Transcription Factor Occupancy of the Insulin Gene in Vivo

EVIDENCE FOR DIRECT REGULATION BY Nkx2.2*

Consensus-binding sites for many transcription factors are relatively non-selective and found at high frequency within the genome. This raises the possibility that factors that are capable of binding to a cis-acting element in vitro and regulating transcription from a transiently transfected plasmid, which would not have higher order chromatin structure, may not occupy this site within the endogenous gene. Closed chromatin structure and competition from another DNA-binding protein with similar nucleotide specificity are two possible mechanisms by which a transcription factor may be excluded from a potential binding site in vivo. Multiple transcription factors, including Pdx-1, BETA-2, and Pax6, have been implicated in expression of the insulin gene in pancreatic β cells. In this study, the chromatin immunoprecipitation assay has been used to show that these factors do, in fact, bind to insulin control region sequences in intact β cells. In addition, another key islet-enriched transcription factor, Nkx2.2, was found to occupy this region using the chromatin immunoprecipitation assay. In vitro DNA-binding and transient transfection assays defined how Nkx2.2 affected insulin gene expression. Pdx-1 was also shown to bind within a region of the endogenous islet amyloid polypeptide, pax-4, and glucokinase genes that were associated with control in vitro. Because Pdx-1 does not regulate gene transcription in isolation, these sequences were examined for occupancy by the other islet transcriptional regulators. BETA-2, Pax6, and Nkx2.2 were also found to bind to amyloid polypeptide, glucokinase, and pax-4 control sequences in vivo. These studies reveal the broad application of the Pdx-1, BETA-2, Pax6, and Nkx2.2 transcription factors in regulating expression of genes selectively expressed in islet β cells.

Transcription of the insulin (INS) gene is restricted to pancreatic islet β cells. The 5′-flanking sequences within 350 base pairs of the transcription start site contain the binding sites for the factors that control cell type-specific expression (1–4). An extensive series of in vitro gel shift and cell transfection experiments indicates that regulation is mediated by islet-enriched DNA-binding transcription factors, including Pdx-1 (5–7), BETA-2 (8), and Pax6 (9). Strikingly, each of these factors is also a key regulator of pancreas development. Thus, homozygous loss of Pdx-1 leads to pancreatic agenesis in mice (10, 11) and humans (12), whereas there are severe defects in islet cell formation in BETA-2 (i.e., β, α, and δ cells; Ref. 13) and Pax6 (i.e. α cells; Ref. 14) null mice.

Islet cell development and function is also affected by other islet-enriched transcription factors (15, 16). For example, insulin is not made in β cells of mice lacking Nkx2.2 (17). Interestingly, this appears to be a rather specific effect as other β cell-enriched markers continue to be expressed (e.g., Pdx-1 and amyloid polypeptide (IAPP). Nkx2.2 and insulin are also co-expressed during embryogenesis, although Nkx2.2 expression is not restricted to β cells in adult islets (17). The mechanism by which Nkx2.2 regulates insulin gene transcription has not been resolved. Indeed, no direct gene targets have yet been reported for Nkx2.2 in the pancreas.

Assays such as in vitro DNA binding, co-transfection of reporter constructs, and transgenic knockout animals provide evidence for the involvement of a factor in transcriptional control. However, such experiments are indirect measures of regulation and do not account for conditions like competition with other binding proteins in vivo, chromatin structure of the endogenous gene, and cascades of regulatory interactions. In contrast, the chromatin immunoprecipitation (ChIP) assay (18–20) is a powerful tool for directly analyzing transcription factor site occupancy in vivo. Importantly, one can determine the physical association of a specific DNA-binding factor with potential control sequences in intact cells with this assay. This technique has been applied to many experimental systems, including in β cells to examine the in vivo occupancy of control sequences within the pdx-1 gene by Pax6 and HNF3β (21), within the glut2 glucose transporter gene by HNF1α (22), and within several β cell genes by Pdx-1 (23, 24).

In this study, the ChIP assay was used to assess the in vivo occupancy of insulin 5′-flanking sequences by Pdx-1, BETA-2, Pax6, and Nkx2.2. In addition to directly demonstrating binding of Pdx-1, BETA-2, and Pax6 to these sequences in intact β cells, Nkx2.2 was also found to interact. Analysis of the insulin 5′-flanking region target sequences by gel shift and transfected insulin-driven reporter assays suggests that Nkx2.2 reduces transcription upon binding at ~140–119 bp in β cell lines. Our findings that BETA-2, Pax6, and Nkx2.2 bind within the transcription control region of other islet-enriched genes also highlights how the ChIP assay can be used to identify targeted genes.

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Michelle A. Cissel‡, Li Zhao‡, Lori Sussel¶, Eva Henderson‡, and Roland Stein‡¶
From the ‡Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tennessee 37232 and the §Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver, Colorado 80262

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MATERIALS AND METHODS

Cell Culture and Transfection Analysis—Monolayers of cultures of insulin-producing pancreatic β cells line, βTC-3, HIT T-15,2,2, and MIN6 were maintained as described previously (25). The –238 WT CAT (chlamraphenicol acetyltransferase) plasmid contains sequences from –238 to +2 bp of the rat insulin II enhancer/promoter region linked to the CAT reporter gene (26). Construction of the insulin mutant (MT) expression vector, –238 CAT – 108,107 MT, has been described (25). The Nkx2.2 binding element mutant was generated in –238 WT CAT using the QuickChange mutagenesis kit (Strategene, La Jolla, CA) with an oligonucleotide containing the –133, –132 mutation (5′-CTTAG-CACCGGCGGTGTGGGAAACTCGAGC-3′; mutated nucleotides are underlined). The LipofectAMINE reagent (Invitrogen) was used to transfect 1 μg of the –238 CAT and Rous sarcoma virus (RSV)/lacZ reporter plasmids. The RSV enhancer-driven LUC expression vector, pRSVLUC, was used as a recovery marker. Extracts were prepared 40–48 h after transfection and assayed for LUC (27) and CAT (28) enzymatic activity. The CAT activity from the test construct was normalized to RSV-driven LUC activity. Each experiment was carried out at least three times.

ChIP Assay—ChIP assays were performed on βTC-3 cells as described (24) with the following modifications. Protein A-Sepharose was replaced with protein A/G-Agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the following antibodies were used: 1 μg of N-terminal (amino acids 1–75) or C-terminal (amino acids 271–283) Pdx-1 whole rabbit polyclonal antiserum; 20 μl of rabbit polyclonal Pax6 IgG (PRB-278P; Covance, Richmond, CA); 5 μl goat polyclonal BET2A IgG (sc-1084; Santa Cruz Biotechnology, Inc.); 25 μl mouse monoclonal Nkx2.2 antibody (74.5A5); Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA). Normal rabbit (sc-2027, goat (sc-2028), and mouse (sc-2025) IgG preparations were obtained from Santa Cruz Biotechnology, Inc., 10 μg of normal IgG from the same species of the experimental antiserum was used as a control. The PCR oligonucleotides used to detect mouse gene control sequences were as follows: INS ( – 378) 5′-GGAAAACGTGGAGAGGTCCAGG-3′ and ( – 46) 5′-CCCCCTGCGACTTTGCTGGTTT-3′; CCK ( – 36) 5′-GAGCTTCATCTTCTGATGCAG-3′ and ( – 36) 5′-CAACTTGGCT-CACCTGGACCTGAGC-3′; PCK ( – 453) 5′-GATTGAGCCACCTCACACGTTG-3′ and ( – 96) 5′-GGGACGGCTTGGATCATAGCC-3′; IAPP ( – 290) 5′-AACCTTACCCTACTTTCGAC-3′ and ( – 36) 5′-CAACTTGGCT-CACCTGGACCTGAGC-3′; pax-4 ( – 15) 5′-CGTGCAGAAGCCTGGGCTGGC-3′ and ( – 1628) 5′-CTTGGCGATGTTGTTGGAACGGG-3′; and 5′-CTTGGCGATGTTGTTGGAACGGG-3′. All PCR cycling parameters were: INS, PCK, IAPP, and CK, 1 cycle of 95°C for 2 min and 30 cycles of 90°C for 30 s, 68°C for 30 s, and 72°C for 30 s; pax-4, 1 cycle of 95°C for 2 min and 30 cycles of 90°C for 30 s, 68°C for 30 s, and 72°C for 30 s. Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from βTC-3 cells as described previously by Zhao et al. (25). The TNT-coupled reticulocyte lysate system (Promega, Madison, WI) was used in vitro to transcribe and translate the Nkx2.2 encoding plasmid, pNkx2.2 (17). Ten μg of nuclear extract protein or 2 μl of in vitro-translated protein was added to the binding buffer containing 10 mM HEPES (pH 7.4), 1 mM EDTA, 10% (v/v) glycerol, 100 mM NaCl, 2 mM dithiothreitol, and 1 μg poly(dI-dC). The double-stranded oligonucleotide probes were labeled with [γ-32P]ATP with T4 polynucleotide kinase. Binding reactions, containing 40 fmol of radiolabeled probe, were incubated at 4°C for 30 min. The competitor DNA was added at 10–50-fold molar excess prior to the addition of probe. Anti-Nkx2.2 antibody was pre-incubated with protein at 4°C for 20 min before addition to the binding reaction. The protein-DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel, which was then dried and exposed to autoradiographic film.

RESULTS

Transcription Factor Occupancy of Insulin Control Region Sequences in Vivo—The ChIP assay was first used to investigate binding of Pdx-1 to 5′-flanking control sequences of the insulin gene in an islet β cell line, βTC-3. Two distinct antibodies to Pdx-1 were used to immunoprecipitate formaldehyde cross-linked chromatin. Insulin 5′-flanking sequences (INS; nucleotides –378/−46) were selectively amplified by PCR from chromatin precipitated by anti-Pdx-1 antisera made to either the N- or C-terminal region, but not from chromatin treated with normal rabbit IgG or in the absence of antiserum (Fig. 1A). In contrast, Pdx-1 antisera did not immunoprecipitate control sequences from the PKC gene (nucleotides –434/−96), which is not expressed in β cells (Fig. 1A). Significantly, the amplified region of the mouse PKC promoter contains two TAT motifs that can compete for Pdx-1 binding in vitro (data not shown). Thus, although Pdx-1 can bind to both the insulin and PKC control sequences in vitro, the ChIP assay demonstrates that only Pdx-1 interacts with the endogenous insulin gene in β cells.

To further characterize the in vivo occupancy of the INS transcription unit, ChIP analysis was performed with antibodies to other potential transcriptional regulators, specifically BET2A (8) and Pax6 (9). Both anti-BET2A (Fig. 1B) and anti-Pax6 (Fig. 1C) antibodies effectively immunoprecipitated INS control sequences from formaldehyde cross-linked βTC-3 chromatin, but not PKC. Neither the INS nor PKC regulatory region was amplified from chromatin isolated in control immunoprecipitations.

Finally, we tested if the endogenous INS promoter is occupied by Nkx2.2, a factor that was proposed to be involved in expression as a result of its dramatic and selective effect on insulin levels in Nkx2.2 null mice (17). Anti-Nkx2.2 antibodies, but not control reactions, immunoprecipitated INS sequences from βTC-3 cells (Fig. 1D), whereas the PKC promoter was not immunoprecipitated by anti-Nkx2.2. The same patterns were observed with antisera to Nkx2.2, BET2A, Pdx-1, and Pax6.
with MIN6 β cells (data not shown). These results demonstrate that Nkx2.2, as well as Pdx-1, BETA-2, and Pax6, is physically associated with endogenous INS control region sequences in β cell lines. The binding detected in vivo for Pax6 (−317/−311 bp, Ref. 9), Pdx-1 (−201/−196 bp, Ref. 6, −85/−70 bp, Ref. 5), and BETA2 (−100/−91bp, Ref. 8) within the insulin gene presumably involves the control sites detected in DNA element gel shift assays.

Identification of an Nkx2.2-binding Site between Nucleotides −140 and −119 in the Insulin Gene—The insulin 5′-flanking region (−378/−46 bp) analyzed in the ChIP studies was found to contain potential Nkx2.2-binding elements at nucleotides −140 to −119 (site 1) and −193 to −172 (site 2) upon comparison to the Nkx2.2 consensus-binding site (29) (Fig. 2A). To determine whether Nkx2.2 binds to these INS elements, gel mobility shift assays were performed with in vitro-translated Nkx2.2 and probes to site 1, site 2, and the consensus element (Fig. 2B). Two major protein-DNA complexes were commonly formed with each element in the Nkx2.2 and control in vitro-translated reactions (see asterisk-labeled complexes in Fig. 2B). Significantly, a unique faster mobility complex was detected in the Nkx2.2 reaction with the INS site 1 and consensus element probes, although not with INS site 2 (Fig. 2B). To determine whether Nkx2.2 was involved in formation of this faster mobility complex, antisera specifically raised against this protein was added to the binding reactions. This complex was selec-

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Nkx2.2 binds to a site between nucleotides −140 and −119 in the insulin gene. A, sequences of the oligonucleotides used in the gel shift assays are shown in comparison with the Nkx2.2 consensus-binding element (29). The core-binding site nucleotides are underlined. B, gel shift assays were performed with radiolabeled probes corresponding to the Nkx2.2 consensus-binding site (lanes 1–3), INS site 1 (lanes 4–6), and INS site 2 (lanes 7–9). In vitro-translated control (C) or Nkx2.2 (NK) protein and Nkx2.2-specific antisera (+) were added to the binding reactions. The positions of the Nkx2.2-binding complex and the antibody super-shifted (SS) Nkx2.2 complex are marked with an arrow; nonspecific binding complexes are marked with asterisks. C, BTC-3 (lanes 1–2) and MIN6 (lanes 3–4) cell nuclear extracts were analyzed for INS site 1 binding in the presence (+) or absence (−) of Nkx2.2-specific antisera. The positions of the Nkx2.2-binding complex and the antibody super-shifted (SS) Nkx2.2 complex are marked with an arrow. D, in vitro-translated Nkx2.2 binding to the INS site 1 (lanes 1–9) and the Nkx2.2 consensus-binding site (Con, lane 10) probe was conducted in absence (−) or presence of a 10–50-fold molar excess of the consensus Nkx2.2-binding site (lanes 2–3), INS site 1 (lanes 4–5), INS site 2 (lanes 6–7), and the INS site 1 −133,132 mutant (lanes 8–9) competitor.

**Consensus Oligo:**

- INS Site 1: `5'-ACCAAGGGAGTTGGGAACT-3'`
- INS Site 2: `5'-CTAAGGTCTGGAGTTTG-3'`

**Site 1 -133,132 MT:** `5'-ACCAAGGGAGTTGGGAACT-3'`

**Nkx2.2 Consensus:**

- `T(T/C)AAGTG(A/G)(C/G)TT`
Role of Pdx-1, BETA-2, Pax6, and Nkx2.2 Transcription Factors

Fig. 3. Nkx2.2-binding site 1 inhibits insulin-driven reporter activity. HIT T-15, βTC-3, and MIN6 cells were transfected with the wildtype (WT) (~238 WT CAT, white bars), Nkx2.2 (~238 CAT −133,132 MT; dark bars), or RIPE3b1/C1 (~238 CAT −108,107 MT, grey bars) mutant versions of the rat insulin II ~238/+2 bp CAT expression plasmid. The rat insulin I and II genes share five of six nucleotide identity with the mouse insulin II genes in the core sequence of the Nkx2.2-binding site 1 and retain gel shift binding (data not shown). CAT results are normalized to the LUC activity from cotransfected pRSVLUC and are reported relative to ~238 WT CAT activity. The asterisk denotes that there was a statistically significant increase between ~238 CAT −133,−132 MT and the WT activity in HIT T-15 (p < 0.0001, Student’s t test), βTC-3 (p < 0.001), and MIN6 cells (p < 0.05). The data was compiled from at least six independently performed transfections.

Fig. 4. Pdx-1, BETA2, Pax6, and Nkx2.2 occupy the control region in vitro of many genes selectively expressed in β cells. Formaldehyde cross-linked chromatin from βTC-3 cells was incubated with antibodies raised to the N terminus or C terminus of Pdx-1 (panel A, lanes 3 and 4), BETA2 (panel B, lane 3), Pax6 (panel C, lane 3), or Nkx2.2 (panel D, lane 3). Immunoprecipitated DNA was analyzed by PCR with control region-specific primers to the mouse IAPP, pax-4, and GK genes. Control reactions were performed as described in legend to Fig. 1.

IAPP and pax-4 control sequences were also immunoprecipitated from βTC-3 cells by anti-BETA2 antibodies but not by control antibodies (Fig. 4B). These results confirm the in vitro studies that indicated that BETA2 was involved in pax-4 expression (37). BETA2 has also recently been shown to bind to the GK promoter in vitro and stimulate expression (38). In addition, antisera to Pax6 (Fig. 4C) and Nkx2.2 (Fig. 4D) selectively immunoprecipitated IAPP, GK, and pax-4 control sequences. Interestingly, the pax-4 promoter has been shown to contain a mutationally sensitive binding site that may be regulated by a factor in the Nkx2 family (39), although the precise binding protein had not been identified. In sum, these results suggest that Pdx-1, BETA2, Pax6, and Nkx2.2 define the core of a β cell-enriched transcription complex.

**DISCUSSION**

Though assays such as in vitro binding, co-transfection with reporter constructs, and transgenic knock-out animals provide evidence for the involvement of a factor in transcriptional control, they do not prove this directly. This is especially problematic for factors, such as Pdx-1, that recognize relatively non-selective binding sites. The consensus Pdx-1-binding element, TAAT(T/G) (40), is expected to occur once in every 512 bp. As control region sequences are often several hundred bases in length, the presence of this motif is not uncommon and would not unequivocally demonstrate Pdx-1 regulation even if it were capable of binding to a cis-acting control element in vitro. Likewise, transfection experiments do not account for the native chromatin structure of endogenous genes, which may preclude physiological regulation by a factor that, nonetheless, can bind in vitro. The ChIP assay combines two highly specific techniques, antibody-specific protein-DNA precipitation and PCR amplification, to demonstrate a physical association between a transcription factor and specific control region se-
Role of Pdx-1, BETA-2, Pax6, and Nkx2.2 Transcription Factors

sequences in intact cells. The ChIP protocol can establish direct interactions of a transcription factor and a gene that are known to be functionally related and, importantly, can also be used to identify interactions with candidate target genes.

Using the ChIP assay, we observed occupancy of insulin 5'-flanking control region sequences in βT3-3 and MIN6 cells by Pdx-1, BET2A, Pax6, and Nkx2.2 (Fig. 1). Our demonstration of Pdx-1 occupancy of insulin control sequences corroborates a recent study that was published while our work was in progress (23). Though there was evidence to suggest a direct role for Pdx-1 (5–9, 13, 41), BET2A (8, 13), and Pax6 (9) in insulin gene expression, it has been unclear whether Nkx2.2 directly or indirectly contributed. Thus, the evidence supporting an involvement was based upon the loss of insulin expression in Nkx2.2 null mice, under circumstances where other β cell markers continued to be expressed (17). However, a control site for Nkx2.2 within the insulin gene had not been characterized previously. Based on the reported consensus Nkx2.2-binding element (29), we have localized an Nkx2.2-binding site between –140 and –119 bp of the insulin gene by in vitro gel shift analysis (Fig. 2). Importantly, this site is mutagenically sensitive in transfection assays, indicating that Nkx2.2 binding can affect insulin gene activity (Fig. 3). Collectively, our results demonstrate that Nkx2.2 can directly regulate insulin transcription in β cells.

One would have expected that Nkx2.2 would have had a positive effect on insulin transcription from the results of the Nkx2.2 gene ablation studies in mice (17). However, Nkx2.2 appears to act as a weak repressor in β cell transfection assays performed with either an insulin enhancer (Fig. 3) or consensus site-driven reporter construct (29). Nkx2.2 is also known to function as a transcriptional repressor in the developing central nervous system, a function necessary in cell fate specification in the ventral neural tube (42). Interestingly, structure-function analysis of the Nkx2.2 protein has identified a strong C-terminal transactivation domain whose function is masked by the highly conserved NK2-specific domain (NK2-SD) in βT3-3 cells (29). As a consequence, it is possible that a co-factor expressed during pancreatic development or a stage-specific protein modification might interfere with the NK2-SD-mediated repressor function of Nkx2.2 and allow it to function as a transcriptional activator during development. In contrast, Nkx2.2 repressor activity would result if β cell lines or mature β cells lack this effector activity. By extension of this reasoning, we presume that Nkx2.2 also inhibits pax-4, GK, and IAPP gene expression upon binding in β cells. Although the Nkx2.2 regulatory site(s) within these genes has not been defined precisely, it is likely to be found among the consensus-like binding sites within their control region sequences (i.e. pax-4, –1940/–1933 bp, –1876/–1869 bp; GK, –542/–536 bp, –486/–479 bp; IAPP, –325/–319 bp, –286/–280 bp, –267/–261 bp, –146/–140 bp).

Having used the ChIP assay to demonstrate binding of Nkx2.2 to several genes expressed in an islet-enriched manner, we extended this study to examine if other key β cell regulatory factors also bound within their regulatory domains. Previously, Pdx-1 had been shown to bind to its own promoter region in the ChIP assay (23, 24). Two distinct antisera raised against Pdx-1 were found to immunoprecipitate the IAPP and pax-4 control sequences. Each of these regions has been reported to be a target of Pdx-1 function (IAPP, Refs. 34, 35, and 41; pax-4, Ref. 37) and was independently shown to be occupied in the ChIP assay (23). However, whereas we observed a clear interaction of Pdx-1 and the GK gene using antisera to both the N and C termini of Pdx-1, another group was unable to detect this association using a different Pdx-1 antiserum (23). This difference highlights an important limitation of the ChIP assay, which despite the power of this technique to firmly establish a physical association between a transcription factor and a control region with immunoprecipitating antisera, a negative result does not provide definitive evidence for the lack of such interactions. Another example involves the inability to detect the occupancy of the GLUT2 promoter by Pdx-1 in the ChIP assay (data not shown and Ref. 23), despite a great deal of evidence supporting a role in expression (15, 16). There are two likely explanations for these negative results. The microenvironment of the GLUT2 control region might preclude the exposure of Pdx-1 antigenic determinants required for immunoprecipitation, possibly due to interactions with adjacent binding proteins. Alternatively, it is possible that Pdx-1 does not directly regulate GLUT2 transcription or does so through elements that are located outside of the analyzed region. Both studies focused on the proximal region of the GLUT2 promoter found to bind Pdx-1 in vitro (data not shown and Ref. 23) and, as a result of methodological limitations, Pdx-1/–GLUT2 interactions that lie at ≥1000 bp away would not have been detected.

Interestingly, the Pdx-1, Pax6, BET2A, and Nkx2.2 regulatory factors of the insulin gene were all found to bind to intact β cells to the regulatory control regions of other genes selectively transcribed in islet cells. How each of these factors precisely mediates control within the context of the insulin, pax-4, IAPP, and GK transcription units is still unclear. As islet cell development was so profoundly affected in the null mutants of these factors, the knock-out studies in mice cannot be used to access how each factor controls β cell expression post-natally under normal or stressed states. Our data strongly suggests that Pdx-1, Pax6, BET2A, and Nkx2.2 represent the core components of a transcription complex of an islet-enriched gene, and presumably also contribute in their expression during development.

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Role of Pdx-1, BETA-2, Pax6, and Nkx2.2 Transcription Factors

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Michelle A. Cissell, Li Zhao, Lori Sussel, Eva Henderson and Roland Stein

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