate the activation of NeuroD1 expression.

To assess whether the induction of NeuroD1 promoter activity was indirectly due to the altered regulation of Nkx2.2 or Ngn3 in the transfected cells, rather than the direct activation of the NeuroD1 promoter elements, we compared Nkx2.2 and Ngn3 expression levels in each transfected cell line. Nkx2.2 protein levels are similar when Nkx2.2 is transfected alone or in combination with Ngn3 (Fig. 3G). However, Nkx2.2 mRNA transcript levels do appear slightly elevated when Ngn3 is present (Fig. 3E), which is consistent with previous studies (31). Conversely, Ngn3 mRNA transcript levels are not affected by the expression of Nkx2.2 (Fig. 3F). Therefore, Nkx2.2 and Ngn3 regulation of NeuroD1 appears to be due to cooperative activation. The co-expression of Nkx2.2 and Ngn3 in the islet progenitor cell population is consistent with the possibility that Nkx2.2 and Ngn3 cooperate to regulate NeuroD1 during embryogenesis (3). The Nkx2.2 K184I DNA binding mutation (Nkx2.2DBDmut), which disrupts the DNA binding activity of Nkx2.2, abrogates the activation of NeuroD1 (Fig. 3C), suggesting that the DNA binding activity of Nkx2.2 is required for the regulation of the NeuroD1 promoter.

Nkx2.2 Occupies the Endogenous NeuroD1 Promoter—To determine whether Nkx2.2 regulates NeuroD1 expression directly, we assessed the association of Nkx2.2 with the NeuroD1 promotor at the embryonic stage when we initially detected a loss of NeuroD1 expression in the Nkx2.2 null pancreata. We performed ChIP assays on pancreatic tissue isolated from e13.5 embryos. The Ins2 and MafA promoters, which are known direct targets of Nkx2.2, were used as positive controls in these studies (27, 30). As shown in Fig. 3H, Nkx2.2 occupies the NeuroD1 promoter at e13.5. Amplification of the NeuroD1 promoter DNA was less efficient than either the Ins2 or MafA promoters, however, calculation of the fraction of bound chromatin as a function of input chromatin demonstrated that each promoter element was precipitated by the Nkx2.2 antibody with similar efficiencies (Fig. 3I). Nkx2.2 was also able to occupy the NeuroD1 promoter in the βTC6 cell line (data not shown).

Two Distinct Promoter Regions Independently Contribute to NeuroD1 Activation by Nkx2.2—We used in silico DNA sequence analysis to identify three potential Nkx2.2 consensus binding sites (31) within the conserved regions of the 2.2-kb NeuroD1 minimal promoter. We identified a single putative Nkx2.2 consensus site (Site 1) at −837 bp, just upstream of the three characterized Ngn3-bound E-box elements, that is conserved in both the mouse and zebrafish promoters (21). We also identified two sites (Site 2 and Site 3) in a 182-bp region (−406 to −224 bp) between the transcriptional start site and the E-box elements. To determine which of the putative Nkx2.2 consensus elements were responsible for Nkx2.2-dependent activation we bisected the NeuroD1 promoter into two parts, each containing the requisite number of E-box elements for regulation by Ngn3 and either the proximal (Site 2 and Site 3) or distal (Site 1) putative Nkx2.2 consensus sites (NDΔ2 and NDΔ3, respectively). Each promoter deletion was introduced into Panc1 cells to assess Nkx2.2- and/or Ngn3-dependent activation. Interestingly, both the proximal 686-bp region (NDΔ2) and the distal 1949-bp region (NDΔ3) could be independently activated by Nkx2.2, with additive activation in the presence of Ngn3 (Fig. 4A). The only DNA shared between NDΔ2 and NDΔ3 is the Ngn3-regulated −686-bp to −240-bp region containing Ebox2 and Ebox3. These results suggest that Nkx2.2 regulates NeuroD1 transcription through two distinct promoter elements. Ngn3 may cooperate with Nkx2.2 activity on both the distal and proximal NeuroD1 promoter regions, independently.

We next determined whether Nkx2.2 regulation was dependent on the Ngn3-mediated activation. Huang and colleagues determined that elimination of two of the three E-box elements significantly diminishes NeuroD1 promoter activation by Ngn3 (21). Consistent with this finding, when we deleted Ebox2 and Ebox3 in the context of the full-length 2.2-kb NeuroD1 promoter (NDΔ1), we abrogated activation by Ngn3. Nkx2.2-mediated activation, however, was not affected. Furthermore, when we deleted the E-box sequences from either the proximal (NDΔ4) or distal (NDΔ5) promoter elements, Nkx2.2-mediated activation was retained at levels comparable to that of the full-length promoter (Fig. 4B). This would suggest that each promoter element can confer maximum NeuroD1 promoter activity in Panc1 cells that ectopically express Nkx2.2 (see “Discussion”). Deletion of Site 1 from the distal promoter element, NDΔ5-site1mut, resulted in a reduction of Nkx2.2 activation to levels similar to that seen on the pGL3Basic vector alone (Fig. 4C), suggesting that Site 1 is responsible for activation by Nkx2.2 on the distal NeuroD1 promoter in Panc1 cells.

To assess the regulation of the individual NeuroD1 promoter elements in β cells, we determined promoter activities of the different promoter deletions in βTC6 cells, which express endogenous Nkx2.2 and NeuroD1. Deletion of each proximal or distal promoter region, alone or in combination with deletion of the E-box regions, resulted in a significant reduction of NeuroD1 promoter activity in the βTC6 cells (Fig. 5). This would suggest that both the proximal and distal promoter regions contain regulatory elements that contribute to the full activity of NeuroD1 in vitro. Interestingly, deletion of the Nkx2.2 consensus core 4 bp (AAGT) within Site 1 (NDfull-site1mut) reduced full-length activity to levels similar to that of complete distal region deletion (NDΔ2, Fig. 5). The Site 1 Nkx2.2 core sequence deletion within NDΔ5 (NDΔ5-site1mut) also completely abrogated promoter activity (Fig. 5). These experiments
suggest that Site 1 plays a predominant role within the regulation of the NeuroD1 proximal promoter and is important for full NeuroD1 activity in βTC6 cells.

*Nkx2.2 Directly Binds to a Subset of Consensus Elements within the NeuroD1 Promoter*—To verify that Nkx2.2 bound the predicted Nkx2.2 consensus elements within the NeuroD1 promoter, we used electrophoretic mobility shift assays (EMSA) with in vitro translated Nkx2.2 or islet cell nuclear extracts, combined with anti-Nkx2.2 antibody to detect the presence of Nkx2.2 within the bound protein complex (Fig. 6 and supplemental Fig. S5). We used the previously published Nkx2.2 consensus binding element as a positive control in all assays (31) (Fig. 6A). Nkx2.2 bound specifically to the Site 1 sequence as shown by incubation with in vitro translated

---

**FIGURE 4. Nkx2.2 activates two separate regions of the NeuroD1 promoter.** Panc1 cells were transfected with the full-length 2.2-kb NeuroD1 promoter region or NeuroD1 promoter deletion constructs and Nkx2.2, Ngn3, or Nkx2.2 and Ngn3, in combination. The NeuroD1 promoter constructs are denoted as follows: (A–C) NDfull (2.2 kb (21)), (A) NDΔ1 (deletion between −686 and −240 bp), NDΔ2 (deletion between −2187 and −686 bp), NDΔ3 (deletion between −240 and −113 bp), (B) NDΔ4 (deletion between −2187 and −240 bp), (B, C) NDΔ5 (deletion between −686 and −113 bp), and (C) NDΔ5-Nk1mut (lacking the Nkx2.2 consensus site). Potential Nkx2.2 binding sites are represented by circles, and E boxes are represented by rectangles. Statistical analysis was performed by using Student’s t tests comparing addition of transcription factor(s) to promoter-alone values. **, *p value < 0.01.
Nkx2.2 and by supershift of the complexes formed from βTC6 nuclear extracts with Nkx2.2 antibodies (Fig. 6B, lanes 3–5). Nkx2.2 from αTC1 nuclear extracts also formed a complex with Site 1 (data not shown). Surprisingly, Nkx2.2 failed to bind the Site 2 or Site 3 sequences in EMSA reactions containing either in vitro translated Nkx2.2 or pancreatic nuclear extracts (supplemental Fig. S5, A–C). For each DNA binding probe, slower migrating complexes were detected using cell extracts; however, Nkx2.2 did not appear to be part of the complexes, as evidenced by the lack of a supershift with Nkx2.2 antibody (supplemental Fig. S5A, lane 4, and supplemental Fig. S5C, lane 4). Furthermore, similar complexes assembled on these sites using the Panc1 extracts, which lack Nkx2.2 (supplemental Fig. S5A, lane 5, and supplemental Fig. S5C, lane 1).

Transcription factors often need supporting proteins/cofactors bound to neighboring binding sites to aid in the recruitment to specific DNA motifs (47–49). Although Nkx2.2 binds to its consensus site in the absence of other proteins, the Site 2 and Site 3 probes may comprise sequences that necessitate the binding of cofactor proteins to the flanking DNA to facilitate Nkx2.2 binding. To test this possibility we generated a longer DNA probe containing additional sequences flanking Site 2 or Site 3 (NDprox, −240 to +90 bp, supplemental Fig. S3). In each of the nuclear extracts larger protein complexes were bound to the NDprox probe than seen with the individual sites; however, none of the complexes appeared to contain Nkx2.2 (supplemental Fig. S6, A and B).

Although there were no other apparent Nkx2.2 consensus sites within the proximal promoter region, it was possible that Nkx2.2 was binding to a cryptic or previously uncharacterized DNA binding site. We generated two additional EMSA probes that covered the remainder of the untested DNA sequences present in the NeuroD1 proximal promoter (NDΔ2): NDE1 (−693 to −450 bp) and NDE2 (−476 to −231 bp, encompassing E-box elements (supplemental Fig. S3). In each case, in vitro translated Nkx2.2 did not bind to sequences within the NDE1 and NDE2 EMSA probes, and Nkx2.2 was not present in the protein complexes formed on these sites from pancreatic nuclear extracts (supplemental Fig. S6, C and D; data not shown). In summary, our data suggest that, although Nkx2.2 regulates both the proximal and distal NeuroD1 promoter elements, Nkx2.2 only functions directly through the distal Site 1 and does not appear to bind directly to either Site 2 or Site 3, and is not recruited within the proximal promoter region.

DNA Footprint Analysis of the Proximal Site 2- and Site 3-Containing NeuroD1 Promoter—In an effort to identify transcription factors that may function downstream of Nkx2.2 to regulate the proximal (NDΔ2) NeuroD1 promoter, we performed in vivo footprinting analysis of the 686-bp promoter region in αTC1, βTC6, and mPAC L20 cell lines. Similar to the Panc1 cell line, the mPAC L20 cells are a pancreatic ductal epithelial cell line that does not express endogenous Nkx2.2 (15). To identify regions of this minimally regulated NeuroD1 promoter element that are differentially occupied in response to Nkx2.2, we focused our attention on in vivo footprinting

FIGURE 5. Two Nkx2.2-regulated NeuroD1 promoter regions are important for maximal activity in β cells. βTC6 cells were transfected with luciferase promoter constructs NDfull, NDΔ1–5, and two constructs that harbored Site 1 consensus core sequence deletions: NDfull-Nk1mut and NDΔ5-Nk1mut. Potential Nkx2.2 binding sites are represented by circles, and E boxes are represented by rectangles. Statistical analysis was performed by using Student’s t tests comparing each construct to NDfull activity or between bracketed constructs. *, p value < 0.05.
sequences that differed between the αTC1 and βTC6 cell lines in comparison to the mPAC L20 cell line. In agreement with our EMSA results, there is no obvious evidence of binding on either DNA strand for Site 2 or Site 3 (supplemental Fig. S7, A and B, and data not shown). Strongly protected areas that are specific to the αTC1 and βTC6 cells were observed upstream of the Site 2 (fp1) and between the Site 2/Site 3 (fp2) (supplemental Fig. S7, A and B). Three additional strongly protected regions that were specific to αTC1 and βTC6 cells were observed upstream from Site 2 and Site 3 (fp3−5) (supplemental Fig. S7, A and B).

In silico analysis of the protected regions using MatInspector (Genomatix) definitions for possible transcription factor binding sites identified several transcription factors as possible regulators of NeuroD1. A predicted binding site for MyT1, a known pancreatic transcription factor (50), was identified within the fp3 region. Putative binding sites for IA1 and HNF4, two additional well characterized pancreatic regulatory factors (51–53), are coincident with the fp4 region, as were possible binding sites for two generally expressed regulatory factors, Sp1 and GABP. The fp5 region also contained a second Sp1 site. The fp4 region includes a potential binding site for an uncharacterized zinc finger protein Znf202, which was not pursued due to lack of information about this protein. To determine whether these factors are able to bind the NeuroD1 promoter, we generated probes specific to either fp1/2/3 or fp4/5 for EMSA analysis. We verified that Sp1 can bind the fp1/2/3 region; however, it appeared to bind the region in the presence or absence of Nkx2.2 (mPAC cells versus αTC and βTC cells; supplemental Fig. S8A). Sp1 did not bind DNA within the fp4/5 region (supplemental Fig. S8B). The remaining factors HNF4, IA1, MyT1, and GABP did not bind to either footprinted region (supplemental Fig. S8C).

In summary, the in vivo footprint data suggest that different protein complexes are able to bind the proximal NeuroD1 promoter region that is regulated by Nkx2.2; however, we have definitively ruled out that Nkx2.2 binds directly to the Site 2 and Site 3 within this region. Unfortunately, we have been unable to identify regulatory factors that function downstream of Nkx2.2 to regulate the NeuroD1 proximal promoter. Future proteomics studies of the protected regions will help identify these binding factors and elucidate the precise mechanism through which Nkx2.2 functions to regulate the proximal region of the NeuroD1 promoter.

DISCUSSION

In this study we have demonstrated that Nkx2.2 is necessary for full activation of NeuroD1 expression in vivo and is necessary and sufficient for activation of NeuroD1 in vitro. The initial observation that NeuroD1 was reduced in the Nkx2.2 null islet was not surprising; many of the cell types thought to normally express NeuroD1 were absent in the Nkx2.2 null mice. However, further examination of NeuroD1 expression in the Nkx2.2 null background revealed that there was not an overall loss of NeuroD1+ cell numbers, but rather a general decrease in NeuroD1 expression levels per cell, regardless of cell type identity. These results suggested that Nkx2.2 plays an important role in the regulation of NeuroD1 expression. Our subsequent analyses indicated that Nkx2.2 is necessary for full activation of NeuroD1 during mouse and zebrafish pancreas development. Furthermore, the in vitro studies in immortalized cell lines identified cooperative activation of the endogenous NeuroD1 gene and a minimal NeuroD1 promoter fragment by Nkx2.2 and Ngn3. Interestingly, the cooperative regulation of NeuroD1 expression is more pronounced with the endogenous NeuroD1 promoter. Ngn3 has previously been shown to directly activate both the Nkx2.2 and NeuroD1 promoters (21, 32). All three factors are co-expressed in the pancreatic progenitor population; however, Ngn3 expression is extinguished once the progenitor cells differentiate into the hormone producing populations. It is therefore possible that Ngn3 and Nkx2.2 cooperate to induce NeuroD1 expression in the progenitor population and then Nkx2.2 contributes to the maintenance of NeuroD1 expression in the hormone-producing endocrine cell populations.

Experimental evidence suggests that Nkx2.2 is required for the maintenance of β cell function; the expression of a dominant repressor derivative of Nkx2.2 results in adult β cell dysfunction and decreased insulin gene expression (29). Our finding that Nkx2.2 is required for full activation of NeuroD1 in mature β cells suggests that Nkx2.2 may function through NeuroD1 to regulate insulin expression and β cell function. Further support for the Nkx2.2-dependent activation of NeuroD1 is the absence of NeuroD1 from the

FIGURE 6. Nkx2.2 directly binds Site 1 DNA in the distal NeuroD1 promoter. The positive Nkx2.2 binding control reaction with known consensus probe (NkBDctl) was assessed for nuclear extract and in vitro Nkx2.2 binding (A). Nkx2.2 protein binding to consensus site Site 1 (B) was assessed by EMSA analysis performed with in vitro translated Nkx2.2 (lane 3) or DNA binding mutant Nkx2.2 (lane 2). Specificity of any protein-DNA complex formed from βTC6 nuclear extract (lanes 4 and 5) was assayed by incubation with anti-Nkx2.2 antibody (lane 5). The Nkx2.2 antibody incubation resulted in a supershift of the Nkx2.2 containing complex (lane 5). TNT, in vitro translated; NE, nuclear extract.
somatostatin-producing population, which also lack endogenous Nkx2.2.

Nkx2.2 expression is also maintained in many of the newly differentiated hormone-producing cell types that do not express NeuroD1, suggesting that, although Ngn3 and Nkx2.2 are capable of activating NeuroD1, other regulatory factors may counter their activity to repress NeuroD1 in the newly differentiated and some islet cell populations. Although it was surprising that NeuroD1 was not expressed in the immature insulin-producing population, the lack of expression is consistent with the NeuroD1 null phenotype, which is not manifested in β cells until late in gestation.

Consistent with the idea that other pancreatic transcription factors may participate in the regulation of NeuroD1, we observed differences in NeuroD1 promoter activities in Panc1 cells versus βTC6 cells. In Panc1 cells transfected with Nkx2.2, the proximal or distal promoter elements can each individually confer full promoter activity (Figs. 4, NDAΔ4 and NDAΔ5). Alternatively, in βTC6 cells, each of these promoter elements is only able to confer partial activity (Figs. 5, NDAΔ4 and NDAΔ5). In addition, the two promoter elements together show an additive effect in their regulation of NeuroD1 expression in βTC6 cells and not Panc1 cells (Figs. 4 and Fig. 5, NDAΔ1). This may suggest that additional factors are present in βTC6 cell, which are not expressed in Panc1 cells, that are important for the appropriate modulation of NeuroD1 promoter activity and influence the ability of Nkx2.2 to activate NeuroD1 transcription through either the distal and proximal promoter elements.

Interestingly, the regulation of the minimal NeuroD1 promoter by Nkx2.2 in immortalized cell lines appears to result from direct and indirect transcriptional activation. Using in vitro and in vivo assays, we were able to demonstrate that Nkx2.2 binds to and activates a consensus element at −837 bp of the NeuroD1 promoter. Although this may be the primary regulatory element through which Nkx2.2 can activate NeuroD1, we also demonstrated that Nkx2.2 can activate NeuroD1 through a more proximal promoter element (−1 to −686 bp). Comprehensive analysis of this region failed to identify sequences that were directly bound by Nkx2.2. Because Nkx2.2 DNA binding activity appears to be necessary for NeuroD1 activation through the proximal promoter, our data suggest that Nkx2.2 regulates one or more members of a protein complex that functions downstream of Nkx2.2 to modulate NeuroD1 activity. Indeed, in vivo footprint analyses defined regions of the proximal NeuroD1 promoter that are differentially occupied in the presence of Nkx2.2; however, the precise regulatory factors that are bound to the NeuroD1 proximal promoter have yet to be identified. Interestingly, the cooperative regulation of NeuroD1 by Nkx2.2 and Ngn3 appears to occur with directly bound Nkx2.2 as well as with factors downstream of Nkx2.2 that apparently bind the −240-bp promoter region. Taken together, Ngn3, Nkx2.2, Sp1, and other yet unknown novel factors likely form a large transcriptional complex on the NeuroD1 promoter that will tightly regulate any transcriptional output. Future proteomic analysis of these protected regions will be explored to identify the transcriptional regulatory factors that function downstream of Nkx2.2 to regulate NeuroD1 activity. It is possible that the bound protein complexes will be cell type-dependent and may provide the cell type-specific modulation of NeuroD1 regulation in different cellular contexts. Notably, the presence of two Nkx2.2-dependent regulatory elements within the NeuroD1 promoter defines the importance of transcriptional NeuroD1 regulation by Nkx2.2.

Acknowledgments—We thank Dr. Jackie Lee (Geront) for providing the pCS2:NeuroD1 2.1-kb promoter plasmid, Michelle Doyle (University of Colorado Health Science Center) for generating the Nkx2.2 binding mutant, and Ann Shim for contributing mRNA from pRAV and pRAV:Nkx2.2 cells. We also thank Jonathon Hill for identifying the Nkx2.2 binding site in the distal NeuroD1 promoter fragment and members of the Sussel laboratory for critical reading of the manuscript. We are greatly indebted to Dr. Lee Niswander for the use of her confocal microscope and allowing K. A. to work in her laboratory space.

REFERENCES

1. Pictet, R., and Rutter, W. J. (1972) Development of Embryonic Endocrine Pancreas, pp. 26–28, Williams & Wilkins, Washington, DC
2. Murtaugh, L. C. (2007) Development 134, 427–438
3. Jørgensen, M. C., Ahnfelt-Rønne, J., Hald, J., Madsen, O. D., Serup, P., and Hecksher-Sørensen, J. (2007) Endocr. Rev. 28, 685–705
4. Collombat, P., Hecksher-Sørensen, J., Serup, P., and Mansouri, A. (2006) Mech. dev. 123, 501–512
5. Habener, J. F., Kemp, D. M., and Thomas, M. K. (2005) Endocrinology 146, 1025–1034
6. Kemp, D. M., Thomas, M. K., and Habener, J. F. (2003) Rev. Endocr. Metab. Disorder 4, 5–17
7. Schwitzgebel, V. M. (2001) Mol. Cell. Endocrinol. 185, 99–108
8. Collombat, P., Hecksher-Sørensen, J., Broccoli, V., Krull, J., Ponte, I., Munding, T., Smith, J., Gruss, P., Serup, P., and Mansouri, A. (2005) Development 132, 2969–2980
9. Gradwohl, G., Dierich, A., LeMeur, M., and Guillemot, F. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 1607–1611
10. Prado, C. L., Pugh-Bernard, A. E., Elghazi, L., Sosa-Pineda, B., and Sussel, L. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 2924–2929
11. Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J., and Melton, D. A. (2008) Nature 455, 627–632
12. Johansson, K. A., Dursun, U., Jordan, N., Gu, G., Beermann, F., Gradwohl, G., and Grapin-Botton, A. (2007) Dev. Cell 12, 457–465
13. Huang, H. P., Chu, K., Nemoz-Gaillard, E., Elberg, D., and Tsai, M. J. (2002) Mol. Endocrinol. 16, 541–551
14. Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., deMayo, F. J., Leiter, A. B., and Tsai, M. J. (1997) Genes Dev. 11, 2323–2334
15. Gasa, R., Mrejen, C., Leachman, N., Otten, M., Barnes, M., Wang, J., Chakrabarti, S., Mirmira, R., and German, M. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 13245–13250
16. Kim, W. Y., Fritzsch, B., Serls, A., Bakel, L. A., Huang, E. I., Reichardt, L. F., Barth, D. S., and Lee, J. E. (2001) Development 128, 417–426
17. Liu, M., Pleasure, S. J., Collins, A. E., Noebels, J. L., Naya, F. J., Tsai, M. I., and Lowenstein, D. H. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 865–870
18. Miyata, T., Maeda, T., and Lee, J. E. (1999) Genes Dev. 13, 1647–1652
19. Itkin-Ansari, P., Marcara, E., Geron, I., Tyberg, B., Demeterco, C., Hao, E., Padilla, C., Ratineau, C., Leiter, A., Lee, J. E., and Levine, F. (2005) Dev. Dyn. 233, 946–953
20. Gasa, R., Mrejen, C., Lynn, F. C., Skewes-Cox, P., Sanchez, L., Yang, K. Y., Lin, C. H., Gomis, R., and German, M. S. (2008) Differentiation 76, 381–391
21. Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M., and Tsai, M. J. (2000) Mol. Cell. Biol. 20, 3292–3307
22. Kitamura, Y. I., Kitamura, T., Kruse, J. P., Raum, J. C., Stein, R., Gu, W., and Accili, D. (2005) Cell Metab. 2, 153–163
23. Andrali, S. S., Sampley, M. L., Vanderford, N. L., and Ozcan, S. (2008) Biochem. J. 415, 1–10
24. Briscoe, J., Sussel, L., Serup, P., Hartigan-O’Connor, D., Jessell, T. M., Rubenstein, J. L., and Ericson, J. (1999) Nature 398, 622–627
25. Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J. L., and Qiu, M. (2001) Development 128, 2723–2733
26. Sussel, L., Kalamaras, J., Hartigan-O’Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L., and German, M. S. (1998) Development 125, 2213–2221
27. Cissell, M. A., Zhao, L., Sussel, L., Henderson, E., and Stein, R. (2003) J. Biol. Chem. 278, 751–756
28. Doyle, M. J., Loomis, Z. L., and Sussel, L. (2007) Development 134, 515–523
29. Doyle, M. J., and Sussel, L. (2007) Diabetes 56, 1999–2007
30. Raum, J. C., Gerrish, K., Artner, I., Henderson, E., Guo, M., Sussel, L., Schisler, J. C., Newgard, C. B., and Stein, R. (2006) Mol. Cell. Biol. 26, 5735–5743
31. Watada, H., Mirzam, R. G., Kalamaras, J., and German, M. S. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 9443–9448
32. Watada, H., Scheel, D. W., Leung, J., and German, M. S. (2003) J. Biol. Chem. 278, 17130–17140
33. Chao, C. S., Loomis, Z. L., Lee, J. E., and Sussel, L. (2007) Dev. Biol. 312, 523–532
34. Westerfield, M., Doerry, E., Kirkpatrick, A. E., and Douglas, S. A. (1999) Methods Cell Biol. 60, 339–355
35. Nasevicius, A., and Ekker, S. C. (2000) Nat. Genet. 26, 216–220
36. Thisse, C., and Thisse, B. (2008) Nat. Protoc. 3, 59–69
37. Bain, J. R., Schisler, J. C., Takeuchi, K., Newgard, C. B., and Becker, T. C. (2004) Diabetes 53, 2190–2194
38. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494–498
39. Bewig, B., and Schmidt, W. E. (2000) BioTechniques 28, 870–873
40. Schisler, J. C., Jensen, P. B., Taylor, D. G., Becker, T. C., Knop, F. K., Takekawa, S., German, M., Weir, G. C., Lu, D., Mirzam, R. G., and Newgard, C. B. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 7297–7302
41. Schisler, J. C., Fueger, P. T., Babu, D. A., Hohmeier, H. E., Tessem, J. S., Lu, D., Becker, T. C., Naziruddin, B., Levy, M., Mirzam, R. G., and Newgard, C. B. (2008) Mol. Cell. Biol. 28, 3465–3476
42. Knuesel, M., Wan, Y., Xiao, Z., Holinger, E., Lowe, N., Wang, W., and Liu, X. (2003) Mol. Cell Proteomics 2, 1225–1233
43. Sigurdsson, M., Clark, D. R., Fitzsimmons, D., Doyle, M., Akerblad, P., Breslin, T., Bilke, S., Li, R., Yeamans, C., Zhang, G., and Hagman, J. (2002) Mol. Cell. Biol. 22, 8539–8551
44. Fitzsimmons, D., and Hagman, J. (1996) Curr. Opin. Immunol. 8, 166–174
45. Kasahara, H., Usheva, A., Aoki, T., Horikoshi, N., and Izumo, S. (2001) J. Biol. Chem. 276, 4570–4580
46. Paule, S., Zecchin, E., Tiso, N., Bortolussi, M., and Argenton, F. (2007) Dev. Biol. 304, 875–890
47. Hollemhorst, P. C., Shah, A. A., Hopkins, C., and Graves, B. J. (2007) Genes Dev. 21, 1882–1894
48. Maier, H., Colbert, J., Fitzsimmons, D., Clark, D. R., and Hagman, J. (2003) Mol. Cell. Biol. 23, 1946–1960
49. Melloul, D., Marshak, S., and Cerasi, E. (2002) Diabetologia 45, 309–326
50. Wang, S., Zhang, J., Zhao, A., Hipkens, S., Magnuson, M. A., and Gu, G. (2007) Mech. Dev. 124, 898–910
51. Mellitzer, G., Bonne, S., Luco, R. F., Van De Casteele, M., Lenn-Samuel, N., Collombat, P., Mansouri, A., Lee, J., Lan, M., Pipeleers, D., Nielsen, F. C., Ferrer, J., Gradwohl, G., and Heimberg, H. (2006) EMBO J. 25, 1344–1352
52. Gierl, M. S., Karoulis, N., Wende, H., Strebel, M., and Birchmeier, C. (2006) Genes Dev. 20, 2465–2478
53. Stoffel, M., and Duncan, S. A. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 13209–13214
54. Ren, B., Robert, F., Wyrick, J. J., Aparicio, O., Jennings, E. G., Simon, I., Zeitlinger, J., Schreiber, J., Hannett, N., Kanin, K., Volkert, T. L., Wilson, C. J., Bell, S. P., and Young, R. A. (2000) Science 290, 2306–2309