The C-Terminal Domain of the β Cell Homeodomain Factor Nkx6.1 Enhances Sequence-Selective DNA Binding at the *insulin* Promoter†

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ABSTRACT: The Hox-like factor Nkx6.1 is required for the formation and secretory function of insulin-producing β cells, and has the capacity to activate or repress the transcription of relevant target genes in a DNA-context dependent manner. A key determinant of transcriptional activity by Nkx6.1 may be its C terminus, which has been suggested to interfere with DNA binding. To determine how the C terminus modulates homeodomain binding, we assessed the nature of Nkx6.1−DNA interactions at the *insulin* promoter. By quantitative gel shift analysis, we demonstrate that although the C terminus of Nkx6.1 mitigates the affinity of the homeodomain for DNA slightly (about 2-fold), it enhances the selectivity of the homeodomain for TAAT DNA sequences nearly 10-fold. By reporter gene analysis, this selectivity is also functionally preserved in mammalian cells *in vivo*. Based on deletional and mutational studies, the sequence selectivity imparted by the C terminus appears to be mediated by a stretch of highly conserved residues between amino acids 318 and 338. Strikingly, these residues impart minimal changes to the secondary structure of the unbound protein as assessed by circular dichroism spectroscopy, suggesting that conformational adjustments of the homeodomain that occur upon binding to DNA may play a more important role in sequence selectivity. The C terminus of Nkx6.1 also functions in a modular fashion, as it can confer similar DNA binding properties when fused to the heterologous homeodomain of Pdx-1. Taken together, our data suggest a model whereby the Nkx6.1 C terminus may function in a regulatory manner by imposing specific functional constraints upon the protein. These constraints may serve to modulate the potential of Nkx6.1 to both recognize target genes and regulate their transcription.

The development of the mammalian pancreas is dependent upon a tightly regulated program of gene expression that is coordinated by the actions of multiple homeodomain-containing transcription factors (*1*, *2*). Targeted disruptions of the genes encoding Pdx-1, Hb9, Pax4, Nkx2.2, and Nkx6.1 in mice have elucidated the roles of these homeodomain proteins in processes beginning with initial pancreatic morphogenesis and ending with the differentiation and maintenance of distinct endocrine cell types within the pancreatic islets of Langerhans (*3*−*9*). Nkx6.1, a Hox-like transcription factor, is a crucial component in the final stages of the differentiation of insulin-producing β cells. Mice deficient for Nkx6.1 display dramatically diminished numbers of β cells within the pancreatic islets, and secrete insulin at only 2% of normal levels (*7*). In the fully differentiated β cell, Nkx6.1 appears to be necessary for the normal insulin secretory response to elevated extracellular glucose (*10*).

To promote proper differentiation and function of β cells within the pancreas, Nkx6.1 is believed to bind to specific A/T-rich DNA sequences in the promoter regions of developmentally and functionally relevant genes, and subsequently regulate transcriptional activity (*11*, *12*). In this regard, Nkx6.1 is known to contain both a transcriptional repression domain (at its N terminus) and a transcriptional activation domain (at its C terminus) (*12*, *13*). Interestingly, the ability of Nkx6.1 to repress or activate transcription appears to rely, at least in part, on the differential recognition of its homeodomain for relevant DNA sequences: repression of transcription has been observed upon binding to 5′-TAAT-3 (or 5′-ATTA-3′)-containing sequences, whereas activation is observed upon interaction with 5′-ATTT-3′-containing sequences (*12*, *13*). Precisely how the homeodomain of Nkx6.1 might recognize and bind to different DNA sequences and whether this recognition is a regulated process are unknown.

Although the homeodomain is a highly conserved protein motif of 60 amino acids, the DNA sequence recognition by different homeodomain proteins can vary considerably. For example, in the β cell Nkx2.2 interacts with high affinity to CAATGT sequences (*14*), whereas Pdx-1 and Nkx6.1 interact primarily with TAAT sequences (*11*, *12*, *15*, *16*). Thus, it has been suggested that specific amino acid differences within and even outside of the homeodomain

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Table 1: Apparent Dissociation Constants of Nkx6.1 and Pdx-1 Protein Constructs for *insulin* A3 Element DNA Probes

protein construct	short name	apparent K_D (nM)[a] wild-type A3 probe (TTAATTAC)	apparent K_D (nM)[a] mutant A3 probe (TTATTTAC)	rel affinity (WT vs MUT)[b]
(231−305)Nkx6.1	HD	1.15 ± 0.11	6.73 ± 0.66	6
(231−364)Nkx6.1	HDC	2.03 ± 0.14	71 ± 2.1	35
(231−318)Nkx6.1	HD318	2.62 ± 0.66	10 ± 0.9	4
(231−338)Nkx6.1	HD338	3.48 ± 0.25	>200	
(231−338)Nkx6.1-(E321R, E322P, D323P, D324R)	HD338mN	2.61 ± 0.96	8.69 ± 0.69	3
(138−213)Pdx-1	PdxHD	2.71 ± 0.93	29.1 ± 0.32	11
(138−283)Pdx-1	PdxHDC	1.08 ± 0.23	11.4 ± 1.32	11
(138−213)Pdx-1/(307−364)Nkx6.1 fusion protein	PdxHD-NkxC	3.71 ± 1.22	>200	

[a] Apparent K_Ds were determined from quantitative EMSA as described in Materials and Methods. For calculation of K_Ds, a minimum of 8 concentrations of protein between 2 and 200 nM were used (and EMSAs were performed on three separate occasions). However, figures only show representative shifts using fewer concentrations as indicated. [b] Relative affinity for the wild-type (WT) A3 probe compared to the mutant (MUT) A3 probe was calculated by dividing the K_D for the MUT probe by the K_D for the WT probe.

significantly impact DNA sequence recognition and, by inference, target gene selection in vivo (*17*−*19*). A clue to understanding the differential sequence recognition by Nkx6.1 may lie in prior studies examining its C-terminal region. This region, which lies outside of the homeodomain, is characterized by a stretch of acidic amino acids and was suggested to diminish the DNA binding affinity of the homeodomain (*12*). Regions that negatively impact DNA affinity have been described for other proteins; however, the biologic significance of such inhibition is largely speculative (*18*, *20*, *21*).

To elucidate the role of the Nkx6.1 C terminus in modulating DNA binding affinity and selectivity, we assessed the nature of Nkx6.1−DNA interactions at the *insulin* promoter, a potential target for Nkx6.1 regulation (*12*, *13*). Using quantitative electrophoretic mobility shift assays (EMSAs)[1] in vitro, we demonstrate that the C terminus of Nkx6.1 slightly diminishes DNA binding affinity, but substantially enhances DNA binding selectivity. The enhancement of DNA binding selectivity by this region appears to be mediated by a discrete stretch of highly conserved residues between aa 318 and 338. Strikingly, these residues impart minimal changes to the secondary structure of the unbound protein as assessed by circular dichroism (CD) spectroscopy, suggesting that conformational adjustments of the homeodomain that occur upon binding to DNA may play a more important role in sequence selectivity. Interestingly, the C terminus of Nkx6.1 appears to function in a modular fashion, as it can confer similar DNA binding properties when fused to the heterologous homeodomain of Pdx-1. Taken together, our data suggest a model whereby the Nkx6.1 C terminus may function in a regulatory manner by imposing specific functional constraints upon the protein. These constraints may serve to modulate the potential of Nkx6.1 to both recognize target genes and regulate their transcription.

MATERIALS AND METHODS

Recombinant Plasmids and Mutagenesis. All plasmids were generated using standard recombinant techniques. All PCR-generated constructs were subsequently verified by sequencing. For protein expression, cDNA fragments encoding various Nkx6.1 and Pdx-1 proteins (see Table 1) were PCR amplified from vectors pBAT12Nkx6.1 (*12*) and pBAT12Pdx-1 (*22*), respectively, and ligated into the *Xho*I and *Nco*I sites of the *Escherichia coli* expression vector pET21d, which contains an in-frame C-terminal 6X-His tag (Novagen). CMV promoter-driven expression plasmids containing cDNAs for (1−305)Nkx6.1 and (1−364)Nkx6.1 and the luciferase reporter driven by the *insulin I* gene promoter were described previously (*12*, *23*).

Mutations of the *nkx6.1* coding sequence and the mouse *insulin I* gene were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The oligonucleotide primers for mutagenesis were as follows (top strands shown, mutations underlined): Nkx6.1 HD338mN mutation, 5′-ACTTCGGAGAACGAGGAGCGGCCCCCGCGGTACAACAAGCCCCTGGAC-3′; *insulin I* A4 mutation, 5′-CATCAGGCCATCTGGTCCCTTATTTAGACTATAATAACCC-3′; *insulin I* A3 mutation, 5′-GGCCATCTGGTCCCTTATTAAGACTAAAATAACCCTAAGAC-3′; *insulin I* A4/A3 mutation, 5′-GGCCATCTGGTCCCTTATTTAGACTAAAATAACCCTAAGAC-3′.

Cell Culture and Transient Transfections. The mouse fibroblast-derived cell line NIH3T3 was maintained in DMEM supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Transient transfections of the NIH3T3 cell line were performed using Transfast reagent (Promega) as described previously (*13*). Luciferase enzyme activity was measured 48 h after transfection using a commercially available kit (Promega) and an FB15 luminometer (Zylux) and normalized to protein concentration. Expression of Nkx6.1 constructs in transfected NIH3T3 nuclear extracts (5 μg) was verified both by immunoblot analysis using the ECL-Plus kit (Amersham Pharmacia) with an Nkx6.1-specific polyclonal antibody (from Dr. M. German) and by EMSA analysis.

Protein Expression and Purification. All protein constructs containing a C-terminal His_6-tag were purified from batch cultures of *E. coli*. Briefly, *E. coli* strain BL21(DE3, pLysS) was transformed with the appropriate pET21d expression construct and grown to an OD_{600} of 0.6−1.0 in 1 L of Luria-Bertani broth (LB) prior to the induction of protein expression. IPTG-induced cultures were then pelleted, resuspended in imidazole lysis buffer (50 mM NaH_2PO_4, pH 8.0, 10 mM

[1] Abbreviations: aa, amino acids; bp, base pairs; CD, circular dichroism; EMSA, electrophoretic mobility shift assay; HD, homeodomain (amino acids 231−305 of Nkx6.1); HDC, homeodomain plus C terminus (amino acids 231−364 of Nkx6.1).

imidazole, 300 mM NaCl, 1 mM DTT, 5% glycerol), and lysed by sonication. The resulting bacterial lysate was applied to a 5 mL bed volume column containing Ni-NTA agarose resin (Qiagen) and washed with imidazole wash buffer (50 mM NaH_2PO_4, pH 8.0, 30 mM imidazole, 300 mM NaCl, 1 mM DTT, 5% glycerol). Nickel-bound protein was eluted using a linear gradient of imidazole (30−250 mM), and fractions (0.5 mL) were analyzed for purity by 12% SDS polyacrylamide gel electrophoresis. Protein fractions demonstrating >95% purity were pooled and desalted using PD10 columns (Amersham Pharmacia) as directed by the manufacturer. Protein concentrations were measured using the Bradford method and confirmed by UV absorption at 275 nm under native and denaturing conditions.

Electrophoretic Mobility Shift Assay (EMSA). To generate the *insulin I* promoter probe for EMSA studies, a 171 base pair (bp) fragment of the mouse *insulin I* 5′ regulatory region (from −126 to −296 bp relative to the transcriptional start site) was PCR amplified from plasmid DNA (pCRmINS1) (*15*) in the presence of α^{32}P-ATP using forward (5′-TTAGCCAAAGATGAAGAAGGTCTC-3′) and reverse (5′-CCTTAACACTTGCCTGGTGC-3′) primers. Short oligonucleotide probes were generated by 5′ end-labeling single-stranded oligonucleotides with T4 polynucleotide kinase and γ^{32}P-ATP and then annealing to an excess of unlabeled complementary strand. DNA binding reactions (in 20 μL volumes) proceeded at room temperature as described previously (*22*) and consisted of varying amounts (as indicated in the figure legends) of purified, desalted proteins in a reaction buffer consisting of 10 mM HEPES (pH 7.9), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 3% Ficoll, and 50 ng/μL poly dI-dC. Reactions were subjected to electrophoresis on a 5% polyacrylamide gel. Quantitation of free and shifted probe complexes was performed using a Typhoon phosphorimager (Molecular Dynamics), and apparent dissociation constants (K_Ds) were calculated based on one-step binding kinetics using following equation:

$$\text{fraction of probe bound} = (B_{max}c/(K_D + c))$$

where B_{max} is the fraction of probe bound, c is the protein concentration, and K_D is the apparent dissociation constant. The oligonucleotide probes used in these experiments were as follows (top strands shown): A3 element consensus probe, 5′-GATCTGACCATTTAATTACCCTTCGTTGACAAGG-3′; A3 element mutant consensus probe, 5′-GATCTGACCATTTATTTACCCTTCGTTGACAAGG-3′.

Circular Dichroism (CD). Far-UV (190−250 nm) CD spectra were obtained on an Aviv 215 circular dichroism spectrometer. Proteins were diluted to 10 μM in 50 mM sodium phosphate buffer, pH 8.0 and subjected to spectroscopic analysis at a constant temperature of 25 °C. Secondary structural deconvolution data were obtained using the CDSSTR algorithm (*24*) located on the DichroWeb Internet server (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) (*25*). Thermal denaturation data were obtained using a Peltier-jacketed cuvette, and ellipticity was monitored at 222 nm.

RESULTS

The C Terminus of Nkx6.1 Enhances DNA Binding Selectivity at the Mouse Insulin I Promoter. The *insulin* gene

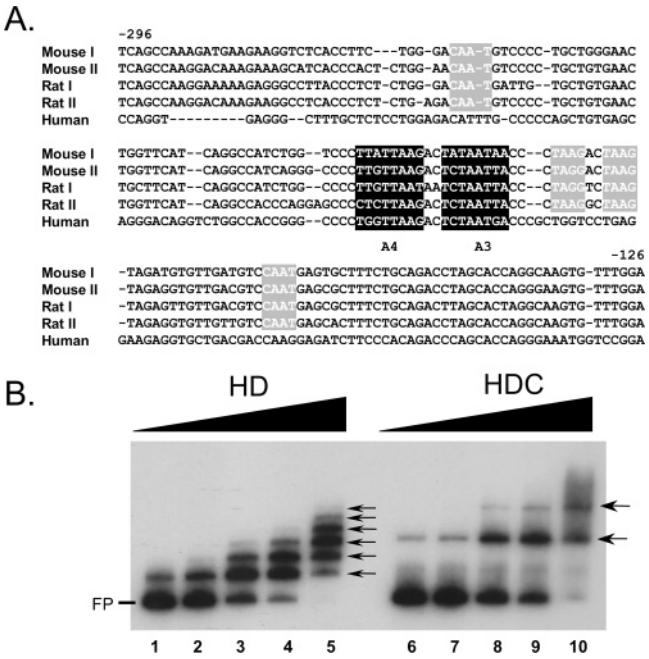


FIGURE 1: The C terminus of Nkx6.1 enhances sequence-selective DNA binding at the *insulin I* promoter in vitro. (A) An alignment of the mouse, rat, and human *insulin* promoters from −296 to −126 bp relative to the transcriptional start site. Black-highlighted sequences indicate the locations of the functionally critical A4 and A3 regulatory elements. Gray-highlighted sequences indicate the locations of nonconsensus A/T-rich DNA elements within the rodent promoter fragments. (B) Representative EMSA demonstrating retardation of the mouse *insulin I* promoter probe upon forming complexes with the Nkx6.1 homeodomain (HD, lanes 1−5) or the homeodomain plus intact C terminus (HDC, lanes 6−10). Arrows on the right identify the shifted complexes. Protein concentrations range from 2 to 200 nM.

in islet β cells has been hypothesized to be a direct downstream target of Nkx6.1 based on the existence of potential binding sites within the insulin promoter and on the finding that an *insulin* reporter gene is repressed in a sequence-dependent manner by Nkx6.1 in transient mammalian transfection studies (*12*). In support of this hypothesis, we recently demonstrated by chromatin immunoprecipitation that Nkx6.1 occupies the endogenous *insulin* promoter in βTC3 insulinoma cells (*13*). To determine the DNA binding characteristics of Nkx6.1 at the *insulin* promoter, we bacterially overproduced and purified Nkx6.1 protein constructs containing either the homeodomain alone ("HD" protein, amino acids (aa) 231−305) or the homeodomain with intact C terminus ("HDC" protein, aa 231−364) and subjected them to EMSA analysis. Because the amino terminal region of Nkx6.1 (aa 1−230) contains a transcriptional repression function and does not appear to contribute to DNA binding (*12*, *26*), we omitted this region from our protein constructs. As a DNA probe for these EMSAs, we used a 171 base pair (bp) regulatory region of the mouse *insulin I*[2] promoter encompassing −126 to −296 bp relative to the transcriptional start site. Notably, two conserved, canonical homeodomain regulatory motifs (TAAT-containing) within this region of the promoter (the A3 and A4 elements, Figure 1A, black-highlighted) (*23*) have been shown to be important for

[2] Note: The *insulin* gene has undergone a duplication event in rodents during evolution, resulting in 2 homologous and coordinately regulated copies of the gene in mice and rats.

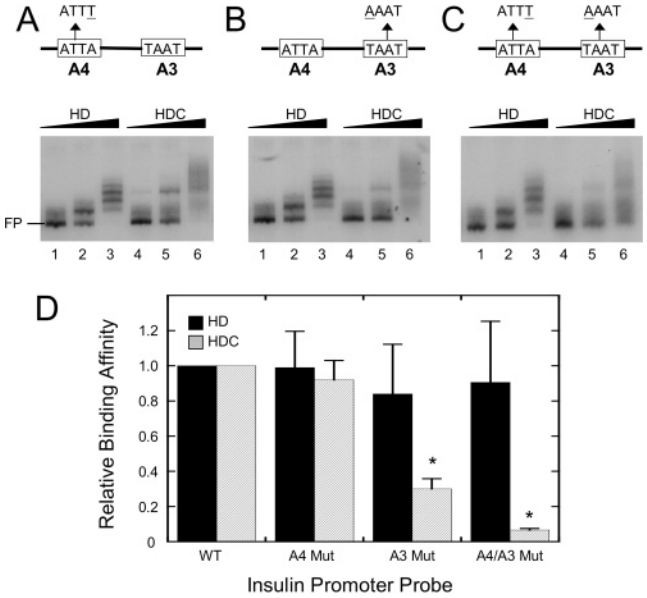


FIGURE 2: The C terminus of Nkx6.1 restricts DNA binding to the *insulin* A3 and A4 regulatory elements in vitro. (A−C) Representative EMSA analyses of the mouse *insulin I* promoter probe containing the mutations shown in the A4 element (A), A3 element (B), or combination A4/A3 elements (C). Lanes 1−3 and 4−6 of each panel represent EMSAs with increasing concentrations of the HD protein and HDC protein, respectively (2, 20, or 200 nM). FP: free (unbound) probe. (D) Relative DNA binding affinities (as determined by phosphorimager analysis) of the HD (solid bars) and HDC (hatched bars) proteins for the mutant *insulin* probes, relative to the affinity observed for the wild-type probe (defined as 1). Data represent the average from 3−5 independent EMSAs. Unpaired, two-tailed Student's *t* tests were performed to determine statistical significance. "*" indicates that the value is statistically different ($p < 0.01$) in comparison to the wild-type probe.

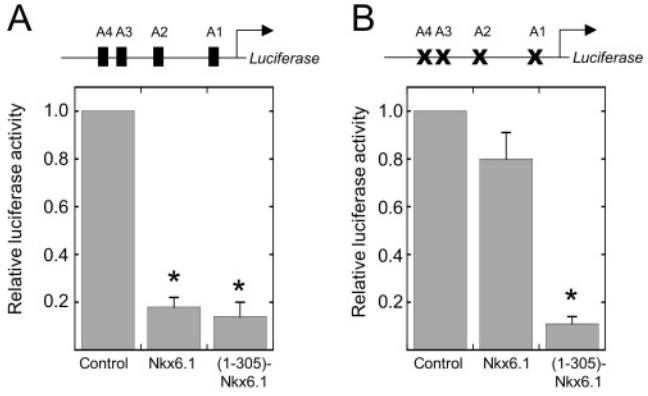


FIGURE 3: The C terminus of Nkx6.1 restricts DNA binding to the *insulin* A box (TAAT-containing) regulatory elements in vivo. Reporter plasmids containing either the wild-type (A) or mutated (B) −410 bp *insulin I* promoter driving *luciferase* were cotransfected with CMV promoter-driven plasmids containing cDNAs encoding either full-length Nkx6.1 or (1−305)Nkx6.1 into NIH3T3 cells as detailed in Materials and Methods. Cells were harvested 48 h after transfection, and extracts were assayed for luciferase activity. Relative luciferase activities were calculated with the activity from cells transfected with the backbone CMV expression plasmid ("Control") defined as 1. Results represent the average of 5 independent transfections. Unpaired, two-tailed Student's *t* tests were performed to determine statistical significance. "*" indicates that the value is statistically different ($p < 0.01$) in comparison to control transfections.

Nkx6.1-mediated transcriptional repression (*12*). Figure 1B shows representative EMSAs using increasing concentrations of the Nkx6.1 HD and HDC proteins and the mouse *insulin I* probe. Strikingly, the HD protein produced six distinct shifted complexes with the probe at the highest concentrations examined (Figure 1B, lane 5). In this regard, the mouse *insulin I* probe we used contains four noncanonical homeodomain-binding DNA sequences (see *gray-highlighted* sequences in Figure 1A) in addition to the two TAAT motifs (A3 and A4 elements), suggesting that these A/T-rich sequences may be bound by the HD protein. By contrast, the HDC protein at equivalent concentrations produced only two distinct shifted complexes with the same probe (Figure 1B, lane 10), consistent with binding to only the canonical A3 and A4 elements. At first glance, these data suggest that the C terminus of Nkx6.1 functions to restrict DNA binding by the homeodomain.

To clarify the sequences in the *insulin* promoter that are bound by the HD and HDC proteins, we mutated (individually and in combination) the A3 (TAATAAC) and A4 (TTATTAAG) sequences to disrupt the canonical core sequences (TAAT or ATTA, respectively) to noncanonical, A/T-rich sequences (TAAA or ATTT, respectively). Figures 2A, 2B, and 2C display representative EMSAs using the A4, A3, and combination A4/A3 *insulin I* probe mutations, respectively. Interestingly, mutation of either or both of these elements did not substantially alter the DNA binding characteristics of the HD protein for the *insulin I* promoter (Figure 2A−C, lanes 1−3). Based on quantitative EMSA analysis, the overall apparent affinity of the HD protein for the *insulin I* probe is not statistically changed upon mutation of the A4 and/or A3 elements (Figure 2D). In striking contrast, the binding affinity of the HDC protein for the *insulin I* probe was reduced by 70% with the A3 mutation and by 95% in the combination A4/A3 mutation (Figures 2B and 2C, lanes 4−6, and Figure 2D). Notably, the HDC construct showed no alteration in overall apparent affinity for the A4 mutant compared to wild-type (Figure 2A, lanes 4−6, and Figure 2D), suggesting that its binding to this element might be cooperatively enhanced by binding to the neighboring A3 element (a phenomenon observed with other homeodomain proteins (*27*)).

To determine whether the DNA binding selectivity of Nkx6.1 is preserved in vivo, we transfected mouse NIH3T3 cells with cDNAs encoding either full-length Nkx6.1 (aa 1−364) or an Nkx6.1 construct without the C terminus (aa 1−305), and examined their effects on the transcription of cotransfected −410 bp *insulin* promoter-*luciferase* reporter genes. For these studies, we retained the N-terminal domain of the Nkx6.1 protein for transcriptional read-out, as it contains the well-characterized transcriptional repression function (*12*, *26*). As shown in Figure 3A, Nkx6.1 and (1−305)Nkx6.1 led to approximately 5- and 8-fold repression of *insulin* promoter-*luciferase* activity, respectively. Because the −410 bp *insulin* promoter harbors 4 TAAT-containing DNA elements (A1, A2, A3, and A4 elements (*23*)), we hypothesized that collective mutation of these elements would mitigate repression by Nkx6.1, but not by (1−305)-Nkx6.1. Figure 3B demonstrates that Nkx6.1 causes no statistically significant repression of the mutated *insulin* promoter-*luciferase* reporter, whereas (1−305)Nkx6.1 still leads to 10-fold repression. Immunoblot and EMSA analyses revealed that expression levels of Nkx6.1 and (1−305)Nkx6.1 were similar in these transfection experiments (data not shown). These data support the hypothesis that the

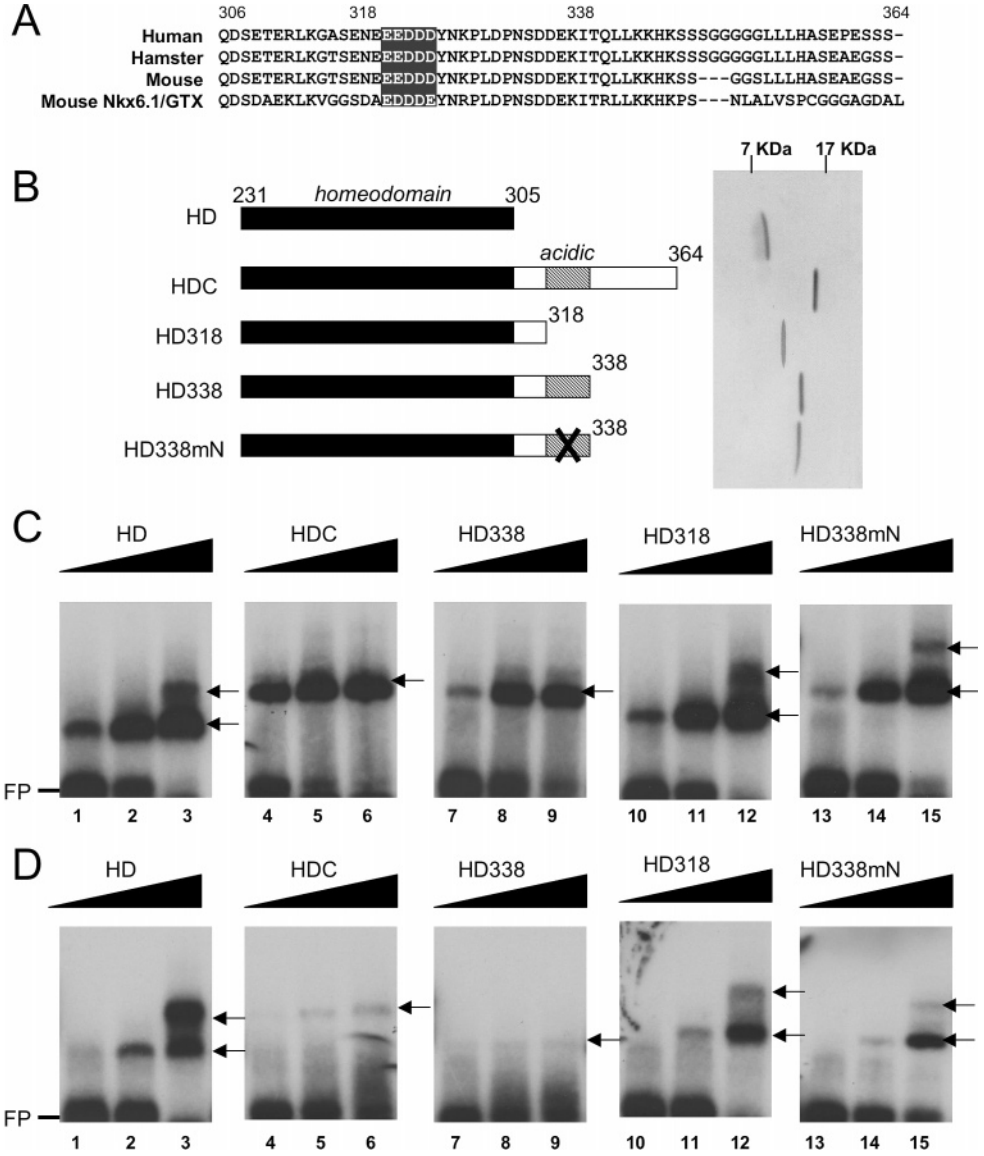


FIGURE 4: A conserved stretch of residues between aa 318 and 338 in the C terminus is necessary for the DNA binding selectivity of Nkx6.1. (A) Alignment of the human, hamster, and mouse Nkx6.1 C termini (from aa 306−364) with the mouse Nkx6.2/GTX C terminus (from aa 218−277). A conserved stretch of acidic amino acids (aa 320−325) is highlighted. (B) Schematic diagrams of the Nkx6.1 constructs used in these studies. The locations of the homeodomain (black region) and acidic residues (hatched region) are indicated. Protein purity (>95%) is demonstrated by the Coomassie-stained 15% SDS−polyacrylamide gel on the right. "X" in HD338mN indicates a mutation of the acidic residues (EEDD) highlighted in panel A to RPPR. (C and D) Representative EMSAs of increasing concentrations (2, 20, 200 nM) of Nkx6.1 constructs (indicated in panel B) complexed with a consensus *insulin* A3 element probe (core sequence: 5′-TTAATTAC-3′) and a mutant *insulin* A3 element probe (core sequence: 5′-TTATTTAC-3′), respectively. Arrows on the right identify the shifted complexes.

C terminus of Nkx6.1 enhances selectivity for TAAT-containing DNA sequences within the *insulin* promoter in vivo.

DNA Binding Selectivity Is Dependent upon a Discrete Stretch of Conserved Residues within the Nkx6.1 C Terminus. The C terminus of Nkx6.1 is well conserved across multiple species and with the closely related transcription factor Nkx6.2/GTX (*28*, *29*) (Figure 4A), supporting the notion that this region serves an important functional role. We have previously demonstrated that a region of acidic amino acids within the C terminus (EEDDD, Figure 4A highlighted) serves to activate transcription in vitro and in vivo under specific contexts (*13*) and to possibly modulate DNA binding affinity (*12*). Closer inspection of the C terminus also reveals additional conserved residues up to, and including, a positively charged group of amino acids (KKHK, see Figure 4A). To determine the extent to which these conserved residues contribute to the DNA binding selectivity of Nkx6.1 at the *insulin* promoter, we compared the apparent DNA dissociation constants (K_Ds) of Nkx6.1 proteins containing either deletions or mutations of the C terminus. Figure 4B depicts the Nkx6.1 protein constructs used in these studies and demonstrates the purity of the proteins following Ni-NTA affinity purification. Figure 4C shows representative EMSAs of each protein in complex with a probe containing a consensus insulin promoter A3 element, 5′-TTAATTAC-3′. Notably, constructs containing residues of the C terminus had slightly lower affinities for this short probe (within about 2−4-fold of the HD protein), consistent with prior observations using Nkx6.1 proteins transcribed/translated in vitro (*12*). Experiments using unlabeled DNA competitor demonstrated that the shifted complexes in Figure 4C were

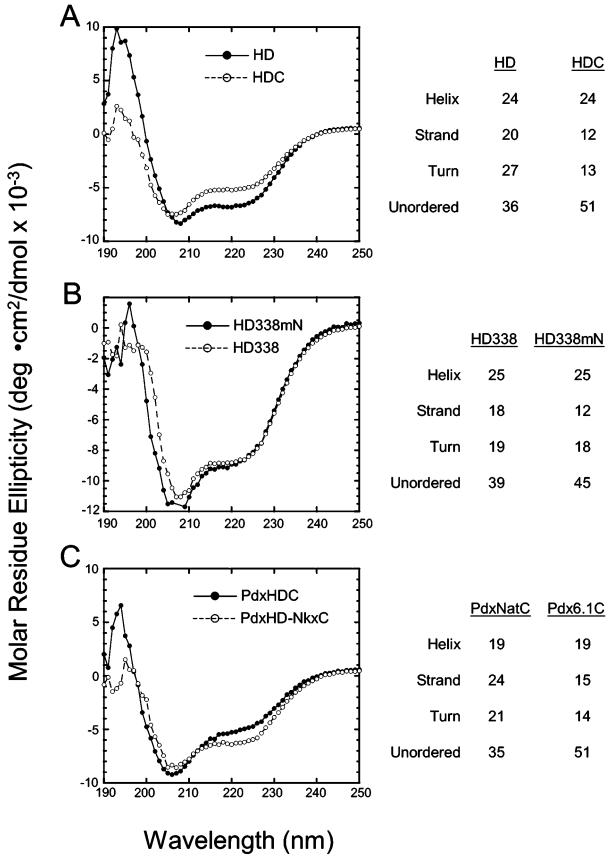
specific for the probe used and not due to random DNA association (data not shown, but see also ref *12*).

When a single bp mutation is introduced into the A3 element to disrupt the TAAT core (5′-TATTTAC-3′), the apparent K_Ds of the Nkx6.1 protein constructs show remarkable differences. Whereas the HD protein showed only a 5−6-fold reduction in apparent affinity for the mutant A3 probe compared to the wild-type (compare Figures 4C and 4D, lanes 1−3, and Table 1), the apparent affinity of the HDC protein for the mutant A3 probe was lower by approximately 35-fold compared to the wild-type (compare Figures 4C and 4D, lanes 4−6, and Table 1). These data are consistent with those observed with the larger insulin promoter fragment (Figure 2), and imply that the C terminus imparts nearly an order of magnitude selectivity to DNA binding by the homeodomain. This selectivity is preserved in the deletional construct (231−338)Nkx6.1 (HD338), which contains a deletion of all C-terminal residues up to the negatively charged region; in this case, minimal (if any) binding was detected to the mutant A3 element (compare Figures 4C and 4D, lanes 7−9, and Table 1).

Strikingly, further deletion of the conserved negatively charged stretch (between aa 319 and 324) to yield (231−318)Nkx6.1 (HD318) resulted in a protein that exhibited only a 4-fold drop in affinity for the mutant A3 probe, similar to results observed with the HD protein (compare Figures 4C and 4D, lanes 10−12, and Table 1). These results suggest that DNA binding selectivity is conferred by the conserved stretch of residues between aa 318 and 338. To confirm this possibility, we next introduced a previously described mutation (*12*) into the negatively charged stretch within the C terminus (EDDD to RPPR) of (231−338)Nkx6.1 (to yield "HD338mN"). This mutation was originally designed to disrupt both charge and potential secondary structure within C-terminal region by introduction of Pro residues. As shown in Figures 4C and 4D (compare lanes 13−15) and Table 1, specific mutation of these negatively charged residues results in loss of DNA binding selectivity, as the HD338mN protein binds to the mutant A3 probe with only a 3-fold decrease in affinity versus the wild-type probe. These results suggest either that these acidic residues specifically are responsible for the DNA binding selectivity of Nkx6.1 or, more generally, that the overall integrity of the conserved residues between 318 and 338 is critical for selectivity.

Of note, in Figures 4C and 4D, those proteins that show diminished selectivity (HD, HD318, and HD338mN) also demonstrate binding to an additional site on the A3 probe (note extra shifted complex); this site likely corresponds to an ATTT sequence located just 5′ of the A3 consensus sequence in this probe. Importantly, this additional complex is not observed for the HDC and HD338 proteins.

The DNA Binding Selectivity of the C-Terminal Region Does Not Appear To Emanate from Substantial Structural Changes to Overall Protein Conformation. We considered two possibilities by which the C terminus might enhance DNA binding selectivity: (1) the negatively charged residues within this region might form salt bridge interactions with the positively charged residues in the recognition helix (helix 3 (*30*)) of the homeodomain, thereby interfering with protein−DNA recognition, and/or (2) the C terminus might impose alterations to protein conformation that affect the nature of amino acid−DNA interactions. To address the first possibility, we performed an EMSA using the mutant A3 probe and the HD protein, and added increasing concentrations of a purified C-terminal peptide fragment (corresponding to aa 307−364). These studies revealed that the C-terminal peptide fragment, added in *trans*, had no effect on the binding of the HD protein to the mutant A3 probe (data not shown); these data suggest that the DNA selectivity by the C terminus likely does not emanate from its direct physical interaction with the homeodomain.



	HD	HDC
Helix	24	24
Strand	20	12
Turn	27	13
Unordered	36	51

	HD338	HD338mN
Helix	25	25
Strand	18	12
Turn	19	18
Unordered	39	45

	PdxNatC	Pdx6.1C
Helix	19	19
Strand	24	15
Turn	21	14
Unordered	35	51

FIGURE 5: Circular dichroism spectroscopy of Nkx6.1 and Pdx-1 proteins. All spectra were recorded at 10 μM protein concentrations at 25 °C, and represent the average of three scans. Deconvolution analysis to estimate percentages of secondary structure (shown on the right of each panel) was performed as described in Materials and Methods. (A) Spectra of (231−305)Nkx6.1 (HD, closed circles) and (231−364)Nkx6.1 (HDC, open circles). (B) Spectra of (231−338)Nkx6.1-(E321R, E322P, D323P, D324R) (HD338mN, closed circles) and (231−338)Nkx6.1 (HD338, open circles). (C) Spectra of (138−283)Pdx-1 (PdxHDC, closed circles) and (138−213)Pdx-1/(307−364)Nkx6.1 fusion protein (PdxHD-NkxC, open circles).

To determine if the DNA selectivity by the C terminus results from its effects on the overall conformation of the protein, we subjected the Nkx6.1 protein constructs used in these studies to circular dichroism (CD) spectroscopy. Figure 5A shows the far UV CD spectrum of the HD and HDC proteins at 10 μM concentration. Deconvolution analysis using the CDSSTR algorithm (*31*) predicts that both proteins exhibit similar proportions of alpha helix, but there is relatively less sheet and turn, and more random coil in the HDC protein (see Figure 5A). To clarify whether these structural differences might account for the difference in DNA selectivity between the two proteins, we performed CD analysis of two other comparable proteins that exhibited similar differences in DNA binding selectivity: the HD338 and HD338mN proteins. As shown in Figure 5B, both of

these proteins exhibited similar spectral characteristics, with no substantial differences in predicted helical, sheet, turn, and coil content. These data suggest that the conformational differences between the HD and HDC proteins in solution likely do not contribute to the observed differences in DNA binding selectivity, but do not rule out the possibility of conformational differences occurring upon binding to DNA. CD-monitored thermal denaturation revealed no apparent differences in structural stability among the proteins examined (with all proteins exhibiting melting temperatures between 47 and 52 °C, data not shown).

The C Terminus of Nkx6.1 Confers Additional DNA Binding Selectivity to a Heterologous Homeodomain. We next asked whether the C terminus of Nkx6.1 could function in a modular fashion by enhancing the DNA binding selectivity of a heterologous homeodomain. For these experiments, we studied the pancreatic homeodomain protein Pdx-1. Similar to Nkx6.1, Pdx-1 is known to bind preferentially to TAAT-containing DNA sequences and to directly regulate transcription of the *insulin* gene by binding to the A elements within the promoter (A3 and A4 (*22*, *32*, *33*)). Figure 6A shows the Pdx-1 protein constructs used in these studies. As shown in the representative EMSAs in Figures 6B and 6C and the corresponding K_Ds in Table 1, the homeodomain of Pdx-1 (PdxHD, aa 138−213) displays an inherent 11-fold selectivity for the wild-type A3 probe compared to the mutant A3 probe (Figures 6B and 6C, compare lanes 1−3; see Table 1). Importantly, the Pdx-1 homeodomain plus its native C terminus (PdxHDC, aa 138−283) demonstrates the same relative selectivity for the two probes (Figures 6B and 6C, compare lanes 4−6; see Table 1), suggesting that (unlike Nkx6.1) the native C terminus of Pdx-1 does not appear to enhance the DNA binding selectivity of the homeodomain. However, when the C terminus of Nkx6.1 is fused to the homeodomain of Pdx-1 to form the chimeric protein PdxHD-NkxC (see Figure 6A), there is a further enhancement of DNA binding selectivity: as demonstrated in Figures 6B and 6C (lanes 7−9) and Table 1, although the PdxHD-NkxC chimeric protein interacts with the wild-type A3 probe, very minimal binding of this protein is observed with the mutant A3 probe. This relative selectivity of the chimeric protein also appears to be preserved on the 171 bp extended fragment of the *insulin* promoter, which contains only two TAAT sequences (A3 and A4 elements). As shown in the EMSA in Figure 6D, the PdxHDC protein interacts with multiple sequences on the 171 bp promoter (as evidenced by the occurrence of at least 5 shifted complexes at 500 nM protein concentration), whereas PdxHD-NkxC interacts with only two sequences. CD analysis revealed that the PdxHD-NkxC protein contained a similar fraction of helix but substantially more unordered structure (at the expense of sheet and turn) compared to PdxHDC (Figure 5C). These structural findings are similar to those observed for the HDC protein (Figure 5A), and suggest that the C terminus of Nkx6.1 does not significantly alter the largely helical nature of the homeodomain, but may itself be somewhat unordered.

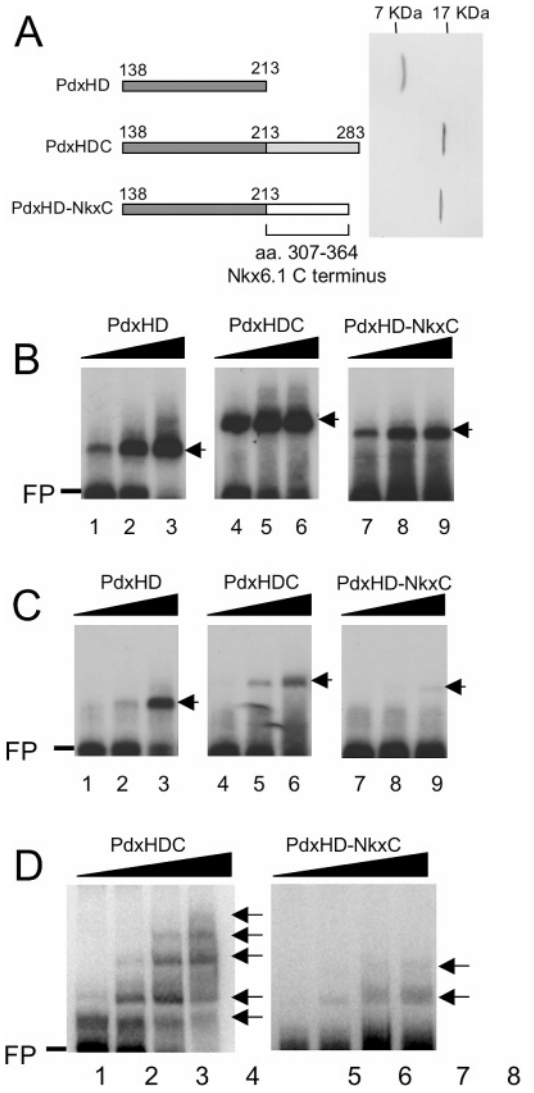


FIGURE 6: The Nkx6.1 C terminus functions as a modular DNA selectivity domain. (A) Schematic diagrams of the Pdx-1 and Pdx-1/Nkx6.1 chimeric proteins used in these studies are shown. Protein purity (>95%) is demonstrated by the Coomassie-stained 15% SDS−polyacrylamide gel on the right. (B and C) Representative EMSAs of increasing concentrations (2, 20, 200 nM) of the protein constructs (indicated in panel A) complexed with a consensus *insulin* A3 element probe (core sequence: 5′-TTAATTAC-3′) and a mutant *insulin* A3 element probe (core sequence: 5′-TTATTTAC-3′), respectively. (D) Representative EMSAs of increasing concentrations (2, 20, 100, 200 nM) of PdxHDC (left panel) and PdxHD-NkxC (right panel) complexed with the 171 bp *insulin I* promoter fragment (shown in Figure 1A). FP indicates free (uncomplexed) probe, and distinct shifted complexes are indicated by the arrows.

DISCUSSION

Sequence-specific DNA binding within promoter regions of genes is the first step in a cascade of events by which transcription factors regulate gene expression. In this context, most homeodomain-containing transcription factors bind to A/T-rich DNA sequences and regulate developmentally and functionally relevant genes in virtually all organ systems. Importantly, the selectivity of homeodomain proteins observed for DNA sequences in vitro often predicts the nature of DNA sequences bound in vivo (*34*). In this study of Nkx6.1, we have identified a unique role for amino acid sequences outside of the homeodomain in enhancing DNA binding selectivity at the *insulin* promoter. Thus, our findings may provide clues as to how some homeodomain transcription factors discriminate between target genes at the level of DNA binding.

The *insulin* gene was suggested to be a target for Nkx6.1 action based upon the dramatically diminished expression of the gene in *nkx6.1*-null mice (*7*), and upon chromatin

immunoprecipitation evidence that Nkx6.1 occupies the endogenous *insulin* promoters in βTC3 insulinoma cells (*13*). Curiously, based upon *insulin* promoter-driven reporter gene analysis, our data in NIH3T3 cells (Figure 3) suggest that Nkx6.1 in fact functions as a natural repressor, not activator, of the *insulin* promoter. These data are consistent with similar studies performed in β cell-derived cell lines (*12*). The seeming dichotomy between these findings and those predicted by the *nkx6.1*-null mice underscores the likely difference in the role of Nkx6.1 in the mature β cell vs the developing β cell. In the mature β cell, Nkx6.1 (perhaps in conjunction with other factors) is likely required for maintaining the level of *insulin* transcription under various metabolic conditions (glucose, amino acids, cAMP, etc.) within precise physiologic limits. In this regard, it was recently demonstrated that Nkx6.1 is necessary (but not sufficient) for the normal physiologic release of insulin in response to elevated extracellular glucose (*10*). On the other hand, the overriding role of Nkx6.1 during development may be to define the more global phenotype of the β cell via regulation of other β cell-specific genes. For example, Nkx6.1-null mice do not express the critical *insulin* gene activator MafA, thereby providing one possible explanation for the absence of *insulin* expression in these animals (*35*).

To identify the nature of DNA sequences within the *insulin* promoter that are bound by Nkx6.1, we examined the DNA binding characteristics of various Nkx6.1 protein constructs. Using an iterative oligonucleotide amplification and selection strategy, we and others demonstrated in prior studies that the homeodomain of Nkx6.1 preferentially binds to 5′-TAATTA-3′-containing DNA sequence in vitro (*11*, *12*); similar sequences (with a TAAT core sequence) are present in the regulatory "A" elements of the *insulin* promoter. However, depending upon the number of iterative selections performed, this type of selection strategy is biased toward the most high-affinity sequences and may miss lower affinity, but physiologically relevant, ones. In this regard, we observed binding in vitro of the Nkx6.1 homeodomain (HD protein) to at least 6 distinct sequences within a 171 bp stretch of the *insulin* promoter, 4 more than predicted by the number of "A" elements present within this region. By contrast, we observed binding of an Nkx6.1 protein construct containing both the homeodomain and the C terminus (HDC protein) to only two distinct sites (with strong preference for the A3 element) on this same fragment of promoter; this suggests a role for the C terminus of Nkx6.1 in providing DNA sequence selectivity. Notably, the selectivity imparted by the C terminus appears to be functionally preserved in vivo, as mutation of "A" elements within the *insulin* promoter abrogates the transcriptional repression by full-length Nkx6.1, but not by an Nkx6.1 protein lacking the C terminus. Based on both direct and competition EMSA studies using isolated A3 elements, we confirmed that even a single bp mutation to disrupt the central TAAT core sequence (to TATT) results in a dramatic reduction in apparent DNA binding affinity in Nkx6.1 protein constructs containing the C terminus (Table 1).

The C terminus of Nkx6.1 is highly conserved across species, and shares substantial homology with the related homeodomain factor Nkx6.2/GTX (*28*, *29*) (see Figure 4). One particularly striking feature of this region is the occurrence of negatively charged residues (accounting for approximately 45% of the residues in this region). Using proteins transcribed/translated in vitro, it was previously suggested that these negatively charged residues diminish the absolute affinity of the homeodomain for TAAT DNA sequences (*12*). Our studies using bacterially purified proteins tend to support this conclusion, although the magnitude by which these residues mitigate affinity appears to be relatively small (no more than 2−4-fold, Table 1). More strikingly, we observed that these negatively charged residues are minimally necessary to enhance the selectivity of Nkx6.1 for TAAT-containing sequences, because their mutation appears to completely restore the ability of the homeodomain to bind to nonconsensus DNA elements in which the central TAAT core is mutated (ATTT). We note that our studies do not rule out the possibility that other conserved residues between aa 318 and 338 are also necessary for the observed DNA binding selectivity. From a teleological perspective, it is possible that the DNA binding selectivity of Nkx6.1 was enhanced (to ensure targeting of very specific genes) at the expense of DNA binding affinity.

From the standpoint of mechanism, the C terminus may contribute to sequence-selective DNA binding by imposing conformational changes to the homeodomain. Based upon X-ray crystallographic data of several homeodomain proteins complexed to DNA, the conserved 60 aa homeodomain contains 3 alpha helices, of which the third (the "recognition helix") appears to function as an important determinant of DNA sequence recognition (*30*). Despite the conserved structural characteristics of the recognition helix, there can be considerable differences in the nature of DNA sequences bound by homeodomain proteins. For example, Nkx6.1 and other Hox-like proteins preferentially recognize TAAT-containing sequences (*34*), whereas the NK2 family members recognize CAAG-containing sequences (*14*, *36*). In this context, it is becoming increasingly clear that amino acids outside the recognition helix (and the homeodomain itself) can contribute to both the affinity and selectivity of homeodomain−DNA interactions (*17*, *19*, *37*, *38*). CD analysis suggests that the C terminal residues of Nkx6.1, though somewhat disordered on their own, do not grossly alter the helical nature of the homeodomain. However, these findings do not rule out the possibility that the C-terminal residues modify the structure of the homeodomain when complexed to DNA. Structural, biochemical, and molecular dynamics studies suggest that amino acid sequences outside of the homeodomain can influence both the secondary structure and positioning of the recognition helix relative to the major groove of DNA, thereby altering both affinity and specificity of protein−DNA interactions (*17*, *18*, *39*).

Interestingly, the ability of Nkx6.1 to differentially repress or activate transcription appears to rely, at least in part, on DNA sequence recognition. Here, and in prior studies (*12*), we have demonstrated that Nkx6.1 acts as a potent transcriptional repressor upon binding to a TAAT-containing sequence. Conversely, transcriptional activation by Nkx6.1 appears to occur upon binding of the factor in vivo to a nonconsensus (G/C)n-ATTT-(G/C)n sequence, as found within the *nkx6.1* promoter itself (*13*). These observations have at least two important implications: first, Nkx6.1 association with non-TAAT-containing sequences might indeed occur in vivo, and could arise as a result of protein−protein interactions with other transcription factors at the

promoter. Binding to nonconsensus sites is a well-known phenomenon by which homeodomain proteins select target genes in vivo (*19*). Second, interactions with consensus vs nonconsensus DNA sequences may represent an important mechanism by which Nkx6.1 represses vs activates gene transcription, respectively. In this context, studies have shown that minor nucleotide variations in DNA sequences can lead to allosteric effects that ultimately modulate cofactor recruitment and subsequent transcriptional rates (*40*). Interestingly, the activation domain of Nkx6.1 maps precisely to the acidic residues within the C terminus (*13*), suggesting that the C terminus may ultimately play a pivotal role in how target genes are both selected and regulated.

Taken together, our studies form a framework for understanding how the biochemistry and biology of Nkx6.1 are interrelated. In the context of β cell differentiation and function, we propose that the C terminus of Nkx6.1 may function in a regulatory manner, assisting with a pattern of target gene recognition and expression that favors β cell formation and glucose-stimulated insulin secretion (*10*). Based upon models involving other pancreatic homeodomain proteins such as Pdx-1, we expect that this regulatory function of the C terminus likely depends on the nature of complexes that Nkx6.1 forms with other transcription factors and cofactors on relevant promoters. Elucidating these complexes will undoubtedly be important in generating a final model of Nkx6.1 regulation in vivo.

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