Interference and Transcriptional Repression Domains*

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β-Cell Differentiation Factor Nkx6.1 Contains Distinct DNA Binding Interference and Transcriptional Repression Domains*

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Raghavendra G. Mirmira‡§¶, Hirotaka Watada‡, and Michael S. German‡§**

From the ‡Hormone Research Institute and §Department of Medicine, University of California, San Francisco, California 94143

β-cell differentiation factor Nkx6.1 is a homeodomain protein expressed in developing and mature β-cells in the pancreatic islets of Langerhans. To understand how it contributes to β-cell development and function, we characterized its DNA binding and transactivation properties. A single copy of the homeodomain of Nkx6.1 binds to a strictly conserved 8-base pair DNA consensus sequence, TTAATTTAC; even minor variations to this consensus reduce DNA binding affinity significantly. Full-length Nkx6.1, however, has markedly reduced DNA binding affinity due to an acidic domain at the carboxyl end of the molecule that functions as a mobile binding interference domain capable of interrupting the interaction between DNA and DNA binding domains of the helix-turn-helix type. When expressed in fibroblast cell lines, Nkx6.1 represses transcription through isolated Nkx6.1 binding sites in β-cell lines, Nkx6.1 specifically represses the intact insulin promoter through TAAT-containing sequences. In Gal4 one-hybrid fusion studies, transcriptional repression maps to a discreet region within the amino terminus. Our findings suggest a model in which Nkx6.1, regulated by interactions through its carboxyl terminus, directs the repression of specific genes in developing and mature β-cells.

The development of the mammalian pancreas requires the concerted action of multiple transcription factors. Targeted disruption of several of these factors, including Pdx1, Nkx2.2, Pax6, Isl1, Beta2/NeuroD, and Pax4 (1–8), has identified a temporal and spatial pattern of transcription factor expression that controls the ordered development of the endocrine cell types that comprise the islets of Langerhans (9, 10). Recently, Nkx6.1 has been identified as a member of this pancreatic network of transcription factors and has been shown to specifically control β-cell differentiation (11).

Nkx6.1 is a homeodomain-containing transcription factor that is initially expressed broadly in the developing pancreatic bud, but eventually becomes restricted exclusively to the β-cells (12, 13). Studies of null mutations of the Nkx2.2 and Nkx6.1 genes in mice have outlined the role for Nkx6.1 in β-cell development. In mice deficient for Nkx2.2, β-cell development is arrested, as demonstrated by the accumulation of cells expressing some markers characteristic of β-cells, such as islet amyloid polypeptide and Pdx1, but lacking other markers, most notably insulin and Nkx6.1 (8). As a result, these mice develop severe hyperglycemia and die shortly after birth. Mice deficient for Nkx6.1 display a dramatic reduction in β-cell numbers, with insulin levels reduced to 2% of wild type, but have near-normal development of all other endocrine and exocrine cell types (11). These studies have demonstrated that Nkx6.1 lies downstream of Nkx2.2 and is required for late steps in the pathway for β-cell differentiation.

Nkx6.1 is a divergent member of the NK family of transcription factors. With the exception of the closely related factor Gtx (Nkx6.2), the homeodomain of Nkx6.1 displays only about 45% identity and 60% homology to the homeodomains of other members of the NK class (14). Gtx (Nkx6.2), a transcription factor expressed in testis and oligodendrocytes but not pancreas, shows striking homology to Nkx6.1 within the homeodomain and COOH-terminal region, but diverges significantly at the NH2 terminus (15, 16). Apart from the homeodomain and NK decapetide (the function of which is unknown), Nkx6.1 has no significant homology to any other transcription factor.

Although it was very recently shown that the homeodomain of Nkx6.1 is capable of recognizing a TAAT-containing DNA sequence (17), nothing is known of how it restricts DNA target specificity, the binding properties of the full-length protein, or its transactivation properties. In an effort to understand Nkx6.1 function during β-cell development, we investigated in detail its DNA binding characteristics and transactivation properties. We confirm that the homeodomain recognizes a TAAT-containing sequence, but demonstrate that flanking base pairs can have significant effects on DNA binding. We also show that the native transcription factor binds to this site in a conditional manner that is dependent upon a discreet binding interference domain (BID)1 in the COOH terminus. Furthermore, we show that Nkx6.1 functions as a potent transcriptional repressor, with repressor activity mapping to sequences in the NH2 terminus. The findings suggest that Nkx6.1, regulated by interactions through the BID, inactivates specific target genes during β-cell differentiation.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids and Mutagenesis—All plasmids were constructed using standard recombinant DNA techniques and constructs generated by PCR were confirmed by sequencing. The Escherichia coli expression vector, pET-NKX6.1.HD, encoding the homeodomain (aa 229–305) of Nkx6.1 was made by amplifying the appropriate coding sequence (17), not shown. The abbreviations used are: BID, DNA binding interference domain; TK, herpes simplex virus thymidine kinase promoter; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; CMV, cytomegalovirus; Gal4-DDB, yeast Gal4 DNA binding domain; aa, amino acid(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

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¶ To whom correspondence should be addressed: Hormone Research Inst., University of California, 513 Parnassus Ave., San Francisco, CA 94143-0534. Tel.: 415-476-9262; Fax: 415-731-3612; E-mail: mgerman@biochem.ucsf.edu.

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fragment of hamster Nkx6.1 by PCR, and then ligating it in frame to the Ndel and SacI sites of the 6X-His vector, pET15b (Novagen).

The GAL4-TK reporter vector was constructed by inserting a 100-bp fragment containing five tandem copies of the yeast GAL4 promoter site immediately upstream of the TK promoter in pFOXLuc2TK (18). The one-hone-pot PCR reactions were performed in 20 μl containing 1× ExtraTaq buffer (2.5 mM MgCl2), 0.1 mM dNTPs, and 250 ng of each of the PCR products. The resulting fragment was cloned into the pCold vector to make the expression plasmid pCold1Nkx6.1.

Plasmids for the expression of intact Nkx6.1 and its fragments in mammalian cells were constructed in the CMV promoter-based vector, pBamHI. Constructs were made by subcloning the appropriate fragments from the GAL4-DBD vectors (pM) into the EcoRI and SacI sites of pBPLI. For expression of proteins in vitro, fragments were subcloned into the same sites of the T7 promoter-based vector, pBTT1. The reporter vectors containing the ~350 bp rat insulin 1 promoter constructs driving the luciferase gene were generated by subcloning the promoter fragments from pOK1 (20) into pFOXLuc. The reporter plasmid pFOXLuc6.1TK was constructed by inserting a 1.5-kb fragment containing five tandem copies of the Nkx6.1 consensus site into the BamHI and BglII sites of pFOXLuc2TK.

pBTT1.Pdx1HBD-BID and pBTT1.Pax6PD-BID encoding the homeodomain of Pdx1 (aa 138–213) and paired domain of Pax6 (aa1–137), respectively, fused to the COOH-terminal amino acids of Nkx6.1 (aa 306–364) were expressed in mammalian cells by transient transfection using transient expression vectors that contained the CMV enhancer, a human β-actin promoter, and an SV40 polyadenylation signal. The resulting plasmids were called pBTT1.Pdx1HBD-BID-Nkx6.1 and pBTT1.Pax6PD-BID-Nkx6.1.

The random oligonucleotide selection was a modification of previously published procedures (21–23), in which a 55-mer fragment was used to select DNA sequences that bound to the Nkx6.1 homeodomain. The selection was performed by using 15 μl of binding buffer (10 mM HEPES, 75 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 3% (v/v) Ficoll) at room temperature for 15 min. 50 μl of a 50% slurry of nickel-nitrilotriacetic acid resin was then added, and the reaction was allowed to proceed to another 15 min at room temperature. The reaction was centrifuged, the supernatant was aspirated, and the resin was washed three times with 1× ExtraTaq buffer, once with 1× ExtraTaq buffer containing 0.5 M dithiothreitol, and once with 0.5 M dithiothreitol. The bound DNA was eluted by incubating the resin with 200 μl of 250 mM imidazole in 20 μl Tris–Cl, pH 8. The eluted 55-mers were then amplified using 20 cycles of PCR, the REV primer, and the FWD primer (5′-GATCCCTTGCTAATCTGAG-3′). 10 μl of this PCR product was directly used as a probe for the next round of binding site selection. Six selection cycles were performed, after which an additional six cycles were performed using an EMSA technique for selection (23). After a total of 12 selection cycles were performed, the resulting 55-mers were subcloned into pCR2.1 (CLONTECH) and sequenced. Sequences were analyzed using a non-gapped algorithm (ClustalW) in MacVector® 6.5 software (Oxford Molecular).

In Vitro Transcription and Translation—Nkx6.1 and its truncated derivatives were expressed in vitro, with or without 35Smethionine labeling, from coding fragments subcloned into pBTT1 using the TNT T7 quick coupled reticulocyte lysate system (Promega).

Electrophoretic Mobility Shift Assays and Binding Competition—Single-stranded oligonucleotide probes were 5′-end labeled with [γ-32P]ATP using T4 polynucleotide kinase. Labeled oligonucleotides were column-purified and annealed to an excess of complementary strands in a buffer and ethanol precipitated, and then deprotected by 1 M NaOH (24). Where in vitro synthesized protein was used, 1 μl of the in vitro reaction mixture was used, and where supershift assays were performed, 1 μl of anti-Nkx6.1 antibody (8) or preimmune serum was also added. The following oligonucleotide probes were used (top strands): Nkx6.1 consensus, 5′-GATCTGAGGATATTCCTCCTGTCAGGAAGG; rat insulin I A3/4, 5′-GATCTGACCATTTAGTTACCCTTCGTTGACAAGG; rat insulin I C2, 5′-GATCTGACCATTTAGTTACCCTTCGTTGACAGG; M2, 5′-GATCTGACCATTTAGTTACCCTTCGTTGACAGG; insulin I C2, 5′-CTGGGAAATGGAGGTTGAAATGCTC; Gal4 upstream activated sequence, 5′-GATCTCGAGAGGTCCTCCTCCCGG.

Binding competition assays followed the principle described in Ref. 21 and were performed under identical EMSA conditions, except that only the Nkx6.1 consensus sequence was labeled with [35S]methionine and varying concentrations (from 10−11 to 10−6 M) of unlabeled competitor sequence was added. Radioactivity in the EMSA gels was quantitated by PhosphorImager analysis (Molecular Dynamics), and the data were modeled using simple one-step binding kinetics by use of Kaleidagraph® (Synergy Software) and the following equation:

\[ \frac{\text{Fraction of probe bound}}{\text{Fraction of probe bound in the absence of competitor}} = \frac{\beta_{\text{max}}}{\beta_{\text{max}} + [\text{ competitor}]} \]

\[ B_{\text{max}} \] is the fraction of probe bound in the absence of competitor, [c] is the apparent dissociation constant. The quantity of (229–305)Nkx6.1 protein (1 ng) was chosen, such that no more than 10% of the total oligonucleotide probe was bound in the absence of competitor.

**RESULTS**

Selection of an Optimal DNA Binding Site for Nkx6.1—The homeodomain has been demonstrated to be a modular DNA binding protein motif of high integrity, such that even in isolation it retains both DNA binding specificity and affinity (25, 26). In order to identify the optimal DNA binding site for Nkx6.1, we therefore expressed in E. coli the homeodomain of Nkx6.1 (amino acids 229–305) fused to 6 tandem histidine residues (His6) at the amino terminus and purified the protein on a nickel resin. This purified protein was used to select for binding of specific DNA sequences from among a library of oligonucleotides of 55 base pairs in length containing a central random stretch of 15 base pairs. The protein and its bound DNA sequences were isolated by adsorption to nickel
resin, and the selected sequences were amplified by PCR and used in a subsequent round of selection.

In the first round of selection, 10 pmol of oligonucleotide were used, which corresponds to approximately 1000 times more than the theoretical number of different sequences that would be present with 15 random base pairs (4\(^{15}\)). Six rounds of selection were performed using the nickel adsorption procedure. An additional six rounds of selection were performed using EMSA, in which the retarded protein-DNA complex was cut and eluted from the polyacrylamide gel, followed by PCR amplification of the oligonucleotide with incorporation of radiolabel. After a total of 12 rounds of selection, the sequences of 23 oligonucleotides were determined. The sequences were subjected to an unguided alignment using the multiple sequence alignment function in the MacVector 6.5 software package (Oxford Molecular, Ltd.). Fig. 1A demonstrates that the oligonucleotide sequences are nonrandomly distributed and contain the consensus sequence TTAATTAC, which is similar to the nucleotide sequences are nonrandomly distributed and contain the consensus sequence TTAATTAC, which is similar to the nucleotide sequences that are known to bind this particular homeodomain (TTAATT(G/A), Ref. 17). Fig. 1B shows that an oligonucleotide containing this consensus clearly binds the Nkx6.1 homeodomain in an EMSA.

The twofold symmetry of the consensus binding site we obtain for Nkx6.1 suggests the possibility that the homeodomain contacts DNA as a dimer. Several lines of evidence, however, demonstrate that the Nkx6.1 homeodomain binds DNA as a monomer: (a) an EMSA in which the homeodomain with and without the His\(_6\) tag (mass difference of approximately 2 kDa) were mixed together did not result in a migrating complex of intermediate molecular weight (Fig. 1B), (b) disruption of the twofold symmetry does not affect the migration of the shifted complex (see Fig. 2, oligonucleotide M1), and (c) NMR and crystal structure data reveal that many other homologous homeodomains bind DNA as monomers (26–28).

**DNA Binding Characteristics of the Homeodomain of Nkx6.1**—The consensus sequence that we have determined here contains the “core” motif, TAAT, which is also found in the DNA binding sites for many other homeodomain proteins, such as En of Drosophila (28), the mammalian Hox proteins (21), and the pancreatic homeodomain protein Pdx1 (29). Since the promoter regions of many genes contain sequences with TAAT motifs that are highly similar to the Nkx6.1 consensus, it is difficult to predict the potential downstream target genes for this (or any other) transcription factor on the knowledge of this core motif alone. Sequences flanking this core are important in the recognition of specific sequences by the murine Hox proteins, En-1, Ubx, and Nkx2.5 proteins (21, 23, 30), thereby restricting target gene selection (31). In an effort to determine if sequences flanking this core have significant effects on the DNA binding affinity of Nkx6.1, we performed EMSAs using oligonucleotides that contain alterations to the consensus motif (Fig. 2). Alterations within the core dramatically reduce binding affinity (oligonucleotides M2 and M3), but alterations flanking the core result in reductions as well (rat insulin I A3/4 and oligonucleotide M1; Fig. 2).

In order to obtain quantitative information on the data presented in Fig. 2, we performed binding competition experiments using unlabeled competitor oligonucleotides and \(^{32}\)P-labeled Nkx6.1 consensus probe, followed by EMSA and PhosphorImager analysis. Table I shows the apparent dissociation constants (\(K_d\) values) for the binding of each of the oligonucleotides to the Nkx6.1 homeodomain, expressed as a percentage of the apparent \(K_d\) for the consensus sequence. The \(K_d\) for the consensus sequence (4 \(\times\) 10\(^{-9}\) M) is consistent with the \(K_d\) values determined for the binding of other homeodomain proteins with DNA (21, 26). Remarkably, the homeodomain displays a nearly 5-fold lower affinity for rat insulin I A3/4 sequence (an insulin promoter element that has been shown to be important in the control of insulin gene expression; see Refs. 19, 20, and 29), notwithstanding that this sequence contains four TAAT cores. Although these cores have multiple flanking base pairs that differ from the ideal Nkx6.1 consensus, it is clear that even a single T \(\rightarrow\) G base pair change in the flanking region (oligonucleotide M1, Table I) is sufficient to decrease binding significantly. Changes within the TAAT core are dramatically less well tolerated, resulting in greater than 10-fold

**Fig. 1.** Nkx6.1 binding site selection and homeodomain binding. Binding site selection was performed with the hamster Nkx6.1 homeodomain (amino acids 229–305) and a set of oligonucleotides containing a 15-base pair random stretch, flanked by PCR primer sites. A, sequenced products from the 12th round of selection are shown aligned by the ClustalW alignment algorithm in MacVector 6.5 software (Oxford Molecular, Inc.). The consensus sequence emerging from the best fit line-up is shown. The underlined base pairs derive from the primer sequence. B, an EMSA using in vitro translated Nkx6.1 homeodomain (amino acids 229–305) without (lane 1) and with (lane 2) an NH\(_2\)-terminal His\(_6\) tag and the \(^{32}\)P-labeled Nkx6.1 consensus sequence probe is shown. Lane 3 shows that no complex of intermediate mobility is observed when homeodomains with and without the His\(_6\) tag are added together in a single EMSA reaction.
decreases in binding affinity (oligonucleotides M2 and M3, Table I).

The COOH Terminus of Nkx6.1 Negatively Regulates DNA Binding Affinity—Although the data presented thus far establish the DNA binding characteristics of the Nkx6.1 homeodomain, they have not addressed the issue of whether the same applies to the native Nkx6.1 protein. For many other mammalian DNA-binding proteins, binding site specificities and affinities of the native protein parallels that of the DNA binding domains alone (22, 32, 33). However, in EMSA studies, we have been unable to detect native Nkx6.1 binding from nuclear extracts of a murine β-cell line (β-TC3 cells) or from fibroblast cell line (NIH3T3 cells) transiently transfected with Nkx6.1 cDNA, notwithstanding that Western blotting demonstrates the presence of Nkx6.1 in these extracts (data not shown). These data could be explained by either our inability to detect a specific migrating complex due to masking by other nonspecifically shifted complexes, or by the formation of a very weak protein-DNA complex that is below the detection limit of our EMSAs. In an effort to resolve this issue, we used various truncations (Fig. 3A) and mutations of the Nkx6.1 protein produced in vitro in EMSA studies with the Nkx6.1

### Table I

| Oligonucleotide | Core sequence       | Relative binding affinity (% of consensus) |
|-----------------|---------------------|--------------------------------------------|
| Nkx6.1 consensus| CATTTAATTACCCT      | 100                                        |
| Rat insulin I A3/4 | AATCTAATTACCCT   | 24 ± 8                                     |
| M1              | AGTTTAATGAGATC      | 42 ± 11                                    |
| M2              | CATTAGTTACCCCT      | 3 ± 0.8                                    |
| M3              | CATTTAGTACCCCT      | 5 ± 2                                      |

*a* Designations for the individual oligonucleotides are the same as those in Fig. 2.

*b* Alignment with the Nkx6.1 consensus sequence is shown. The full sequences of the oligonucleotides are provided under “Experimental Procedures.” The underlined residues identify changes in the sequence relative to the consensus.

*c* Affinities of the individual oligonucleotides for the homeodomain of Nkx6.1 were determined by competition EMSA, followed by Phosphorilager analysis, as detailed under “Experimental Procedures.” The apparent dissociation constant ($K_d$) for the consensus oligonucleotide is $4.7 ± 1.7$ nM; the relative affinities of the other oligonucleotides are expressed as a percentage of this value. Data represent a mean ± standard error (S.E.) of at least three separate determinations.

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**Fig. 2.** Binding of the Nkx6.1 homeodomain. An EMSA using the Nkx6.1 homeodomain (amino acids 229–305) and various oligonucleotides (indicated above each lane) were incubated with 5 ng of homeodomain protein for 15 min at room temperature, then subjected to electrophoresis on a 5% polyacrylamide gel. The binding sequences of the oligonucleotides are shown in Table I. The free probe and retarded complex (representing oligonucleotide bound to the homeodomain of Nkx6.1) are indicated.

**Fig. 3.** The COOH terminus of Nkx6.1 inhibits DNA binding. Nkx6.1 and various truncations were produced in vitro by rabbit reticulocyte lysate and subjected to EMSA analysis on a 5% polyacrylamide gel. A, a schematic representation of Nkx6.1 and its truncated derivatives is shown. B, EMSA was performed with 1 μl of the in vitro translated protein indicated and incubated with 32P-labeled Nkx6.1 consensus oligonucleotide. Control represents the rabbit reticulocyte lysate incubated with empty vector. The arrows identify the positions of the specifically shifted complexes corresponding to protein-bound probe. On this native gel, Nkx6.1 Δ3 appears to migrate similarly to Nkx6.1, likely as a result of significant loss of negative charge accompanying deletion of the COOH terminus. C, a 4–20% gradient SDS-PAGE analysis demonstrates that the proteins studied (labeled with [35S]methionine) exhibited appropriate molecular weights and were produced at approximately equivalent levels in vitro, based on the relative molar fraction of methionine residues present.
In Fig. 3B, native Nkx6.1 produced in vitro binds very weakly to the consensus binding site, and no binding can be detected for an Nkx6.1 protein lacking the NH2-terminal 228 amino acids. Since the homeodomain (amino acids 229–305) binds DNA avidly (Fig. 3B, lane 4) as expected, the COOH-terminal region of the protein encompassing amino acids 306–364 must inhibit binding to the consensus site. As shown in Fig. 3B (lane 5), when these amino acids are deleted from the native protein, DNA binding is restored. The COOH terminus (amino acids 306–364) therefore functions as a DNA binding interference domain (BID).

Construction of Nkx6.1 mutants: A, comparison of the amino acid sequence of the COOH terminus of Nkx6.1 (aa 306–364) with the COOH terminus of Gtx/Nkx6.2 (aa 218–277). Identical and highly conserved residues are shown in bold, and the boxed residues indicate the positions of mutations A and B. B, native Nkx6.1 and mutants A and B were translated in vitro, and 1 µl of each translation mixture was used in an EMSA with 32P-labeled Nkx6.1 consensus oligonucleotide, with or without addition of 1 µl of anti-Nkx6.1 antibody as indicated. The arrows indicate the positions of the specific complexes formed by binding of the Nkx6.1 proteins to the probe. A nonspecific complex, a result of proteins in the in vitro lysate, migrates close to the specifically shifted complexes. C, a 4–20% SDS-PAGE analysis demonstrates that all three proteins were produced at approximately equivalent levels, as judged by 35S-methionine incorporation.

Mutations in the Nkx6.1 BID increase DNA binding activity. A, comparison of the amino acid sequence of the COOH terminus of Nkx6.1 (aa 306–364) with the COOH terminus of Gtx/Nkx6.2 (aa 218–277). Identical and highly conserved residues are shown in bold, and the boxed residues indicate the positions of mutations A and B. B. C, native Nkx6.1 and mutants A and B were translated in vitro, and 1 µl of each translation mixture was used in an EMSA with 32P-labeled Nkx6.1 consensus oligonucleotide, with or without addition of 1 µl of anti-Nkx6.1 antibody as indicated. The arrows indicate the positions of the specific complexes formed by binding of the Nkx6.1 proteins to the probe. A nonspecific complex, a result of proteins in the in vitro lysate, migrates close to the specifically shifted complexes. C, a 4–20% SDS-PAGE analysis demonstrates that all three proteins were produced at approximately equivalent levels, as judged by 35S-methionine incorporation.

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Fig. 4A shows the primary structure of the Nkx6.1 COOH terminus, and demonstrates that it contains a large stretch of acidic amino acids. Extensive alignments to the GenBank sequence data base reveal that the COOH terminus bears no significant homology to other proteins, with the exception of the closely related transcription factor Gtx (also known as Nkx6.2; Ref. 15). In order to determine if structural features within this region account for inhibition of DNA binding, we constructed two separate mutations, A and B, as indicated in Fig. 4A. Both mutations eliminate and partially reverse negative charges within the COOH terminus, but mutation A also disrupts a putative α-helix (as predicted by a Chou-Fasman analysis). Fig. 4B shows the results of EMSA studies using in vitro produced wild-type and mutant proteins (Nkx6.1mA and Nkx6.1mB) and the Nkx6.1 consensus sequence probe. When compared with the wild-type sequence, both mutant proteins show dramatic increases in binding affinity, with Nkx6.1mA showing slightly higher affinity than Nkx6.1mB. Importantly, all three proteins are produced at approximately equivalent levels, as judged by 35S-methionine incorporation (Fig. 4C). Although slight mobility differences are seen for the three proteins on native gel (presumably due to differences in net charge), disruption of these shifted bands by anti-Nkx6.1 antibody in Fig. 4B unambiguously identifies them as the Nkx6.1 proteins.

The Nkx6.1 BID Inhibits Heterologous DNA Binding Domains—Although the primary structural features of the BID (amino acids 306–364) are unique to the Nkx6 family, we
tested its ability to interfere with the DNA binding of heterologous transcription factors. We produced chimeric proteins in vitro with the BID fused to three structurally distinct DNA binding domains (the Pdx1 homeodomain, the Pax6 paired domain, and the yeast GAL4 DNA zinc finger binding domain) and tested for DNA binding by EMSA (Fig. 5).

Fig. 5 (lane 1) shows that, although (143–283)Pdx1 (a Pdx1 truncation that includes the homeodomain and native COOH terminus) exhibits appropriate binding to a TAAT-containing probe (the Nkx6.1 consensus), binding activity is abolished when the COOH terminus of Pdx1 is replaced by the COOH-terminal BID of Nkx6.1 (lane 2). As with native Nkx6.1, when mutation A (see Fig. 4A) is introduced into this Pdx1-BID fusion protein, binding activity is entirely restored (Fig. 5A, lane 3). The Pax6 paired domain has no sequence homology to the homeodomain (although it does have a similar helix-turn-helix tertiary structure when bound to DNA; Ref. 34) and binds an unrelated DNA sequence (4, 22). Interestingly, the Nkx6.1 BID also prevents binding by the Pax6 paired domain (Fig. 5A, lane 5). In contrast to both Pdx1 and Pax6, however, when the DNA binding domain of Gal4 (a zinc finger transcription factor; Ref. 35) is fused to the BID, binding activity does not decrease (Fig. 5A, lanes 6 and 7).

The Nkx6.1 BID Alters DNA Binding Affinity but Not Specificity—To test the binding site specificity of native Nkx6.1, its affinity for alterations to the consensus site was compared with the homeodomain alone. Fig. 6 shows that, although its absolute binding affinity is greatly reduced relative to the homeodomain alone, the native protein binds with the same relative affinities to the consensus probe and to probes with minor alterations to this sequence. Importantly, Nkx6.1mA and Nkx6.1mB also display parallel binding specificities for these same probes, suggesting that the mutations in these proteins do not change specificity. Although data do not demonstrate that DNA binding specificity is entirely unaffected in the native protein compared with the homeodomain alone, they strongly argue that it is DNA binding affinity that is primarily reduced in the native protein.

**Fig. 6.** The COOH terminus of Nkx6.1 does not affect DNA binding specificity. EMSA was performed with the in vitro produced proteins indicated and 32P-labeled Nkx6.1 consensus oligonucleotide (Consensus), 32P-labeled rat insulin I A3/A4 sequence (A3/A4), or 32P-labeled oligonucleotide M3 (M3) (see Table I). The free probe is indicated, and the arrow shows the position of the specific complexes corresponding to protein-bound probes.

**Fig. 7.** Transcriptional repression by Nkx6.1 is dependent upon site-specific DNA binding. A, various amounts of expression vector (Nkx6.1 or Nkx6.1mB) were cotransfected into NIH3T3 cells with 1 µg of Nkx6.1 reporter vector (containing five tandem Nkx6.1 binding sites upstream of TK-luciferase, shown above) or control reporter vector (TK-luciferase, without Nkx6.1 binding sites). Relative luciferase activities are calculated with the activity of cells transfected with the backbone expression vector without insert (pBAT12) set at 1. In order to highlight differences in repression, data are shown as -fold repression, the inverse of relative luciferase activity. B, a Western blot using anti-Nkx6.1 antibody was performed after separation by 4–20% gradient SDS-PAGE of nuclear extracts of NIH3T3 cells transiently transfected with expression vectors encoding no protein (control), Nkx6.1, or Nkx6.1mB. The arrow indicates the position of the native and mutant proteins (approximately 40 kDa).

**Nkx6.1 Functions as a Transcriptional Repressor**—To assess the transcriptional function of Nkx6.1, we performed cotransfection experiments in mammalian cell lines using expression vectors encoding the Nkx6.1 or Nkx6.1mB proteins, and a reporter vector containing five tandem copies of the consensus Nkx6.1 binding site upstream of TK-luciferase. Fig. 7A shows that native Nkx6.1 represses transcription from the 5X binding site reporter to a maximum of about 3-fold in NIH3T3 cells.
Fig. 8. Repression activity of Nkx6.1 maps to the NH₂ terminus. A Gal4 reporter construct consisting of five tandem copies of the Gal4 upstream activated sequence upstream of TK-luciferase (0.8 µg) was cotransfected with 0.8 µg of expression plasmid encoding the individual Gal4 fusion constructs indicated schematically on the left. The positions of the NK decapetide (NK Deca) and homeodomain (HD) are indicated. Data were corrected for transfection efficiency by use of a cotransfected CMV promoter-driven β-galactosidase plasmid (0.4 µg). Relative luciferase activities are calculated with the activity of cells transfected with the isolated Gal4-DBD (uppermost construct shown) set at 1. In order to highlight the magnitude of repression, data are shown as -fold repression, the inverse of relative luciferase activity. ** indicates that the magnitude of repression shown is significantly different from the Gal4-DBD alone (p < 0.05) by t test analysis. -fold repression is shown for the indicated NH₂- and COOH-terminal truncations of Nkx6.1 in NIH3T3 and β-TC3 cells. B, -fold repression is shown for constructs containing NH₂-terminal fragments of Nkx6.1 in NIH3T3 cells and β-TC3 cells.

Notwithstanding the fact that it is expressed at dramatically lower levels than native Nkx6.1 (Fig. 7B), the BID mutant, Nkx6.1mB, has a greater repressive effect, possibly due to its greater DNA binding affinity.

To determine if Nkx6.1-directed transcriptional repression can function through a heterologous DNA binding domain, and to map the Nkx6.1 repression activity, several one-hybrid mammalian expression constructs with portions of Nkx6.1 fused to the Gal4-DBD were tested in mammalian cotransfection experiments with a reporter plasmid containing five copies of the Gal4 DNA binding site upstream of TK-luciferase (Fig. 8). Western blots and EMSAs confirmed that the fusion constructs were expressed at comparable levels and were capable of binding DNA (data not shown).

Consistent with the results obtained with native Nkx6.1 binding to its consensus binding site, the full-length Nkx6.1 fusion protein represses transcription about 35-fold in NIH3T3 cells (Fig. 8A). As shown in Fig. 8A, all constructs containing at least amino acids 101–268 reduced transcription maximally. Notably, the Nkx6.1 BID (the COOH-terminal amino acids 306–364) does not affect transcription. Since Nkx6.1 expression is restricted to β-cells, we performed the identical cotransfection experiments in the β-cell line, β-TC3, as shown in Fig. 8A. Notwithstanding a lower magnitude of repression, results for all constructs parallel those obtained in NIH3T3 cells.

To determine if amino acids within the NH₂-terminal portion of Nkx6.1 are sufficient to direct transcriptional repression, additional Gal4 fusion constructs were tested as shown in Fig. 8B. It is readily apparent that amino acids 91–268 can cause transcriptional repression in mammalian cells to nearly the same magnitude as the full-length protein. Notably, the NK decapetide (aa 91–100), a sequence that resembles a Groucho corepressor interaction motif (36), contributes about 2–3-fold to transcriptional repression in these truncated proteins.

Nkx6.1 Represses Transcription through an Intact Promoter—To test the ability of Nkx6.1 to repress transcription through an intact promoter, cotransfection studies were performed in β-TC3 cells using the well characterized −350 bp rat insulin I promoter (20), which contains several potential Nkx6.1 binding sites, including the A3/4 site used in Fig. 2 (37). Fig. 9A demonstrates that native Nkx6.1 can repress the insulin promoter approximately 15-fold over base-line level, whereas it has minimal effects on the TK and CMV promoters. Fig. 9B shows that this repression by Nkx6.1 is mitigated by deletion of the amino-terminal domain (Nkx6.1Δ1), consistent with the findings in the Gal4 one-hybrid experiments. Moreover, since the homeodomain alone (Nkx6.1Δ2) has little effect on transcription, it is clear that repression by Nkx6.1 does not result merely from competition for binding sites with activators of the insulin promoter (e.g. Pdx1).

To determine if the transcriptional effect of Nkx6.1 on the insulin promoter is mediated through binding to one or more of the TAAT-containing sites, cotransfection experiments were performed with insulin promoters mutated at each of these sites (Fig. 9C). Interestingly, although individual mutation of any single TAAT site does not significantly affect transcriptional repression, mutation of all three sites simultaneously decreases repression dramatically, suggesting that repression of the insulin promoter by Nkx6.1 can be mediated by any of the three sites.

**DISCUSSION**

In this study, we present data on the unique DNA binding characteristics and transactivation properties of the β-cell differentiation factor, Nkx6.1. Nkx6.1 employs several strategies for identifying its genetic targets. The binding site selection studies demonstrates that the homeodomain binds to a highly specific, 8-base pair DNA sequence that contains a classic homeodomain binding core, TAAT. This binding core differs substantially from that of the Nkx2 family of homeodomain proteins, which bind the sequence (T/C)AAG (23, 38). The reason for this difference in sequence recognition can be attributed to a key amino acid difference that occurs at position 54 of the respective homeodomains (Thr in Nkx6.1 and Tyr in the Nkx2 proteins; Ref. 39). We show that Nkx6.1 cannot recognize the Nkx2 consensus. We demonstrate further that base pairs flank-
through TAAT sequences.

1 shown above each panel, were cotransfected with 0.25 mg of expression vector (encoding either Nkx6.1 or the truncated proteins shown). Relative luciferase activities are calculated with the activity of cells transfected with the control vector (without insert cDNA) set at 1. In order to highlight differences in repression, data are shown as -fold repression, or the inverse of relative luciferase activity.

Fig. 9. Nkx6.1 specifically represses the insulin promoter through TAAT sequences. 1 μg of each of the reporter constructs, as shown above each panel, were cotransfected with 0.25 μg of expression vector encoding either Nkx6.1 or the truncated proteins shown. Relative luciferase activities are calculated with the activity of cells transfected with the control vector (without insert cDNA) set at 1. In order to highlight differences in repression, data are shown as -fold repression, or the inverse of relative luciferase activity.

A, effect of Nkx6.1 on the TK, CMV, and rat insulin 1 gene (+350 bp to +1 bp) promoters. B, effect of Nkx6.1 deletional constructs (as defined in Fig. 3A) on the rat insulin 1 gene promoter. C, effect of Nkx6.1 on the insulin promoter containing mutations at each or all of three TAAT-containing sites (A3/4, A2/C1, and A1).
other genes that themselves function as transcriptional repressors.

Taken together, these studies demonstrate that Nkx6.1 can selectively target genes for suppression based on a tightly regulated DNA binding mechanism. Since TAAT sequences are common in gene promoters, unregulated repression of genes through these sequences could prove detrimental. On the other hand, if target selection is restricted by the flanking sequences, and the BID prevents DNA binding in the absence of modifying signals, transcriptional regulation can be very selective, explaining the restricted function of Nkx6.1 in vivo.

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Raghavendra G. Mirmira, Hirotaka Watada and Michael S. German

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