THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 45, pp. 31236–31248, November 6, 2009

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

Received for publication, July 28, 2009, and in revised form, September 8, 2009 Published, JBC Papers in Press, September 15, 2009, DOI 10.1074/jbc.M109.048694

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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 coordinately 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[2] 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 relocalization 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

* This work was supported, in whole or in part, by National Institutes of Health Grant U01 DK072504 (to L. S.), NIH supplement to UO1DK072504 (to C. T.), and NIH β Cell Biology Consortium Grant 5U01DK072473 (a collaborative Bridging Project, to L. S. and C. B. N.). This work was also supported by American Diabetes Association Regular Research Grant, Juvenile Diabetes Research Foundation Regular Research Grant 1-2005-188 and the Naomi Berrie Diabetes Center (to L. S.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S8 and Table S1.

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[2] The abbreviations used are: e, embryonic day; ChIP, chromatin immunoprecipitation; PP, pancreatic polypeptide; Bis-Tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; Ad, adenovirus; CMV, cytomegalovirus; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay.

(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 *Nkx2.2* 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 (UCDHSC) 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-

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-amylase (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-ghrelin (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 house 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). *nkx2.2a* morpholino sequence is as follows: 5′-GTAGGGTATACTTACATGAGTATTG-3′. Gene Tools standard control morpholino was used for control experiments. The zebrafish *nkx2.2a* 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 (*nkx2.2a*) and NotI linearization (*neuroD*). Whole mount *in situ* hybridization was performed as described previously (36). Yolks were 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 PflMI and NdeI; for NDΔ2, the −2190/−686 region was excised with SacI and PflMI; for NDΔ3 a proximal 128-bp region was excised with NdeI 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 PfmlI and MluI maintaining the most proximal 113 bp upstream of the transcriptional start site, including the endogenous TATA box. NDfull-nk1mut and NDΔ5-nk1mut 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-*Nkx2.2BDmut*, 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 passively lysed followed by analysis with the Dual-luciferase Reporter Assay (Promega) and plate luminometer, Monolight 3096 (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 difluoride membrane, and the membrane was blocked in 5% milk for 30 min, incubated with anti-Nkx2.2 (1:100, 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 (GCCACGAATTGACCAAGTGA) was used to prepare the siRNA adenovirus (Ad-*siNkx2.2*(283) 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(283), AdCMV-GFP, or Ad-siRNA control was used per transduction. Cells were harvested at 24 and 48 h post-transduction, and total RNA was recovered and assayed as described above.

pRAV:Nkx2.2 Transduction—Full-length *Nkx2.2* cDNA was cloned into the pRAV retroviral expression vector (42). High titer infectious retrovirus stocks were generated by transfecting pRAV and pRAV:*Nkx2.2* into BOSC23 cells as described previously (42). NIH3T3 and PANC1 cell lines were treated with each retrovirus and harvested 48 h post-transduction. Total RNA was recovered and assayed as described above.

ChIP—Pancreas tissue was collected from 45 e13.5 mouse embryos. Pooled pancreata was cross-linked for 10 min at room temperature in 1% formaldehyde followed by glycine quenching for 5 min at room temperature. All 45 pancreata were pooled and Dounce-homogenized, and cells were lysed in 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 10% Nonidet P-40 to release the nuclei. Nuclei were collected and lysed in 1% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 8.1. Chromatin fragmentation was performed using a BioRuptor (Diagenode) that sonicated samples on high for 10 min total with 30-s on and 30-s off intervals. A 12.5% fraction of the chromatin was set aside to verify the fragmentation range of 200 bp to 1 kb. Cross-linked, fragmented chromatin was diluted with 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.0, 167 mM NaCl, and pre-cleared with protein A/G-agarose beads (Santa Cruz Biotechnology) prior to splitting into two samples for mouse IgG2b-kappa control (4 μg, Abcam) and anti-Nkx2.2 (25 μl, DSHB) overnight immunoprecipitations at 4 °C. Washes and elutions of immunoprecipitates were carried out as previously described (27, 30). Two rounds of ligation-mediated PCR were performed with half of each initial immunoprecipitated sample. Briefly, immunoprecipitated chromatin was blunt-ended with T4 DNA polymerase (New England Biolabs), annealed linkers were ligated at 16 °C overnight, first round of amplification with linker primers (OJW102/OJW103) was 20 cycles of PCR, and starting with 50 ng of first round DNA per sample the second round of amplification also included 20 cycles of PCR (Ren *et al.* (54)). After ligation-mediated PCR amplification, PCR reactions using PuReTaq Ready-to-go PCR beads (Amersham Biosciences) were setup for the *MafA* region 3, and *Ins2* −378/−46 promoter regions, as previously described (27, 30) as well as the *NeuroD1* promoter (−400/−23-bp) primers (supplemental Table S1, 28 cycles, annealing temperature = 65 °C). PCR products were resolved by electrophoresis on 1.5% agarose gels. ChIP signal-to-input ratios were determined based on band intensity for each promoter analyzed.

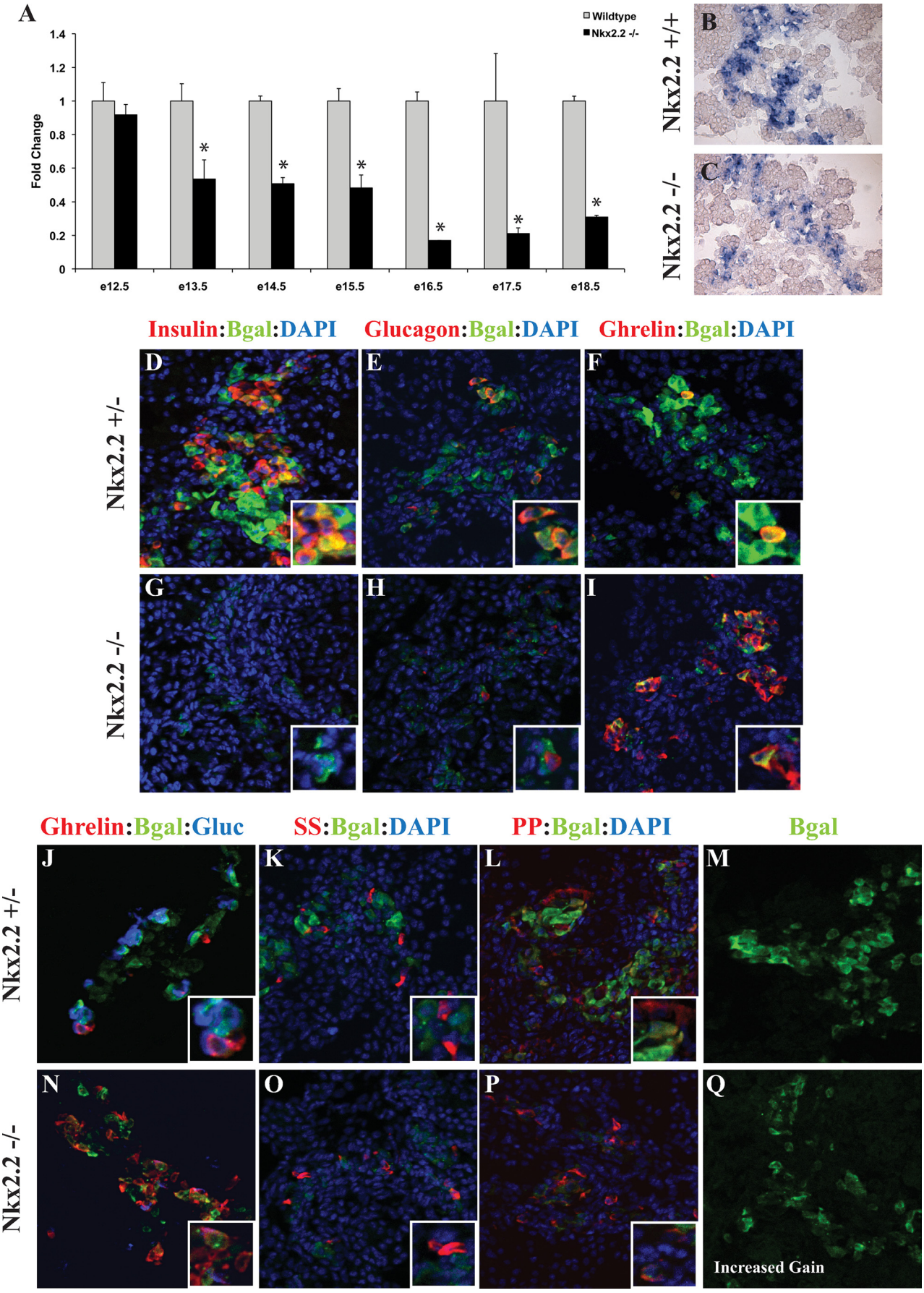
EMSA—Nuclear protein lysates from Panc1, αTC1, and βTC6 cells were prepared using the Nuclear Extract Kit (Active Motif). Electrophoretic mobility shift assay (EMSA) reactions involving nuclear extracts contained either 5 or 15 μg of protein. *In vitro* translated proteins were transcribed initially from pcDNA3-*Nkx2.2* or pcDNA3-*Nkx2.2DBDmut* using the TnT Coupled Reticulocyte Lysate System (Promega), and protein synthesis was verified by Western blotting for Nkx2.2 (data not shown). For EMSA reactions using *in vitro* translated protein, 5 μl from the reticulocyte reaction was used. EMSA experiments were adapted from previous studies (43, 44). EMSA probes were radioactively end-labeled using Klenow polymerase (New England Biolabs) to incorporate [α-^{32}P]dCTP and 25,000 cpm of each probe included per EMSA reaction. EMSA probe sequences were designed as listed in supplemental Table S1. EMSA reactions were incubated at 4 °C for 30 min and included the following conditions: 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM dithiothreitol. For Nkx2.2 supershifts, 1 μl of monoclonal ascites antibody (DSHB) was used per reaction. The *Nkx2.2* DBD mutant was generated using site-directed mutagenesis (QuikChange mutagenesis kit, Stratagene) to change a lysine 184 to isoleucine. The mutation was modeled after the corresponding mutation in Nkx2.5 (L183I) that was demonstrated to disrupt binding of the homeodomain to its cognate DNA (45).

In Vivo DNA Footprinting—DNA methylation of intact mPAC L20, αTC1, and βTC6 cells using dimethyl sulfate and ligation-mediated PCR were performed as previously described (43). Briefly, mPAC L20, αTC1, and βTC6 cells were all grown to 80% confluency on two 15-cm plates each. One set of cells was harvested, and genomic DNA was isolated for *in vitro* methylation and depurination to prepare G and G+A reference ladders of the *NeuroD1* promoter region. The second set of intact cells was treated with 0.1% dimethyl sulfate, in RPMI media plus 10% fetal bovine serum, for 2 min at 37 °C prior to genomic DNA isolation, and cleavage with piperidine. For ligation-mediated PCR of the proximal *NeuroD1* promoter region, 250 ng of *in vivo* methylated DNA and control methylated DNA were used with the NDprox sense strand footprinting primer set described in supplemental Table S1 and the following conditions: first-strand synthesis, 1 cycle; annealing temperature, 63 °C; amplification, 20 cycles; annealing temperature, 65.9 °C with LMPCR1 primer; labeling, 2 cycles, and annealing temperature, 70.2 °C. Amplified and labeled products were resolved by electrophoresis on 6% denaturing polyacrylamide gels and detected on x-ray film (Kodak) as well as by using a Molecular Dynamics Storm 960 PhosphorImager (Amersham Biosciences).

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. 1*A*). 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


A
Wildtype
Nkx2.2 -/-
Fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
e12.5
e13.5
e14.5
e15.5
e16.5
e17.5
e18.5
B
Nkx2.2 +/+
C
Nkx2.2 -/-
Insulin:Bgal:DAPI
Glucagon:Bgal:DAPI
Ghrelin:Bgal:DAPI
D
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I
Nkx2.2 -/-
Ghrelin:Bgal:Gluc
SS:Bgal:DAPI
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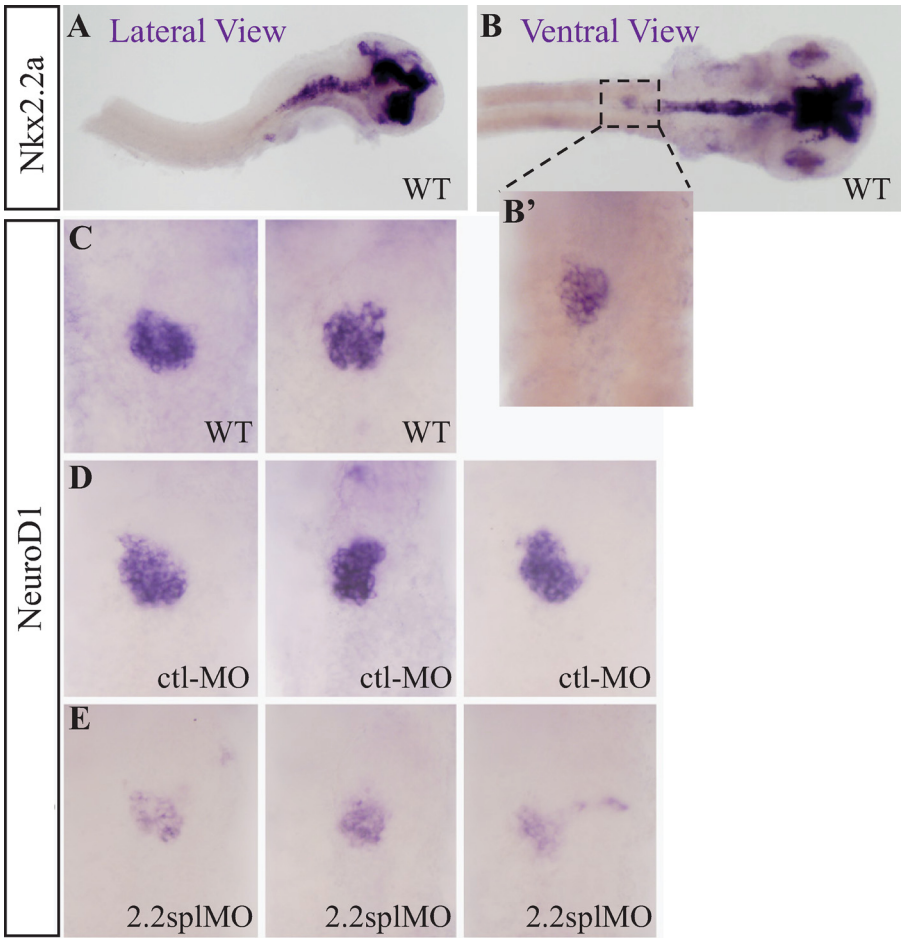

FIGURE 2. **Nkx2.2 regulation of *NeuroD1* expression is conserved in zebrafish.** *In situ* hybridization of wild-type 48-hours postfertilization zebrafish embryos for *Nkx2.2a* (*A*, *B*, and *B'*). *In situ* hybridization of 48-hours postfertilization zebrafish embryonic pancreas for *NeuroD1* (*C–E*) showing a decrease in *NeuroD1* expression in *Nkx2.2* splice morphant embryos (*E*) and either wild-type (*C*) or control morphant embryos (*D*).

type. In e14.5 wild-type mice, β-galactosidase staining shows that *NeuroD1* is predominantly expressed in glucagon- and ghrelin-producing cells, but is largely excluded from the other hormone producing populations, including the immature β cells (supplemental Fig. S1). By e16.5, 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. 2*E*).

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-trans-

FIGURE 1. ***NeuroD1* expression is decreased in the *Nkx2.2* null pancreas.** Shown is quantitative PCR analysis of *NeuroD1* mRNA levels at successive developmental time points during pancreas organogenesis. $Nkx2.2^{-/-}$ levels (*black bars*) are represented by -fold change in comparison to wild type (*A*). Statistical significance determined with a Student *t* test comparing each individual embryonic age group; *, *p* value <0.05. *Error bars* represent ±S.E. RNA *in situ* hybridization comparing *NeuroD1* mRNA expression in e15.5 wild-type (*B*) and $Nkx2.2^{-/-}$ pancreata (*C*). Immunofluorescence staining of embryonic pancreata in e16.5 $Nkx2.2^{+/-}$;$NeuroD1^{+/lacZ}$ (*D–F* and *J–M*) and $Nkx2.2^{-/-}$;$NeuroD1^{+/lacZ}$ (*G–I* and *N–Q*). *NeuroD1* expression is represented by β-galactosidase staining (*green*, *D–Q*). Insulin (*red*, *D* and *G*), glucagon (*red*, *E* and *H*; *blue*, *J* and *N*), ghrelin (*red*, *F*, *I*, *J*, and *N*), PP (*red*, *L* and *P*), and somatostatin (*red*, *K* and *O*) are shown (confocal images: magnification, 45×; *insets*: magnification, 90×). β-Galactosidase expression in the *Nkx2.2* null tissue was captured with a gain setting 3-fold above control samples to demonstrate expression is maintained, but at decreased levels (compare *Q* to *M*).

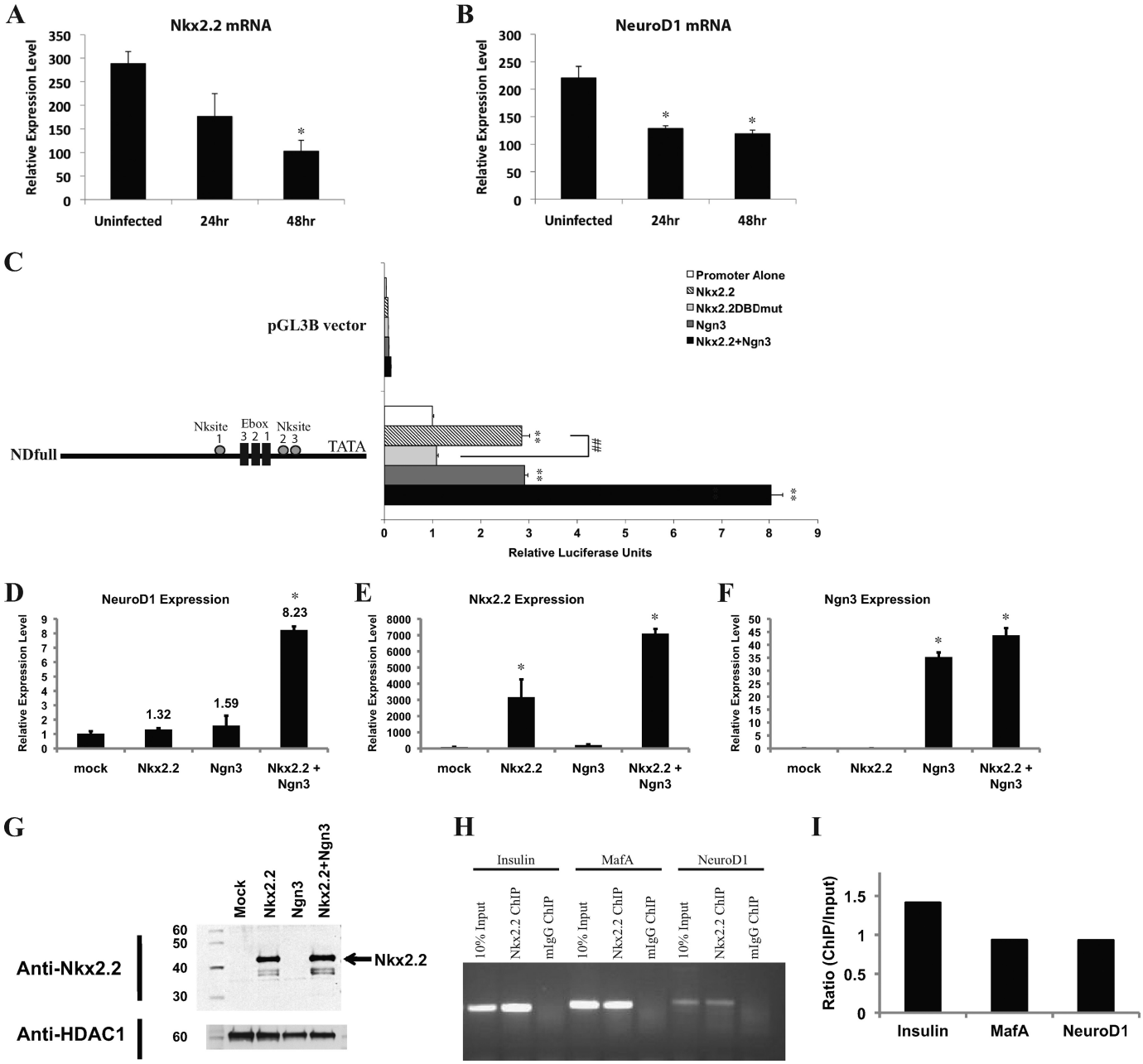

FIGURE 3. **Nkx2.2 regulates endogenous *NeuroD1* and a minimal *NeuroD1* promoter.** βTC6 cells were transduced with adenovirus containing an siRNA(283) directed against *Nkx2.2*. Endogenous *Nkx2.2* mRNA expression levels (*A*) and *NeuroD1* mRNA expression levels (*B*) were measured at 48 and 72 h post transduction. Values were normalized to cyclophilin B. *Asterisks* denote statistically significant decreases between the treated and untreated samples as assessed by Student's *t* test: *, $p < 0.05$. Panc1 cells were transfected with either pGL3B vector alone or pGL3B containing the 2.2-kb *NeuroD1* promoter and *Nkx2.2*, the *Nkx2.2* binding site mutant (Nkx2.2DBDmut), *Ngn3*, or *Nkx2.2* and *Ngn3*, in combination (*C*). Sites are represented by *circles*, and E-box elements by are represented by *rectangles*. Values are normalized to *Renilla* luciferase. *Asterisks* denote statistically significant changes in luciferase activity upon addition of a regulatory factor as assessed by a student *t* test: **, $p < 0.01$; ##, $p < 0.01$ for Nkx2.2 compared with Nkx2.2DBDmut. Alterations in endogenous *NeuroD1* mRNA levels were determined (*D*). Nkx2.2 protein (*G*) and mRNA (*E*) levels were assessed, as were *Ngn3* mRNA levels (*F*). *Asterisks* denote statistically significant changes in expression levels: *, $p < 0.05$. Formaldehyde cross-linked e13.5 chromatin was incubated with antibodies specific to Nkx2.2. Immunoprecipitated DNA was analyzed by PCR with primers specific to *NeuroD1*, insulin, and *MafA* (*H*). For controls, PCR was performed with total input DNA (1/10 dilution, *Input*) and DNA immunoprecipitated with mouse IgG (*mIgG*). Ratios between ChIP and 10% input were compared between promoter primer sets in the ChIP experiment shown here (*I*).

duction (Fig. 3*A*) and resulted in a corresponding significant decrease in endogenous *NeuroD1* transcripts by 50% (Fig. 3*B*). 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 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 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. 3*C* and 4*A*). In addition, we observed additive activation of the *NeuroD1* promoter when *Nkx2.2* was co-transfected with *Ngn3* (Figs. 3*C* and 4*A*). Similarly, endogenous *NeuroD1* mRNA expression was activated when *Nkx2.2* was transfected or virally transduced into Panc1 or NIH3T3 cell lines (Fig. 3*D* and supplemental Fig. S4). Furthermore, co-expression of *Nkx2.2* and *Ngn3* resulted in synergistic activation of endogenous *NeuroD1* transcript (Fig. 3*D*), 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. 3*G*). However, *Nkx2.2* mRNA transcript levels do appear slightly elevated when Ngn3 is present (Fig. 3*E*), which is consistent with previous studies (31). Conversely, *Ngn3* mRNA transcript levels are not affected by the expression of *Nkx2.2* (Fig. 3*F*). 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. 3*C*), 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* promoter 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. 3*H*, 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. 3*I*). 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. 4*A*). 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. 4*B*). 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. 4*C*), 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

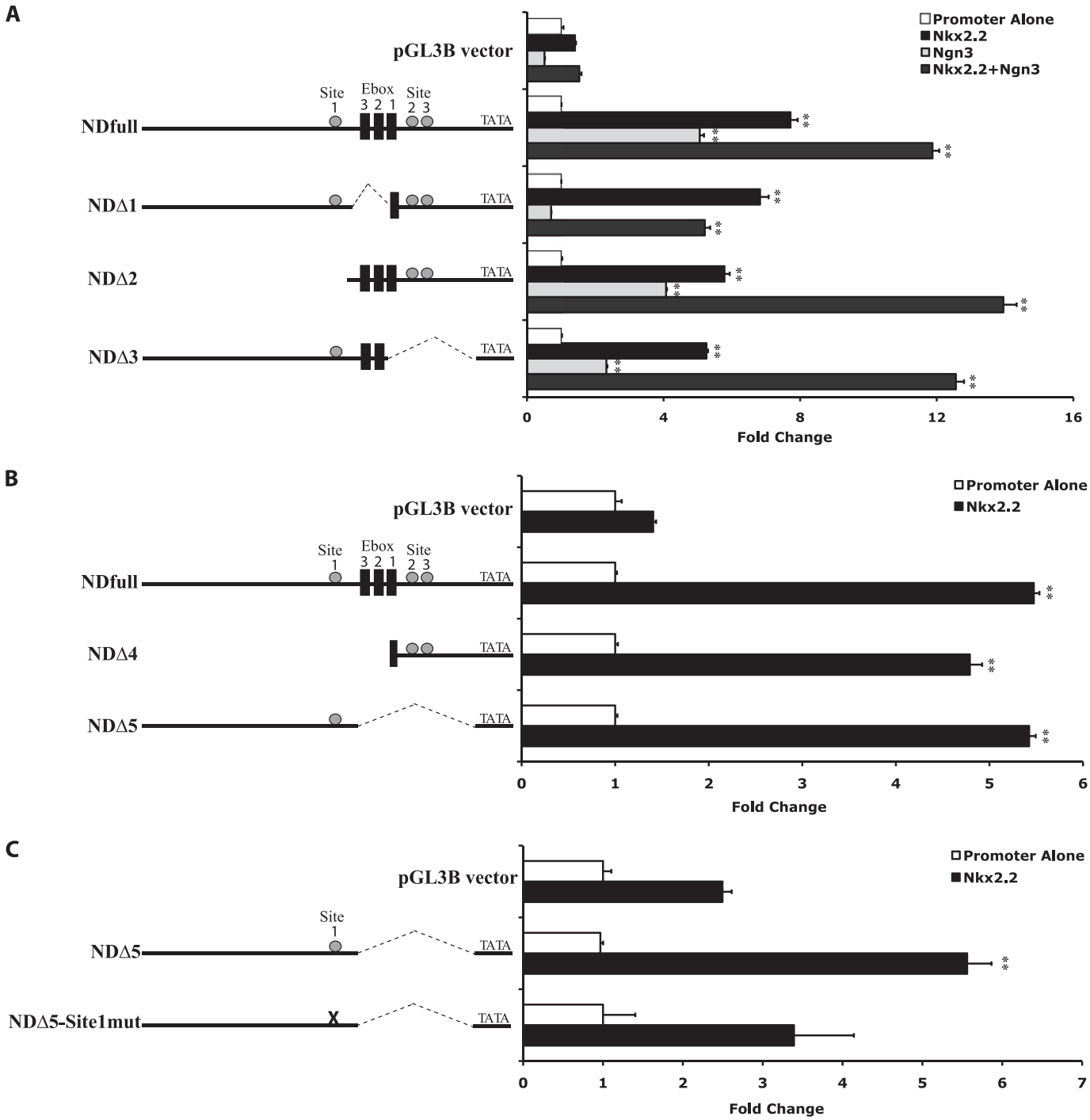

FIGURE 4. **Nkx2.2 activates two separate regions of the *NeuroD1* promoter.** Panc1 cells were transfected with the full-length 2.2-kb *NeuroD1* promoter region or *NeuroD1* promoter deletion constructs and Nkx2.2, Ngn3, or Nkx2.2 and Ngn3, in combination. The *NeuroD1* promoter constructs are denoted 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.

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. 6*A*). Nkx2.2 bound specifically to the Site 1 sequence as shown by incubation with *in vitro* translated

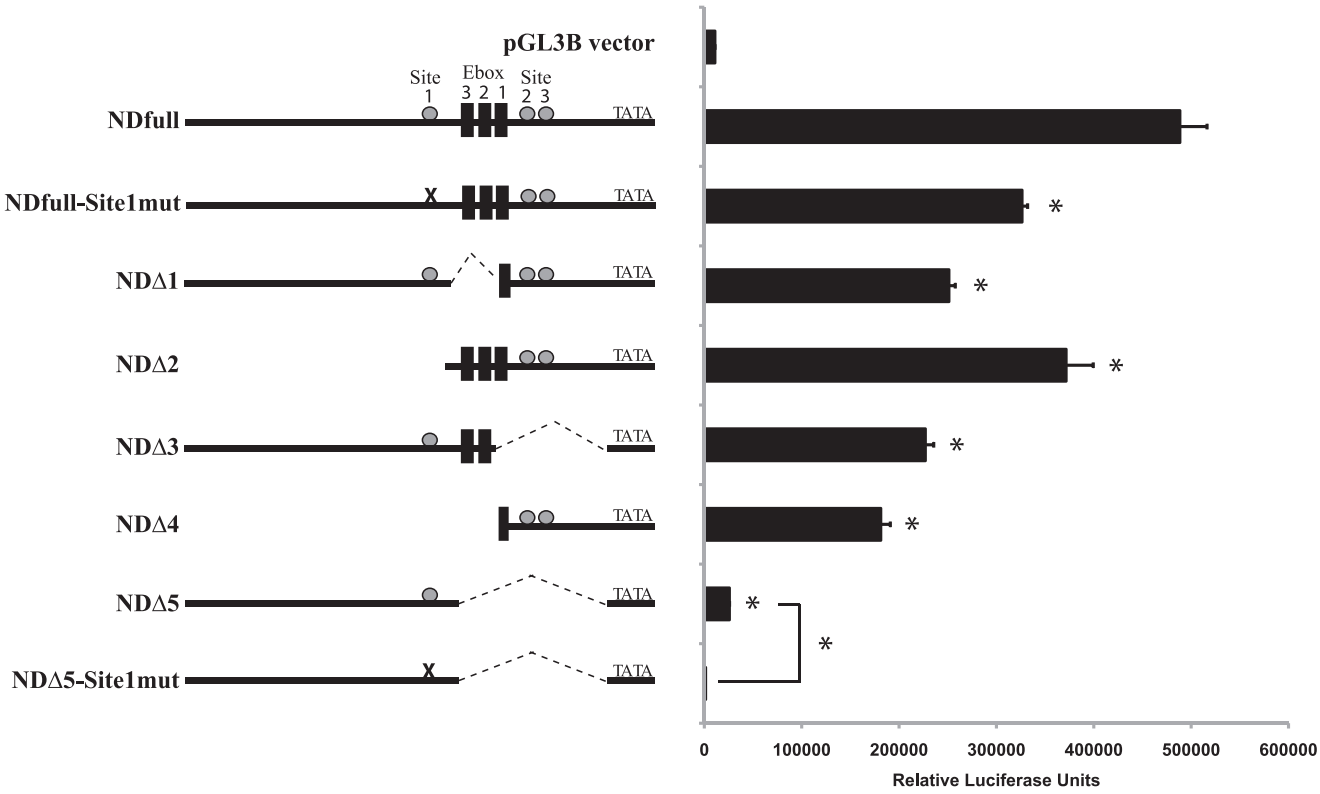

FIGURE 5. **Two Nkx2.2-regulated *NeuroD1* promoter regions are important for maximal activity in β cells.** βTC6 cells were transfected with luciferase promoter constructs NDfull, NDΔ1–5, and two constructs that harbored Site 1 consensus core sequence deletions: NDfull-Nk1mut and NDΔ5-Nk1mut. Potential 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.

Nkx2.2 and by supershift of the complexes formed from βTC6 nuclear extracts with Nkx2.2 antibodies (Fig. 6*B*, *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. S5*A*, *lane 4*, and supplemental Fig. S5*C*, *lane 4*). Furthermore, similar complexes assembled on these sites using the Panc1 extracts, which lack Nkx2.2 (supplemental Fig. S5*A*, *lane 5*, and supplemental Fig. S5*C*, *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* footprint

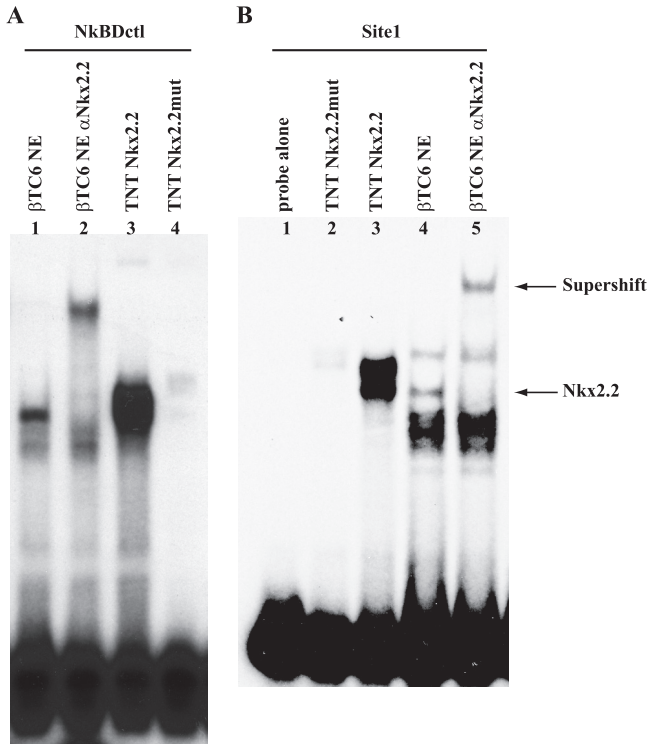

FIGURE 6. **Nkx2.2 directly binds Site 1 DNA in the distal *NeuroD1* promoter.** The positive Nkx2.2 binding control reaction with known consensus probe (*NkBDctl*) was assessed for nuclear extract and *in vitro* Nkx2.2 binding (*A*). Nkx2.2 protein binding to consensus site Site 1 (*B*) was assessed 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.

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. S8*A*). Sp1 did not bind DNA within the fp4/5 region (supplemental Fig. S8*B*). The remaining factors HNF4, IA1, MyT1, and GABP did not bind to either footprinted region (supplemental Fig. S8*C*).

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

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 (Fig. 4, *NDΔ4* and *NDΔ5*). Alternatively, in βTC6 cells, each of these promoter elements is only able to confer partial activity (Fig. 5, *NDΔ4* and *NDΔ5*). 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, *NDΔ1*). 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 the 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 (Geron) 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.

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