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Characterization of Pancreatic Transcription Factor Pdx-1 Binding Sites Using Promoter Microarray and Serial Analysis of Chromatin Occupancy*[S]

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The homeobox transcription factor Pdx-1 is necessary for pancreas organogenesis and beta cell function, however, most Pdx-1-regulated genes are unknown. To further the understanding of Pdx-1 in beta cell biology, we have characterized its genomic targets in NIT-1 cells, a mouse insulinoma cell line. To identify novel targets, we developed a microarray that includes traditional promoters as well as non-coding conserved elements, micro-RNAs, and elements identified through an unbiased approach termed serial analysis of chromatin occupancy. In total, 583 new Pdx-1 target genes were identified, many of which contribute to energy sensing and insulin release in pancreatic beta cells. By analyzing 31 of the protein-coding Pdx-1 target genes, we show that 29 are expressed in beta cells and, of these, 68% are down- or up-regulated in cells expressing a dominant negative mutant of Pdx-1. We additionally show that many Pdx-1 targets also interact with NeuroD1/BETA2, including the micro-RNA *miR-375*, a known regulator of insulin secretion.

Nearly 800,000 new cases of diabetes mellitus are diagnosed every year in the United States. Although insulin replacement remains the mainstay of treatment, considerable effort has been devoted to developing approaches for cell-based therapy (1). Through these efforts, it may become possible to introduce cells into diabetic patients that not only produce insulin, but also respond appropriately to metabolic signals that regulate insulin production, processing, and secretion. Accomplishing this goal demands a better understanding of the essential determinants of the beta cell phenotype. Whereas no single transcription factor can be considered to be the sole determinant of beta cell function, the homeodomain-containing factor Pdx-1 (Ipf1/Idx-1/Stf-1/MODY4) has been shown to be a major regulator of beta cell function. Initially characterized because of its role in regulating insulin and somatostatin promoters (2–4), Pdx-1 is now known to be critical for many aspects of pancreatic development (5–9). Indeed, Pdx-1 is being tested for its ability to generate beta cells from embryonic stem (10) and liver cells (11–15) for use in beta cell transplants. Additionally, Pdx-1 is expressed in adult beta cells and thus is a potential target for therapeutics. In fact, a newly approved anti-diabetic drug, exendin-4, functions in part through an intracellular signaling cascade activating Pdx-1 (16–20).

For a transcription factor required for pancreatic development (6), and whose conditional deletion in mice leads to diabetes (8, 21), surprisingly little is known about Pdx-1 target genes. In beta cells, Pdx-1 is reported to directly control insulin gene expression (2) as well as the expression of the genes encoding glucose transporter 2 (*Slc2a2*) (22), islet amyloid polypeptide precursor (23), *Pax4* (24), synaptotagmin 1 (25), and *Pdx-1* itself (26, 27). Like many other homeodomain factors, Pdx-1 also represses expression of some target genes. One such example is glucagon. Normally expressed in pancreatic alpha cells, glucagon may be under direct repressional control by Pdx-1 in beta cells (8, 28). However, the number of target genes is certainly much greater, as *Pdx-1*$^{+/-}$ mice display pleiotropic phenotypes (5, 7).

With the aid of genomic sequencing and associated technologies, it is now known that the number of transcripts (29–31) and genetic regulatory domains (32, 33) is far greater than previous estimates. To identify new Pdx-1 binding sites, including sites in novel regulatory regions, we have used chromatin immunoprecipitation (ChIP)[2] (34) and a new promoter microarray produced by the Beta Cell Biology Consortium (Mouse PromoterChip BCBC-5B). In addition to promoter ele-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data and Tables S1–S4.

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[2] The abbreviations used are: ChIP, chromatin immunoprecipitation; Dox, doxycycline; GO, gene ontology; GST, genomic signature tag; Pdx-1, pancreatic and duodenal homeobox protein-1; SACO, serial analysis of chromatin occupancy; qRT-PCR, quantitative real-time PCR; RT, reverse transcriptase; DN, dominant negative; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum.

ments, this array contains enhancers and highly conserved regions that may contain novel regulatory elements or transcripts. Additionally, genomic elements are included that represent micro-RNAs, an abundant class of regulatory RNAs that also have implications for gene regulation. Lastly, the microarray contains putative regulatory regions identified through serial analysis of chromatin occupancy (SACO) (35), a technique that combines ChIP with a modification of LongSAGE (Serial Analysis of Gene Expression) (36). Review of the target gene list indicates that Pdx-1 binds to regulatory elements in genes involved in virtually every aspect of the insulin secretory process in beta cells, from glucose uptake and metabolism to insulin processing and release. The data provide a comprehensive framework for understanding *Pdx-1* knock-out mouse models that display beta cell dysfunction and systemic glucose intolerance (5, 7, 8, 21).

It is highly likely that Pdx-1 binding sites are embedded within larger genetic regulatory domains containing multiple transcription factor binding sites. To test this idea, we made a second SACO library from NIT-1 cells using antibodies directed against NeuroD1/BETA2. NeuroD1 is a critical determinant of pancreatic endocrine cell differentiation (37), and both Pdx-1 and NeuroD1 regulate insulin expression (2, 38). The results from this parallel screen reveal a high degree of target overlap between these two factors and suggest that the identified binding sites may denote regulatory modules (39, 40) that are occupied by multiple factors controlling gene expression in a combinatorial fashion. One co-regulated target that we identified is the micro-RNA, *miR-375*, believed to negatively regulate insulin secretion (41). This result uncovers a previously unknown genetic regulatory pathway involving micro-RNA in pancreatic beta cells and provides a model wherein Pdx-1 and NeuroD1 control both insulin gene expression and its secretion via *miR-375*.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse insulinoma NIT-1 cells were grown in Kaighn's modification of Ham's F-12 medium (F12K) (ATCC, Manassas, VA), which contains 1.5 g/liter sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen). The inducible DN-Pdx-1 rat insulinoma INS-1 cell line (lacking the first 79 amino acids) was produced by C. B. Wollheim and H. Wang (28). Stable and control INS-1 cells were both grown in RPMI medium (Invitrogen) supplemented with 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10% FBS, penicillin, and streptomycin. Cells were grown under selection with 50 μg/ml G418 sulfate (Promega, Madison, WI) and 50 μg/ml hygromycin (Invitrogen) and were induced by 300 ng/ml doxycycline (Dox) (Sigma) for 4 days, with media and drugs changed after 2 days. All cells were grown at 37 °C in a humidified chamber in 5% CO_2.

ChIP—Media was removed from confluent 10-cm plates of NIT-1 cells ($\sim 5 \times 10^6$ cells per plate) and replaced with 1% paraformaldehyde in 1× phosphate-buffered saline for 15 min at room temperature. The plates were washed twice with 1× phosphate-buffered saline on ice and harvested in ice-cold buffer consisting of 100 mM Tris-HCl (pH 9.4) and 10 mM dithiothreitol. Cells were centrifuged at 1000 × *g* at 4 °C for 5 min followed by a 1× phosphate-buffered saline wash. Cell pellets were lysed in 600 μl of buffer containing 20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100, and 1× protease inhibitor mixture (Roche Applied Science) and then sonicated to an average size of 2 kb using a bath sonicator (5 × 30 s, 140-W pulses with 30-s rest intervals in ice water) (Misonix, Inc., Farmingdale, NY). Samples were centrifuged and supernatants (400 μg of protein) were pre-cleared for 1 h at 4 °C with protein A-Sepharose (Invitrogen) that was previously blocked with bovine serum albumin (0.25%) and glycogen (0.24 mg/ml). Samples were centrifuged and supernatants were incubated overnight at 4 °C with 0.75 μl ($\sim$25 μg) of rabbit anti-Pdx-1 antiserum raised against the N terminus (5), 1 μl of C-terminal anti-Pdx-1 antiserum (Chemicon International, Temecula, CA), or 1 μg of purified rabbit anti-NeuroD1 raised against amino acids 122–165 (42). An equivalent amount of control rabbit antiserum (Sigma) was added to IgG samples and incubated overnight. Anti-Pdx-1 and NeuroD1 complexes were captured with 50 μl of protein A-Sepharose (50% slurry) for 2 h at 4 °C, then sequentially washed with lysis buffer 4 times for 10 min each, LiCl buffer (10 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate) once for 10 min, and TE buffer (50 mM Tris-HCl, pH 8.1, 1 mM EDTA), once for 30 min and again for 5 min. Elution buffer (100 mM $NaHCO_3$ and 1% SDS) was added directly to the beads and the immunocomplexes were dissociated in two sequential washes of 200 μl each for 15 min at room temperature. The bead supernatants were pooled and incubated at 65 °C overnight to reverse the formaldehyde cross-linking. The samples were extracted with 25:24:1 phenol:chloroform:isoamyl alcohol (Invitrogen) and nucleic acids were precipitated with ethanol. Each ChIP sample was resuspended in 100 μl of 10 mM Tris-HCl (pH 8.0).

Mouse Pancreatic Islet Preparation—Pancreata from eight C57BL mice aged 4 to 8 months were injected with 1 ml of cold Hanks' balanced salt solution (HBSS, Invitrogen) containing 0.3 mg/ml Liberase R1 (Roche Applied Science). Pancreata were then dissected and minced before incubating in a total volume of 5 ml of HBSS at 37 °C for 25 min to allow digestion to occur. Digestions were stopped with the addition of 45 ml of HBSS containing 10% FBS. Tissue suspensions were centrifuged at 1000 × *g* for 1 min, supernatants removed, and the pellets were resuspended in 25 ml of HBSS + 10% FBS. Pancreatic islets were visualized by addition of 0.5 mg/ml diphenylthiocarbazone (Sigma) and were placed individually into fresh HBSS and then into RPMI media containing 5 mM glucose, 10% FBS, and penicillin/streptomycin supplements. Approximately 400 islets were maintained overnight at 37 °C in a humidified chamber in 5% CO_2 before performing the ChIP assays.

Reverse Transcription—INS-1 control and DN-Pdx-1 expressing cells (5×10^5) were plated onto 12-well plates and treated with or without 300 ng/ml doxycycline for 4 days. Total RNA was isolated by extraction with TRIzol reagent (Invitrogen), and 500 ng was used for first-strand cDNA synthesis in a reaction containing 50 ng of random primers, 500 μM dNTPs, 1× first-strand buffer, 10 mM dithiothreitol, 200 units of Molo-

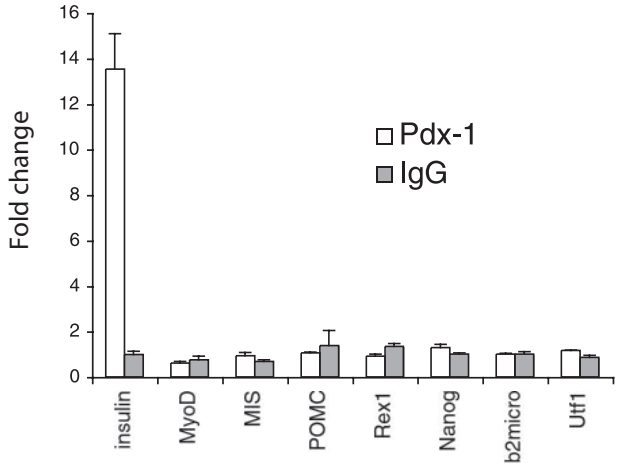

FIGURE 1. **Specific immunoprecipitation of insulin promoter chromatin by Pdx-1 antiserum.** Pdx-1-DNA complexes were isolated by ChIP from mouse insulinoma NIT-1 cells. Normal rabbit antiserum was used as a nonspecific antibody control. ChIP DNA was extracted, purified, and used in a PCR assay with primers encompassing the insulin promoter or primers designed against a panel of genes not expressed in NIT-1 cells.

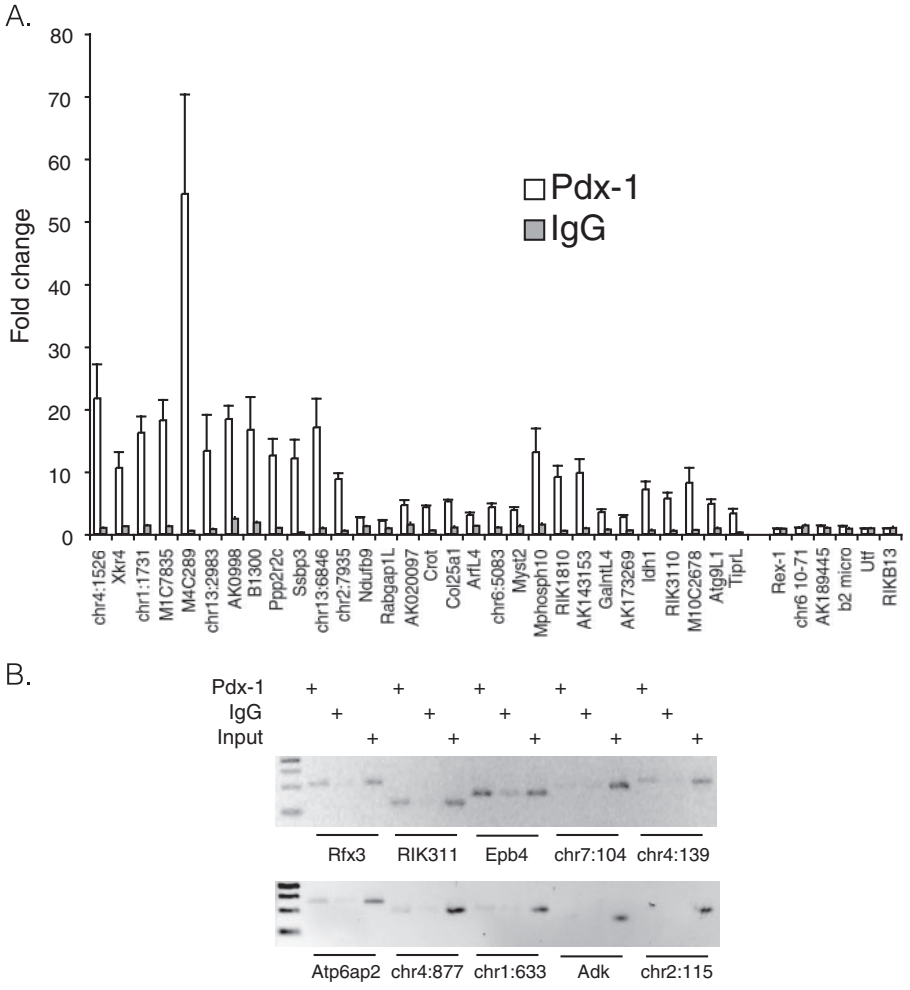

FIGURE 2. **Confirmation of high confidence Pdx-1 binding sites.** *A*, of the 583 candidate target genes, 30 were randomly selected for confirmation by repeat ChIP assay and qPCR analysis. DNA from three independent experiments was used in qPCR, and most primer sets were designed to the mean of the 1-kb sequence spotted onto the array. 100% of the tested sites were confirmed, as their Pdx-1 signal was 2-fold or greater than their IgG signal and 2-fold or greater than a panel of negative genes not expressed in NIT-1 cells. The six primer sets at the right of the figure represent negative controls. *B*, a subset of target genes were confirmed by ChIP assay from isolated mouse pancreatic islets. Pdx-1 and IgG ChIP assays were performed on paraformaldehyde-fixed islets from 8 mice. Standard PCR for the Pdx-1 and IgG ChIPs, as well as ChIP input DNA, were performed on the targets indicated.

ney murine leukemia virus reverse transcriptase (Invitrogen), and 20 units of RNasin ribonuclease inhibitor (Promega) in a 20-μl reaction volume. Samples were diluted 1:5 before using in quantitative PCR.

Quantitative PCR—ChIP DNA or cDNA (2 μl) was analyzed in a 10-μl real-time PCR (qPCR) containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 1× SYBR Green, 0.1 unit of Platinum *Taq* DNA polymerase (all PCR reagents from Invitrogen), and 1 μM primers (IDT, Coralville, IA). Primers were designed using Primer3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (43) using default parameters excepting: rodent and simple repeat mispriming library was used; product size was selected as 50–150 bp; optimal primer size was 23 nucleotides; primer T_m was between 65 and 70 °C, with an optimal T_m of 68 °C; maximal self-complementarity was 4.00; and maximal poly-x was 3. For ChIP confirmations, input sequences for Primer3 analysis were 400-bp regions surrounding the mean of the array or SACO loci. In a few cases, additional primers were designed outside this 400-bp window. PCR was performed using a PTC-200 DNA engine cycler with a CFD-3200 Opticon detection system (MJ Research, Inc., Waltham, MA) for one cycle at 95 °C (1 min) and 45 cycles of 94 °C (15 s) and 68 °C (40 s). Standard curves for each primer set were serial dilutions of ChIP input DNA that had been processed in a similar manner to ChIP DNA. Quantitative ChIP data were derived from the standard curve, and expressed as "-Fold change," in which the sample value was divided by the average of six control values, multiplied by 100. Pdx-1 positive signals were considered to be 2-fold above the average control value, and 2-fold above the average IgG ChIP signal. For RT-PCR, "percent change" was calculated from samples treated with or without Dox. PCR products were further analyzed on 4% NuSieve GTG low-melt agarose gels (Cambrex Bio Science, Inc., Rockland, ME). Only single-band PCR products were considered for analysis. Primer sequences are listed in supplemental Table S1.

Construction of Promoter Microarray—The Mouse PromoterChip BCBC 5B (www.cbil.upenn.edu/EPConDB/) contains over 18,000 1-kb tiles amplified from mouse genomic DNA, and includes promoters, enhancers, conserved sequences, micro-RNAs, and Pdx-1 SACO elements. The array contains 5100 proximal promoters, as determined from full-length cDNA

TABLE 1

Functional assignments for abbreviated group of Pdx-1 target genes based upon GO

Pdx-1 target genes were assigned functions based upon the GO categories of molecular function, biological process, or cellular component, and then classified into functional categories.

Gene name	Gene description
I. Cell adhesion	
Cdh24	Cadherin-like 24
Negr1	Neuronal growth regulator 1
Pak1	p21-activated kinase 1
Parvb	Parvin, β
II. Cell cycle	
Anapc5	Anaphase-promoting complex 5
Ccnb1	Cyclin B1
Ccrk	Cell cycle-related kinase
Cdk8	Cyclin-dependent kinase 8
III. Cell growth/death	
Bcl7b	B-cell CLL/lymphoma 7B
Casp3	Caspase 3
Fadd	Fas-associated via death domain
St18	Suppression of tumorigenicity 18
IV. Cytoskeleton	
Epb4.1	Erythrocyte protein band 4.1
Mark2	MAP affinity-regulating kinase 2
Myh9	Myosin, heavy polypeptide 9
Pfn2	Profilin 2
Sntb2	Syntrophin, basic 2
Sntg1	Syntrophin, γ1
V. Exocytosis	
Pclo	Piccolo
Rph3a	Rabphilin 3A
Syn1	Synapsin I
Syt7	Synaptotagmin VII
Syt11	Synaptotagmin XI
Syt13	Synaptotagmin XIII
VI. Hormone processing	
Pcsk1	Proprotein convertase 1
Pcsk2	Proprotein convertase 2
VII. Intracellular trafficking	
Arfl4	ADP-ribosylation factor 4-like
Chm	Choroidermia
Ica1	Islet cell autoantigen 1
Rab10	RAB10, member RAS family
Rab21	RAB21, member RAS family
Rab3ip	RAB3A interacting protein
Tom1l2	Target of myb1-like 2
Trappc2	Trafficking protein particle complex 2
Vamp8	Vesicle-associated membrane 8
Vapb	VAMP-associated protein B and C
VIII. Metabolism	
Atp5b	ATP synthase, F_1 complex, β
Atp5g2	ATP synthase, F_0 complex, c2
Eno1	Enolase 1
G6pc2	Glucose-6-phosphatase, catalytic, 2
Glud1	Glutamate dehydrogenase 1
Gyk	Glycerol kinase
Hmgcr	HMG-coenzyme A reductase
Mdh1	Malate dehydrogenase 1
Ndufb8	NADH dehydrogenase 1, β8
Pccb	Propionyl-CoA carboxylase, β
Pgam1	Phosphoglycerate mutase 1
Pla2g6	Phospholipase A2, group VI
Txn1	Thioredoxin 1
IX. Nuclear	
Crsp2	Cofactor required for Sp1, subunit 2
Hes6	Hairy and enhancer of split 6
Id3	Inhibitor of DNA binding 3
Isl1	Islet-1
Klf7	Kruppel-like factor 7
Mybl2	Myeloblastosis oncogene-like 2
Myst2	MYST histone acetyltransferase 2
Myt1	Myelin transcription factor 1
Neurod1	Neurogenic differentiation 1
Nkx2-2	NK2 transcription factor related
Pax6	Paired box gene 6
Pbx1	Pre-B-cell leukemia factor 1
Pdx-1/Ipf1	Insulin promoter factor
Trp53	Transformation related protein 53
X. Signal transduction	
Frap1	FK506-binding protein 12-rapamycin associated protein 1
Fyn	Fyn proto-oncogene
Il1r1	Interleukin 1 receptor, type I
Impa1	Inositol (myo)-1(or 4)-monophosphatase 1
Inpp5f	Inositol polyphosphate-5-phosphatase F
Pde4b	Phosphodiesterase 4B
Pde10a	Phosphodiesterase 10A
Pi4k2b	Phosphatidylinositol 4-kinase 2b
Pik3c2g	Phosphatidylinositol 3-kinase, C2 domain, γ
Pik4cb	Phosphatidylinositol 4-kinase, catalytic, β
Plcl3	Phospholipase C, η1
Ppp2r2c	Protein phosphatase 2, regulatory subunit B, γ
Prkca	Protein kinase Cα
Prkce	Protein kinase Cϵ
Psen2	Presenilin 2
XI. Transporter activity	
Abcc8	ATP-binding cassette, subfamily C, member 8
Atp6v0a1	ATPase, H^+ transporting, lysosomal V0 subunit A1
Cacna1c/Ca_v1.2	Calcium channel, voltage-dependent, L type, α1C subunit
Cacna1h/Ca_v3.2	Calcium channel, voltage-dependent, T type, α1H
Kcnj11/Kir6.2	Potassium inwardly rectifying channel, subfamily J, member 11
Slc2a3	Facilitated glucose transporter 3
Slc5a1	Sodium/glucose cotransporter 1

libraries and Reference Sequences (RefSeqs), and 7728 enhancers, defined as a 1-kb sequence containing the most putative transcription factor binding sites (based upon a combined TRANSFAC hit (*p* value) and human-mouse conservation score) within 5 kb of the transcriptional start site. The array also includes putative regulatory sequences highly conserved between human and pufferfish (1172 tiles) (44) and pancreatic-specific genes conserved between human and chicken (318 tiles) (45). 615 tiles span micro-RNA and surrounding sequences. Lastly, 1981 tiles were added that are potential regulatory regions as identified through the Pdx-1 SACO assay.

ChIP-on-Chip Assay—Pdx-1 and IgG ChIP DNA from two independent experiments, each performed in duplicate, were amplified by ligation-mediated PCR and fluorescently labeled as described (46). Labeled DNA was hybridized to the Mouse PromoterChip BCBC-5B.

SACO Assay—The Pdx-1 SACO library was made as described (35). Briefly, sonicated ChIP DNA was first amplified by ligation-mediated PCR, then digested with NlaIII (New England Biolabs, Beverly, MA) leaving an overhanging CATG. Adapters with corresponding CATG overhangs that contained a recognition site for MmeI (New England Biolabs) were then ligated. MmeI cleaves 20–22-bp downstream from its binding site, leaving a dinucleotide overhang. The digested fragments were then self-ligated to form "ditags." The Mme adapters were cleaved using NlaIII, concatamerized, and subcloned into pZERO-2 (Invitrogen). Sequencing of concatamers was performed by Rexagen Corp. (Seattle, WA).

Statistical Analysis—Detailed descriptions of the procedures are provided in the MIAME supplement. In brief, after examination of QA/QC metrics and normalization, a regularized *t* test was used to determine enrichment using a level of significance of 0.05. Putative targets were identified as those elements meeting both the statistical criteria as well as a biological

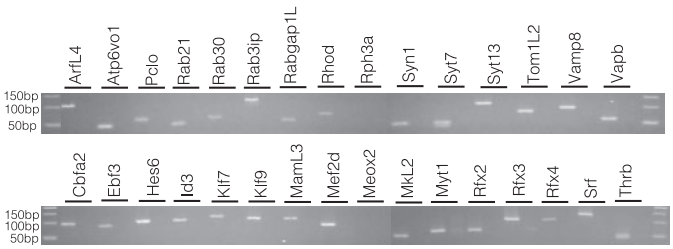

FIGURE 3. **Newly identified Pdx-1 target genes are expressed in beta cells.** RT-PCR was performed on randomly selected target genes involved in intracellular trafficking and exocytosis (*top panel*) and transcription co-activators (*bottom panel*). RT-PCR either with (*first lane of pair*) or without (*second lane of pair*) reverse transcriptase are shown.

threshold (*i.e.* fold > 1.4). For all tables, q-values are reported (47). The q-value of a test measures the proportion of false positives incurred (the false discovery rate) when that particular test is called significant. Annotation of the array was done using the mouse (mm8) assembly. Elements were mapped to target genes using the Entrez Gene Identifier. Categorical data analysis was performed to examine if particular TRANSFAC motifs, Gene Ontology (GO) categories, or KEGG pathways were over-represented in the candidate target gene list compared with the expected frequency of those categories based on the entire array.

RESULTS

Quality of Pdx-1 ChIP—To test the quality of the Pdx-1 ChIP, we measured the signal obtained on the well characterized Pdx-1 target gene, insulin. Pdx-1 ChIPs were performed using NIT-1 insulinoma cells, a mouse beta cell line, and the results were analyzed using qPCR. Fig. 1 demonstrates the specificity of the Pdx-1 antiserum, as there was an ~14-fold enrichment for the insulin promoter using anti-Pdx-1 *versus* IgG antiserum. As an additional control, genes not expressed in this cell line were also examined, demonstrating that the Pdx-1 antibody does not precipitate nonspecific sequences.

Analysis of Pdx-1 Genomic Binding Sites—A collection of high confidence binding sites was identified by hybridizing the Pdx-1 ChIP DNA and control IgG DNA to the Mouse Promoter-Chip BCBC-5B microarray. A high confidence binding site was defined as having a -fold change over IgG greater than 1.4 and p value <0.05, as well as meeting the QA/QC metrics for the array (based on PCR flags). 817 elements on the array were identified as high confidence sites (supplemental Table S2). This included 298 mouse-human conserved elements, 230 elements from the Pdx-1 SACO library, 152 proximal promoter elements, 107 human-pufferfish conserved elements, 22 human-chicken conserved elements, and 8 elements that were within 5 kb of micro-RNAs (supplemental Table S2). We analyzed the Pdx-1 positive elements for occurrences of a Pdx-1 binding motif as identified by the TRANSFAC data base (48). We found that this motif was highly enriched ($\chi^2 = 47.05$, $df = 1$, $p < 1 \times 10^{-5}$), when compared with all array tiles (subtracting out the SACO elements because they are already considered to be enriched for Pdx-1 motifs), providing additional evidence that the sites identified are valid Pdx-1 target sites.

To begin to assign functional significance to the Pdx-1 binding sites, we mapped the elements to 583 gene targets (based on unique Entrez Gene ID) after duplicate genes were removed. To confirm whether these high confidence binding sites were authentic, we randomly selected 30 for repeat ChIP assays using specific primers. 100% of the sites were confirmed by repeat ChIP assay and qPCR analysis (Fig. 2*A*). As qPCR analysis is more sensitive than the hybridization signal obtained from microarray analysis, in this case a positive signal was considered to have a -fold change of 2.0 above a nonspecific IgG ChIP and a -fold change of 2.0 above a selection of negative control genes. Negative control genes were selected because they were transcriptionally silent in NIT-1 cells, and the data are displayed at the far right in Fig. 2*A*. As an additional control, we repeated the ChIP assays using Pdx-1 antiserum that recognizes another epitope and obtained the same confirmation rate (data not shown). Furthermore, we performed ChIP assays for Pdx-1 and

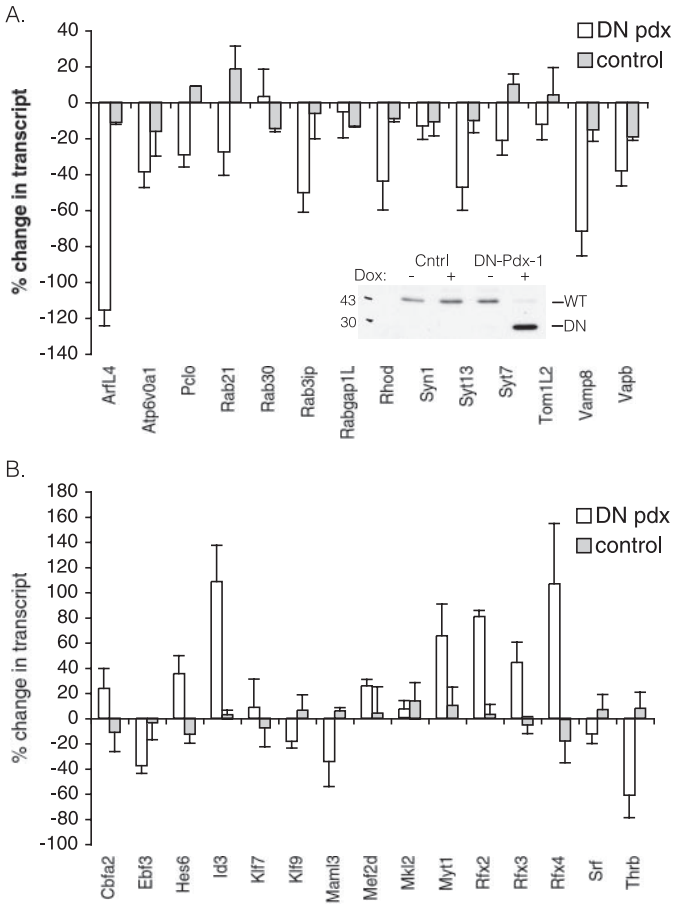

FIGURE 4. **Pdx-1 regulates the transcription of many newly identified targets.** *A*, targets involved in intracellular trafficking and exocytosis are predominantly down-regulated by DN-Pdx-1 induction. Rat insulinoma INS-1 cells expressing a stably inducible DN-Pdx-1 construct and INS-1 control cells were treated with or without 300 ng/ml Dox for 4 days to induce expression of DN-Pdx-1 and were analyzed by qRT-PCR. 14 random genes falling into the categories of intracellular trafficking and exocytosis were selected for this analysis. Data are from three independent experiments, each of which was repeated in triplicate. Data are normalized to 18 S RNA and are graphed as percent change in transcript between samples treated with or without Dox. Genes that would normally be activated by Pdx-1 show down-regulation in this assay. *B*, Pdx-1 target genes that encode transcriptional activators are both up- and down-regulated by DN-Pdx-1 induction. As above, 15 genes in this category were randomly selected for analysis. qRT-PCR was performed on samples from 3 independent experiments as detailed above. *Inset* shows an anti-Pdx-1 Western blot using cell lysates from parental or DN-Pdx-1 inducing cells, in the absence or presence of 300 ng/ml Dox.

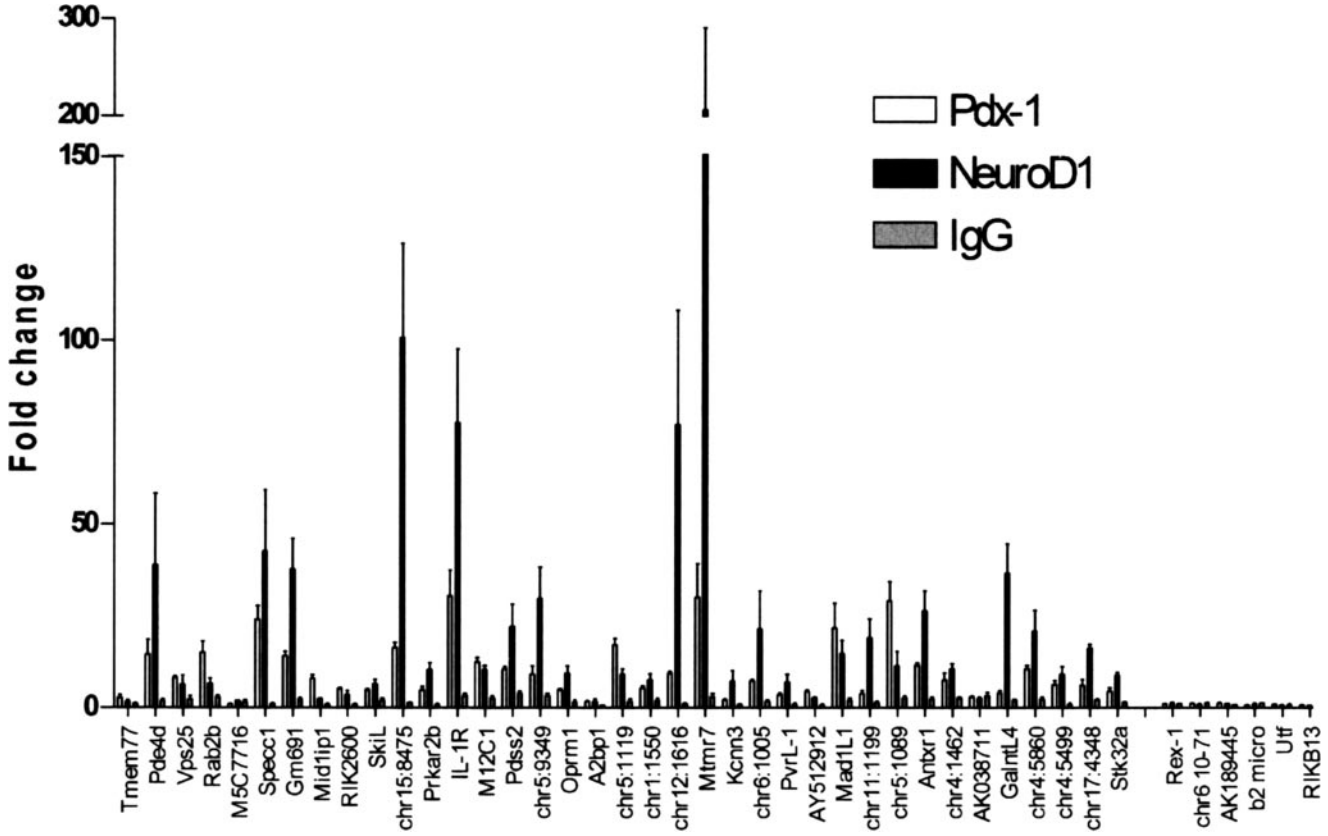

FIGURE 5. **Confirmation of Pdx-1 and NeuroD1 shared targets.** Of the 440 candidate target genes, 37 were randomly selected for confirmation by repeat ChIP assay and qPCR analysis. DNA from three independent experiments was used in qPCR. Primer sets were designed against the mean of the Pdx-1 and NeuroD1 GSTs. 92% (34/37) of the Pdx-1 and 89% (33/37) of the NeuroD1 tested sites were confirmed, as their ChIP signal was 2-fold or greater than their IgG signal and 2-fold or greater than a panel of negative control genes not expressed in NIT-1 cells. The six primer sets at the right of the figure represent the negative controls.

IgG using isolated mouse pancreatic islets and confirmed the majority (7/10) of targets tested (Fig. 2*B*).

Pdx-1 Target Genes Control Critical Functions of Beta Cell Biology—To categorize the properties of the genes associated with Pdx-1 binding sites, we utilized the GO biological process classification (www.geneontology.org) (Table 1 and supplemental Table S3). Genes associated with metabolism-related GO categories were significantly over-represented in the candidate target list. These categories included primary and cellular metabolism, as well as regulation of metabolism (all p values $< 1 \times 10^{-4}$). Other significantly over-represented GO categories include cell organization and biogenesis ($p = 0.0019$), regulation of cellular physiological process ($p = 0.0024$), cell division ($p = 0.0053$), cell development ($p = 0.011$), and establishment of localization ($p = 0.021$). Members of two pathways were also significantly over-represented in the candidate gene list: maturity onset diabetes of the young (KEGG MMU04950) and *n*-glycan biosynthesis (KEGG MMU00510) (both p values < 0.05).

Pdx-1 Regulates Transcription of Identified Target Genes—To test the functionality of the newly identified Pdx-1 binding sites, we measured transcript levels of 31 protein-coding genes located within 5 kb of a high confidence Pdx-1 binding site. Two categories of genes were selected based on GO classifications: transcription co-activators and mediators of intracellular trafficking and exocytosis. Genes in each category were randomly selected and analyzed by reverse-transcriptase PCR (RT-PCR) in rat insulinoma INS-1 cells. 29 of 31 genes were expressed, suggesting that the sites of Pdx-1 binding are highly correlated with transcription of the associated gene (Fig. 3).

To address whether these transcribed genes are under control of Pdx-1, we measured transcript levels in the presence or absence of a doxycycline-inducible dominant-negative Pdx-1 inhibitor (Fig. 4*A*, *inset*) (28, 49). This dominant-negative inhibitor (DN-Pdx-1) lacks the N-terminal transactivation domain and thus interferes with endogenous WT Pdx-1 activity by competing for promoter sites. Stable induction of this mutant protein in INS-1 cells reduces the expression of known Pdx-1 target genes and impairs agonist-stimulated insulin secretion (28, 49). To test whether DN-Pdx-1 induction influenced the expression of high confidence Pdx-1 target genes, we performed qRT-PCR on control and DN-Pdx-1 INS-1 cells treated for 4 days with or without doxycycline. Of 14 target genes functioning in exocytosis and intracellular trafficking, 9 were significantly down-regulated by DN-Pdx-1 (Fig. 4*A*), indicating that these genes are activated by WT Pdx-1. Of 15 target genes functioning as transcription co-activators, expression changes after doxycycline treatment were variable, with several genes being significantly up-regulated as well as several that were down-regulated (Fig. 4*B*). These data demonstrate that the majority of genes bound by Pdx-1 are also regulated in a Pdx-1-dependent manner in this cell culture system.

Identification of Pdx-1 Binding Sites

Pdx-1 and NeuroD1 Shared Targets—Pdx-1 and NeuroD1 cooperate to regulate transcription of the insulin gene (50, 51). Whether the binding sites for these two factors coexist in other genes has not been extensively examined, however. To address this question, we sequenced a NeuroD1 SACO library in parallel to the Pdx-1 SACO library. Both libraries were prepared from NIT-1 cells, allowing us to identify DNA sequences that were occupied by both factors. Pdx-1 and NeuroD1 ChIP DNA was processed into ~21-bp genomic signature tags (GSTs) as described by Impey *et al.* (35) and these GSTs were mapped in relation to each other. Pdx-1 and NeuroD1 GSTs that fell within 500 bp of each other were confirmed at a high rate (~90%) in repeat ChIP assays (Fig. 5). We next located target genes that were within 5 kb of either GST in our high confidence data set. In total, 440 RefSeq genes and micro-RNAs were found that had binding sites for both factors (Table 2 and supplemental Table S4).

Interestingly, one gene targeted by both factors is the micro-RNA, *miR-375*. Two distinct NeuroD1 binding domains were identified, the first located 500 bp upstream from the micro-RNA 5′ end, and the second located 1700 bp downstream (Fig. 6). We considered the first domain to be the proximal promoter, whereas the second domain a distal enhancer. Two potential NeuroD1 binding sites (E-boxes) are located in the proximal promoter region spanned by the ChIP primers. Likewise, the distal enhancer region also contains two conserved E-boxes that are spanned by the downstream ChIP primers (Fig. 6). Pdx-1 binding was also detected at both the proximal promoter and the distal enhancer (Fig. 6), however, no canonical Pdx-1 binding motifs were present at either site, raising the possibility that binding was through a non-canonical site or through an indirect mechanism.

TABLE 2

Functional assignments for abbreviated group of Pdx-1 and NeuroD1 shared target genes based upon GO

Target genes were assigned functions based upon the GO categories of molecular function, biological process, or cellular component, and then classified into functional categories. Abbreviated list of shared targets of Pdx-1 and NeuroD1 is shown.

Secretion	Transporters	Transcription	Micro-RNAs
Ap3d1	Abcc8	Foxo3a	miR-191
Exoc8	Ca_v 3.2	Id3	miR-222
Syngr4	Glut5	Nfkb1	miR-375
Syt13	Kcnh6	Stat3	miR-425
SytL3	Ryr1	Stat5a	miR-488

DISCUSSION

Characterization of the family of Pdx-1 and NeuroD1 genomic targets has many implications for the understanding and treatment of diabetes. Some of these genes may be potential drug targets. Moreover, the ability to convert embryonic stem and liver cells into the pancreatic lineage may be facilitated by knowledge of the transcriptional and signaling networks in beta cells (52–55). Finally, this analysis may identify new genetic markers for use in isolating pancreatic progenitors and mature beta cells (56). With these goals in mind, we have identified 583 new Pdx-1 candidate target genes in NIT-1 cells using a combined approach of hybridization and sequence-based technologies. We found that 29 of 31 Pdx-1 target genes tested had detectable transcript levels in INS-1 cells (Fig. 3). Expression of the majority was altered by induction of a dominant-negative version of Pdx-1 (Fig. 4). Furthermore, we have identified 440 genomic loci that contain linked Pdx-1 and NeuroD1 binding sites by aligning GSTs from two SACO libraries in parallel. This analysis suggests that a significant fraction of Pdx-1 and NeuroD1 binding sites exist within regulatory modules, where they may control gene expression in a synergistic manner.

The list of transcriptional targets presented here is intended to aid in the understanding of Pdx-1 and NeuroD1 biology. Results from Brissova *et al.* (5) using isolated islets from *Pdx-1*$^{+/-}$ mice point to impaired beta cell metabolism and Ca^{2+} signaling, whereas Johnson *et al.* (7) demonstrated defects in islet architecture and increased islet cell apoptosis. The Pdx-1 targets presented here may help explain the basis for these phenotypes. For example, GO classification indicated that metab-

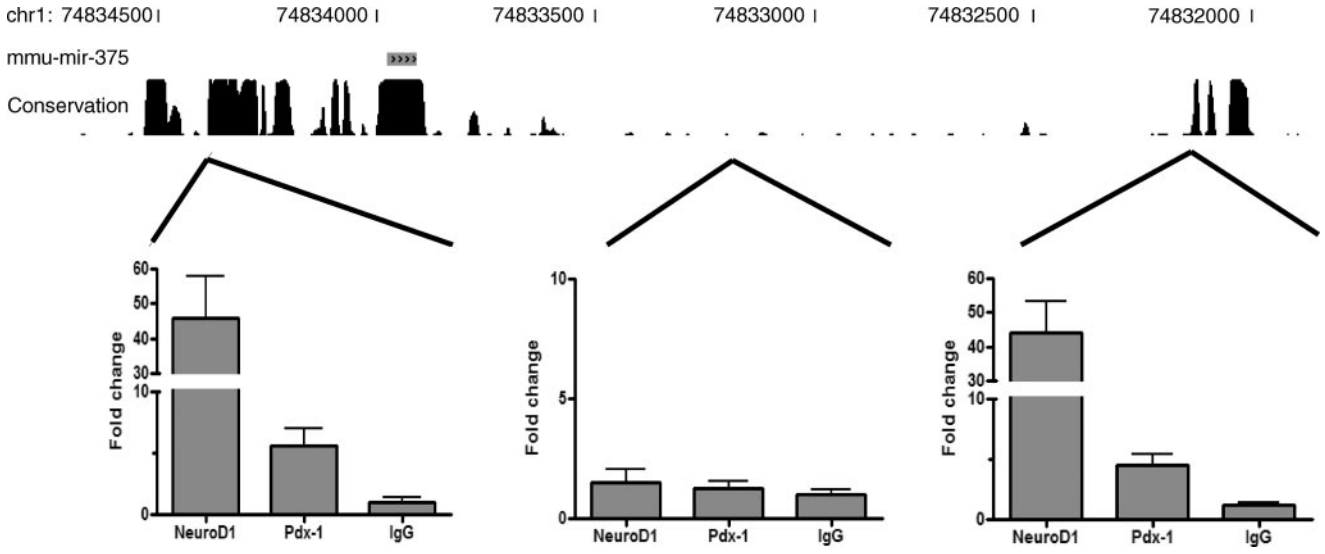

FIGURE 6. **NeuroD1 and Pdx-1 bind to the *miR-375* locus.** The *miR-375* locus is graphically displayed showing chromosome position, *miR-375* location, and UCSC Genome Brower conservation track. NeuroD1, Pdx-1, and IgG ChIP assays were performed from NIT-1 cells and qPCR was carried out using primers against the areas indicated. The data are graphed as -fold change and represent the -fold enrichment over a panel of six negative control genes that are not expressed in NIT-1 cells. The data were confirmed in mouse insulinoma MIN6 cells (data not shown).

olism-related genes were significantly over-represented, providing further evidence that Pdx-1 is critical for maintaining the proper metabolic state of beta cells (57). Our data also implicates Pdx-1 in regulating genes involved in Ca^{2+} signaling, for example, protein kinase C isoforms (*PKC* α and ϵ) and voltage-dependent calcium channels (Ca_v*1.2*, *1.4*, and *3.2*). Additionally, several target genes are involved in synthesis of inositol 1,4,5-triphosphate, a second messenger critical for opening intracellular Ca^{2+} channels. These include phosphatidylinositol 4-kinases (*PI4KB* and *PI4K2B*) and phospholipase Cβ. Still other Pdx-1 target genes may explain, in part, the increased apoptosis seen in *Pdx-1*$^{+/-}$ islets (7). Of note, one target is the transcription factor p53, a master regulator of cell growth arrest and cell death (58). Also, several target genes are involved in caspase signaling, including interleukin-1 receptor, Fas-associated death domain, and the effector caspase, *Casp3* (59). Misregulation of these genes may sensitize the cell to apoptotic stimuli. Identification of these Pdx-1 targets provides a genetic framework for understanding the *Pdx-1*-deficient mouse models.

Pdx-1 expression has been tested for its ability to convert embryonic stem cells (10) and liver cells (11–15) into beta cells. Data from our study and others (52–55) suggest that to re-enforce commitment to a particular lineage, multiple transcription factors may be required. For example, we have found that the homeobox protein Pax6 is a target of Pdx-1 (Table 1) and, in turn, Pax6 has been reported to regulate Pdx-1 (60). This relationship defines a multicomponent feedback loop that potentially can provide long-term stability to the system (54, 61–63).

As another example, we have identified a potential feed-forward circuit between Pdx-1 and NeuroD1. This type of network may impart sensitivity to sustained, as opposed to transient, inputs (54, 61–63). We discovered that Pdx-1 binds to the NeuroD1 promoter (Table 1), and in turn show that both Pdx-1 and NeuroD1 may cooperate to drive downstream expression of 440 additional genes as identified by our SACO analyses (supplemental Table S4). In support of this hypothesis, we identified several potential Pdx-1 binding sites 3.5 kb upstream from the *NeuroD1* transcriptional start site that are conserved between humans and fish.

One of the downstream target genes of both NeuroD1 and Pdx-1 is the micro-RNA, *miR-375*. This micro-RNA has been shown to negatively regulate insulin secretion in mouse insulinoma MIN6 cells (41) but to date no studies have investigated the regulation of micro-RNAs in pancreatic beta cells. We show here that there are at least two regions of the *miR-375* locus, termed the proximal promoter and distal enhancer, which are bound by both NeuroD1 and Pdx-1 (Fig. 6). As these two transcription factors are critical for beta cell development (64), we anticipate that *miR-375* will also play a role in beta cell development and may be controlled temporally during embryogenesis by NeuroD1 and Pdx-1. Future work will focus on the expression patterns of these three regulators during beta cell differentiation.

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