The transcription factor Pdx1 is expressed in the pancreatic β-cell, where it is believed to regulate several β-cell-specific genes. Whereas binding by Pdx1 to elements of β-cell genes has been demonstrated in vitro, almost none of these genes has been demonstrated to be a direct binding target for Pdx1 within cells (where complex chromatin structure exists). To determine which β-cell promoters are bound by Pdx1 in vitro, we performed chromatin immunoprecipitation assays using Pdx1 antiserum and chromatin from β-TC3 cells and Pdx1-transfected NIH3T3 cells and subsequently quantitated co-immunoprecipitated promoters using real-time PCR. We compared these in vivo findings to parallel immunoprecipitations in which Pdx1 was allowed to bind to promoter fragments in in vitro reactions. Our results show that in all cells Pdx1 binds strongly to the insulin, islet amyloid polypeptide, glucagon, Pdx1, and Pax4 promoters, whereas it does not bind to either the glucose transporter type 2 or albumin promoters. In addition, no binding by Pdx1 to the glucokinase promoter was observed in β-cells. In contrast, in in vitro immunoprecipitations, Pdx1 bound all promoters to an extent approximately proportional to the number of Pdx1 binding sites. Our findings suggest a critical role for chromatin structure in directing the promoter binding selectivity of Pdx1 in β-cells and non-β-cells.

The expression of genes in a cell type-specific manner is largely dependent upon the restricted expression patterns of the transcription factors that control those genes. Nowhere is cell type-specific gene expression more evident than in the β-cells of the pancreatic islets of Langerhans, where several genes including insulin, glucokinase, glucose transporter type 2 (Glut2), and islet amyloid polypeptide (IAPP) demonstrate β-cell-specific expression patterns. Although a network of transcription factors likely directs the overall expression of these β-cell genes (1–4), one particular factor, Pdx1 (also known as STF1, IDX1, and IPF1 (5, 6)), has recently emerged as one of the most important candidates orchestrating β-cell gene regulation.

Pdx1 expression has been demonstrated to occur in most cell types that comprise the developing pancreas (5, 7), and as such, targeted disruption of the Pdx1 gene in mice results in animals that lack pancreas formation (8, 9). However, Pdx1 expression in the mature pancreas is most prominent in the β-cell (6, 7), where it is believed to regulate several β-cell-specific genes. For example, the β-cell-specific disruption of the mouse Pdx1 gene results in animals that develop late-onset diabetes with impairments in insulin, IAPP, and Glut2 expression (10, 11). In addition, several functional studies (transient and stable transfections into cell lines, followed by reporter gene analysis) have addressed the role of Pdx1 in β-cell gene regulation; taken together, these studies point to an important role for Pdx1 in the regulation of the insulin (10, 12, 13), Glut2 (11, 14), IAPP (10, 15–17), glucokinase (18, 19), Pdx1 (20), Pax4 (21), and glucagon (10, 11, 15) genes.

Whereas the functional data described above are important in establishing a link between Pdx1 and the expression of specific genes, these studies do not address the issue of whether Pdx1 is involved in a transcriptional complex that directly controls the expression of these genes. Only circumstantial evidence suggests direct involvement; specific elements within the promoter regions of these β-cell-specific genes have been demonstrated to be bound by Pdx1 in in vitro electrophoretic mobility shift assays (EMSAs) (12, 14, 17, 19–21). Pdx1 is a homeodomain-containing transcription factor and demonstrates sequence-specific DNA binding (to TAAT-containing motifs), similar to the homeobox genes of Drosophila (22, 23). Because Pdx1 functions as an activator of gene transcription, it is therefore believed to activate gene expression by binding to upstream TAAT sequences (24).

Critical evidence that is lacking to establish Pdx1 as an immediate regulator of β-cell gene expression includes the demonstration that Pdx1 is physically associated with β-cell gene promoters in vivo. An emerging theme in transcriptional regulation is the influence of chromatin structure within the nuclear environment on both DNA binding by transcription factors as well as the resultant regulation of transcription (25, 26). The highly compacted nature of chromatin could conceivably hinder accessibility to some DNA binding sites but not to others, notwithstanding that these sites may display equal affinity for a given factor in vitro (27, 28). Thus, we cannot necessarily predict from studies in vitro which promoters Pdx1 binds in vivo.

To determine which promoters Pdx1 might directly regulate in vivo, we performed chromatin immunoprecipitation (ChIP) assays using Pdx1 antiserum and chromatin from various cell lines and followed this by quantitative real-time PCR to deter-
mine the relative distribution of co-immunoprecipitated promoters. We performed these studies in vivo using a non-β-cell line (NIH3T3) and a β-cell line (β-TC3) as well as in an in vitro setting for comparison. We demonstrate that Pdx1 is associated with several, but not all, β-cell-specific genes in vivo. We show further that there is a significant difference in the binding distribution of Pdx1 to promoters in vitro and in vivo as well as in β-cells and non-β-cells. Our findings suggest a model in which the chromatin structure of a given cell type plays an important role in restricting target promoter binding by Pdx1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—The mouse cell lines β-TC3 and α-TC1.6 were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin/streptomycin. The mouse fibroblast cell line, NIH3T3, was maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 1% penicillin/streptomycin. For transient mammalian cell transfections, 5 × 10⁵ NIH3T3 cells were plated on 60-mm plates 1 day before transfection. A total of 8 μg of either the cytomegalovirus promoter-driven expression vector pBAT12Plux (12), or the parent vector without insert (pBAT12) were mixed with 12 μl of Transfast (Promega), and transfections were performed according to the manufacturer’s protocol. Cells were harvested for chromatin immunoprecipitation assays or Western blots ~48 h later.

EMSA—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 strand. EMSA buffers and conditions were described previously (28). Where supershift assays were performed, 1 μl of Pdx1 antiserum (provided by Dr. M. German) was also added. Oligonucleotide fragments were derived from elements of each promoter known to be important in the transcriptional regulation of that gene and that contained a Pdx1 binding motif (TAAT). The albumin promoter oligonucleotide was selected based entirely upon the presence of a TAAT motif. The following oligonucleotide probes were used (top strands shown): mouse insulin promoter (A3/A4 element (12, 30)), 5’-CTTATAGACATAATACCTAACCTAAGACTA-3’; mutated mouse insulin promoter (A3/A4 element), 5’-CTTATACATAGATGATACCTAACCTAAGACTA-3’; mouse glut2 promoter (Glut2TAAT motif (14)), 5’-ATACACTGACCTTAATAAAGCCTAAGACTA-3’; mouse glucokinase promoter (UPF1 element (31)), 5’-GAACAAACCCAGTTTACCTTACGAGAAG-3’; mouse glucokinase promoter (UPF1 element (31)), 5’-GAACAAACCCAGTTTACCTTACGAGAAG-3’; mouse glucokinase promoter (UPF1 element (31)), 5’-GAACAAACCCAGTTTACCTTACGAGAAG-3’; mouse glucokinase promoter (UPF1 element (31)), 5’-GAACAAACCCAGTTTACCTTACGAGAAG-3’; mouse glucokinase promoter (UPF1 element (31)), 5’-GAACAAACCCAGTTTACCTTACGAGAAG-3’; mouse glucokinase promoter (UPF1 element (31)), 5’-GAACAAACCCAGTTTACCTTACGAGAAG-3’. Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed as described in the literature (33) with some modifications. A total of 1 × 10⁶ NIH3T3 cells (from 3 confluent 10-cm plates) or 1× 10⁷ β-TC3 cells (from 2 confluent 10-cm plates) were treated with 1% formaldehyde by adding 0.27 ml of 37% formaldehyde directly to 10 ml of culture medium. After incubating in formaldehyde for 10 min at room temperature, glycine was added to a final concentration of 0.125 M. The cells were then suspended in 0.6 ml of lysis buffer (50 mM Tris-Cl, pH 8.1, containing 1% Triton X-100, 0.1% deoxycholate, 150 mM NaCl, and 5 mM EDTA) plus protein inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and aprotinin) and subjected to sonication (using a Fisher Scientific model 60 sonic dismembrator with a microtip at a setting of 10). Ten 5-s sonication pulses were required for NIH3T3 cells, and 15 5-s pulses were required for β-TC3 and α-TC1.6 cells to shear chromatin to 1000-bp fragments. The effectiveness of shearing was confirmed by incubating a 10-μl aliquot of the extract at 65 °C for 3 h (to reverse cross-links) and subsequently subjecting it to electrophoresis on a 1% agarose gel. 0.25 μl of the clarified extracts were diluted to 1 ml in lysis buffer containing protease inhibitors and then incubated with either 5 μl of anti-Pdx1 antiserum or normal rabbit serum (25 μl was also divided into separate aliquots and stored for later PCR analysis as 10% of the input extract). Incubations occurred overnight at 4 °C on a rocking platform, after which 40 μl of protein A-agarose slurry (Santa Cruz Biotechnology) and 2 μl of a 10 mg/ml herring sperm DNA solution (Sigma) were added, and incubation was continued an additional 1 h.

The agarose was pelleted by centrifugation, and the pellets were washed consecutively with 1 ml of lysis buffer, lysis buffer plus 500 mN 1 mM NaCl, lysis buffer plus 0.25 μl LiCl, and Tris/EDTA. DNA and protein were eluted from the pellets by incubating the pellets 2 times in 0.25 ml of elution buffer (0.1 M NaHCO₃ with 1% SDS and 20 μg/ml herring sperm DNA, and DNA-Protein cross-links were reversed by incubating at 65 °C for 3 h. DNA and protein were ethanol-precipitated overnight at −20 °C. The precipitated samples were pelleted and dissolved in proteinase K buffer (10 mM Tris-Cl, pH 7.5 with 1% SDS) and incubated with 1 μl of RNase-free protease K (Roche Molecular Biochemicals) at 37 °C for 1 h at 55 °C. The samples were extracted once with phenol/chloroform and ethanol-precipitated overnight at −20 °C. Samples were pelleted, washed with 70% ethanol, and dissolved in 100 μl of Tris/EDTA. 3-μl aliquots were used for each real time PCR reaction to quantitate co-immunoprecipitated promoter fragments (see below).
Promoter Binding by Pdx1

Fig. 1. Pdx1 binds to diverse promoter elements in vitro. NIH3T3 cells were transfected with either a vector containing the Pdx1 cDNA or no insert, and nuclear extracts from these cells were prepared and used in EMSAs with $^{32}$P-labeled oligonucleotides corresponding to various promoter elements (as indicated above each lane). The sequences for the oligonucleotides are provided under “Experimental Procedures.” For each promoter element, EMSAs using both empty vector-transfected (−) and Pdx1 vector-transfected (+) nuclear extract are shown. For the insulin promoter A3/A4 element, a supershift (SS) assay was also performed (lane 3) using antibody (Ab) against Pdx1 to verify the shifted complex corresponding to Pdx1 binding. All promoter elements show specific Pdx1 binding (to various extents), with the exception of the insulin A3/A4 mutant (in which TAAT sites were mutated to TAGT). A large background band is observed in the EMSA with the insulin A3/A4 mutant probe due to factors endogenously expressed in NIH3T3 cells.

was performed for each sample after PCR amplification to ensure that a single product of expected melt curve characteristics was obtained. Western Blot Analysis—Nuclear extracts from NIH3T3, β-cell, and α-TC1.6 cells were prepared from single, confluent 10-cm plates of cells according to methods described previously (35). 5 μg of nuclear extract was subject to Western blot analysis after electrophoresis on a 4–20% gradient SDS-polyacrylamide gel using Pdx1 antiserum. Western blots were visualized using the ECL-Plus® system (Amersham Biosciences). Western blots from immunoprecipitated samples proceeded similarly, except that 20 μl of protein after elution (in 100 μl of Laemmli buffer) from the protein A-agarose was used in the analysis.

RESULTS

Pdx1 Binds to TAAT-containing DNA Sequences in Vitro—Several studies document the sequence-specific DNA binding properties of Pdx1 (12, 13, 36). To demonstrate that Pdx1 can bind to key promoter elements from a variety of β-cell-specific genes, we performed EMSAs (Fig. 1) using Pdx1 protein from transfected NIH3T3 nuclear extract and duplexed oligonucleotide fragments corresponding to known regulatory elements of the insulin, Glut2, and glucokinase genes. Fig. 1 shows that Pdx1 binds to upstream promoter elements from these genes and also demonstrates that when the TAAT sequences in the insulin promoter are mutated by a single base pair (TAAT to TAGT), DNA binding by Pdx1 is completely abolished (Fig. 1, lanes 4 and 5).

Fig. 1 also shows that Pdx1 is capable of binding to promoter elements from genes not expressed in the β-cell (Albumin and Glucagon, lanes 8–11), suggesting that Pdx1 binding to DNA elements in vitro is dependent primarily upon the presence of binding sites. Importantly, it should be noted that variations do exist in the extent of binding to each of these promoter elements, due presumably to variations in the DNA sequences that flank the TAAT motif (37). The data in Fig. 1 are unchanged when Pdx1 protein is obtained from β-cell nuclear extract (β-TC3 cells) or from an in vitro translation system using rabbit reticulocyte lysate (data not shown).

Chromatin Immunoprecipitation of Pdx1-associated Promoters—Because the data in Fig. 1 demonstrate poor promoter selectivity by Pdx1 in vitro, we sought to determine the distribution of Pdx1 promoter binding in vivo by use of the ChIP assay. The ChIP assay is a recently developed method that has typically been used to assess the association of abundant histone complexes with specific genes (33). When the ChIP assay is coupled to PCR-based detection methods, however, the association of far less abundant transcription factor complexes with specific genes can be reliably assessed (38). Fig. 2a shows the general scheme of the ChIP assays. Assays were performed using chromatin from three cell types: (a) NIH3T3 cells (a mouse fibroblast-derived cell line, which serves as a non-β-cell line control for our studies) transfected with either a vector containing the Pdx1 cDNA (referred to as “NIH3T3/Pdx1”) or empty vector (NIH3T3/EV) before ChIP; the β-TC3 and α-TC1.6 cells were untreated. The in vitro reactions consisted of nuclear extracts from NIH3T3/Pdx1 and NIH3T3/EV cells and 1 fmol of cloned promoter fragments in a 200-μl volume as detailed under “Experimental Procedures.” Note that although the in vitro reaction was immunoprecipitated similar to the cells, it was not treated by sonication since the promoter fragments were already in the 100–250-bp size range. b, Western blot demonstrating the expression of Pdx1 in NIH3T3/Pdx1 cells and native β-TC3 cells but not in NIH3T3/EV and α-TC1.6 cells. MW, molecular mass. c, 1% ethidium bromide-stained agarose gel demonstrating progressive shearing of chromatin from NIH3T3 cells with increasing 5-s cycles of sonication. Average chromatin length was about 1 kilobase pair (kb). The data for β-TC3 and α-TC1.6 cells were similar, except that 15 cycles were required to obtain the same average chromatin length (data not shown). d, Western blot demonstrating that Pdx1 (arrow) is recoverable from NIH3T3/Pdx1 cell extract after immunoprecipitation using Pdx1 antiserum but not using normal rabbit serum. The data are identical for immunoprecipitation from β-TC3 cell extract (not shown). The large background band likely represents immunoglobulin. Ab, antibody.

To compare the in vivo ChIP results from the cell lines, immunoprecipitation assays were also performed in an in vitro environment, where nuclear extracts from NIH3T3/Pdx1 and NIH3T3/EV cells were incubated with an equimolar mixture of
Promoter Binding by Pdx1

TABLE I

Identification of the promoter fragments and their corresponding primer sequences used for PCR amplification

| Promoter       | Amplified region | Number of Pdx1 binding sites (TAAT) | Reference                  | Primer sequences                                                                 |
|----------------|------------------|-------------------------------------|----------------------------|---------------------------------------------------------------------------------|
| Albumin        | Distal TAAT-containing region | 2                                  | 5'-TGGGAAAAACTGGGAAAAACCATC-3' | 5'-CAGCTCTCATACACTCCTGCTG-3'                                                     |
| Mouse I insulin| −126 to −296     | 2                                   | 5'-TCAGCCCAAGATGAAGAAGTCTGC-3' | 5'-TCCAACATTTGGCTTGGTC-3'                                                       |
| Glut2          | −523 to −738     | 3                                   | 5'-ATCTGGCTCGCCTCATCTTCTTG-3' | 5'-CCCCGAGTCITTTGCTGTCTATTGAG-3'                                                |
| IAPP           | −97 to −190      | 2                                   | 5'-TCACCACACCAAGCCACTAC-3'    | 5'-ATGGTTTCTGCGCTCCATTAC-3'                                                     |
| Glucagon       | (region 1)       | 0                                   | 5'-CTGAAAAAGAGATGACAAAGT-3'   | 5'-GGAACGCTGTCAGACAAGGAGAGACAC-3'                                              |
| Glucagon       | (region 2)       | 2                                   | 5'-TCCAAACTGCCCCCATTCCAC-3'   | 5'-GGGTGGGAGCTTAATTGCT-3'                                                       |
| Pdx1           | −2471 to −2598   | 2                                   | 5'-TGGCTCGGAAAGGCTTCTTG-3'    | 5'-CCATCGGTTGCGCTTAATTGACTATG-3'                                               |
| Glucokinase    | −112 to −255     | 2                                   | 5'-TGCATACACCAAGGCACTGAC-3'   | 5'-GGGAAACCTGGGGACTGATGC-3'                                                     |

a Where indicated, the numbers represent the position of the amplified fragment relative to the known transcriptional start site deduced for the gene.

cloned, 100–250-bp fragments of the promoters analyzed in this study (see Table I). The primary difference between the in vitro reactions and the in vivo experiments is that the promot- ers in vitro are not associated with histones or other modifying proteins and, therefore, have no chromatin-like compaction. Because the Pdx1 in these in vitro samples was from nuclear extract, we assume that any ubiquitous interacting proteins that might affect DNA binding by Pdx1 are also present in these reactions.

The cell lines and in vitro reactions were treated with 1% formaldehyde to cross-link proteins to chromatin, and the cell line samples were subsequently sonicated to shear the chromatin to an average of length of 1000 bp (Fig. 2c). All samples were then subjected to immunoprecipitation using either Pdx1 antisera or normal rabbit serum. To confirm that Pdx1 was successfully immunoprecipitated from samples containing the protein, we performed Western blots for Pdx1 on the final immunoprecipitated samples as shown in Fig. 2f.

Pdx1 Associates with Only a Subset of Promoters in NIH3T3 cells in Vivo but Predictably to All TAAT-containing Promoters in Vitro—Immunoprecipitated samples were subjected to quantitative real-time PCR using primers to amplify the β-cell gene promoters mouse I insulin, Glut2, IAPP, glucokinase, Pdx1, and Pax4 with non-β-cell gene promoters glucagon and albumin. Primer pairs (Table I) were designed to amplify specific promoter regions that are either believed to be bound and regulated by Pdx1 (promoters for mouse I insulin, Glut2, IAPP, Pdx1, Pax4, and glucokinase) (13, 14, 17, 19–21) or contain important regulatory regions with potential Pdx1 binding sites (promoter for glucagon (32). As a negative control for in vitro studies, the albumin promoter fragment was chosen as a random genomic element containing Pdx1 binding sites but not believed to be regulated by Pdx1. As a negative control for in vitro studies, a fragment of the glucagon promoter (referred to as glucagon region 1, see Table I) was chosen, which contained no TAAT sequences. Fig. 3 shows that PCR amplification of all promoter fragments from sheared NIH3T3 genomic DNA results in homogenous products of expected molecular weights.

Co-immunoprecipitated promoters were analyzed by quantitative, real-time PCR using SYBR Green I methodology. SYBR Green I is a intercalating dye that stoichiometrically incorporates into double-stranded DNA, resulting in fluorescence emission at 530 nm during early cycles of PCR that is proportional to the amount of DNA template present in the sample (41). As an example, Fig. 4 shows the results (in duplicates determinations) of the amplification of the mouse I insulin promoter from NIH3T3/Pdx1 and NIH3T3/EV cells after ChIP. The data demonstrate that the NIH3T3/Pdx1 chromatin that has been immunoprecipitated using Pdx1 antiserum reaches the “threshold fluorescence” value ~3 cycles before control immunoprecipitations (which include NIH3T3/Pdx1 chromatin immunoprecipitated with normal rabbit serum and NIH3T3/EV chromatin immunoprecipitated with Pdx1 antiserum or normal rabbit serum). This finding implies that there is an 8-fold (i.e. 2-fold) enrichment of insulin promoter in the NIH3T3/Pdx1 immunoprecipitation compared with NIH3T3/EV immunoprecipitations. Each value was normalized to the total input DNA (i.e. total genomic DNA) to correct for variations in input quantity of DNA before immunoprecipitation (as detailed under “Experimental Procedures”). The results shown in Fig. 5 summarize the real-time PCR results for each of the promoters considered in this study. The results shown in panel a are for the in vitro ChIP analysis of NIH3T3/Pdx1 and NIH3T3/EV cells, whereas panel b shows the results of the in vitro ChIP analysis using nuclear extracts from these cells.

The data in Fig. 5 reveal several important features of promoter binding by Pdx1. (a) In vivo (panel a), Pdx1 shows 8–25-fold greater association with the insulin, IAPP, glucokinase, glucagon, and Pax4 promoters than controls, whereas it displays no statistically significant association with the Glut2 and albumin promoters (notwithstanding interaction of Pdx1 with Glut2 and albumin promoter elements in vitro, see Fig. 1). Although binding to the Pdx1 promoter did not reach statistical
Fig. 4. Quantitative real-time PCR. Real-time PCR profiles for the amplification of the mouse I insulin promoter are shown for a representative ChIP assay in which chromatin from NIH3T3/Pdx1 and NIH3T3/EV cells was immunoprecipitated using either Pdx1 antiserum or normal rabbit serum. The data (shown in duplicate determinations) demonstrate the early exponential increase in fluorescence at 530 nm as a result of SYBR Green I incorporation into the amplifying insulin promoter fragment. Pdx1 ChIP represents duplicate curves from chromatin from NIH3T3/Pdx1 cells that have been immunoprecipitated with Pdx1-antiserum; Control ChIPs represent duplicate curves from immunoprecipitations from NIH3T3/Pdx1 chromatin using normal rabbit serum and from NIH3T3/EV chromatin using Pdx1 antiserum or normal rabbit serum. Input represents curves from total genomic DNA from NIH3T3/Pdx1 and NIH3T3/EV cells before immunoprecipitation. The cycle at which the amplification curve reaches threshold fluorescence (TF), the threshold cycle, is used to determine the relative amounts of promoter in each sample (as detailed under “Experimental Procedures”).

significance in the in vivo experiments, the data suggest a trend toward binding (greater than 7-fold over control). (b) In the in vitro reaction, Pdx1 binds to nearly all promoters but to an extent approximately proportional to the number of TAAT-containing sites within the promoter fragments; thus, the results show the greatest association (15–25-fold) with Glut2 and Pax4 promoters (containing 3 and 4 TAAT sites, respectively), no association with the glucagon (region 1) promoter (no sites), and intermediate levels of association (4–10-fold) with all other promoters (2 sites). (c) Significant differences in promoter association are observed between the in vivo and in vitro conditions; specifically, the Glut2 and albumin promoters show significantly greater association with Pdx1 in vitro than in vivo (about 5- and 3-fold higher, respectively), whereas the IAPP promoter shows greater association with Pdx1 in vivo than in vitro (about 4-fold greater). These findings suggest that chromatin structure in vivo may limit the accessibility of some promoters to Pdx1 binding while facilitating the accessibility of others (see “Discussion”). Of note, Pdx1 displays a significant (13-fold) level of association with the glucagon (region 1) fragment in vivo, notwithstanding that this region contains no TAAT sequences. Because the DNA in the in vivo experiments was sheared to 1000-bp fragments, this result is likely due to the binding of Pdx1 to TAAT sequences just outside of the 125-bp PCR-amplified region. As expected, the data in Fig. 5 show that no significant coimmunoprecipitation of promoters occurs from NIH3T3/EV cells or nuclear extract (since no Pdx1 protein is expressed).

Promoter Targeting by Pdx1 in β-TC3 Cells—To determine the distribution of promoter binding by endogenous Pdx1 in a β-cell-derived cell line, we performed ChIP assays in β-TC3 cells. As a negative control for these studies, we also performed ChIP assays in α-TC1.6 cells, an α-cell-derived cell line that does not express Pdx1 (see Fig. 2b). Fig. 6 demonstrates that in β-TC3 cells, Pdx1 strongly associates with the insulin, IAPP, and Pdx1 promoters (about 20–30-fold over controls) and moderately associates with the glucagon and Pax4 promoters (about 8–15-fold over controls). As in NIH3T3 cells, Pdx1 shows no significant association with the Glut2 and albumin promoters (1.5- and 1.2-fold over controls, respectively). In sharp contrast to NIH3T3/Pdx1 cells, however, Pdx1 demonstrates very little binding to the glucokinase promoter (about 3.5-fold over control, not statistically significant). As expected in these studies, α-TC1.6 cells show no coimmunoprecipitation of any of the promoters studied.

Promoter Binding by Pdx1 in Vivo Depends upon Cell Type—To directly compare the promoter binding distributions by Pdx1 in NIH3T3/Pdx1 cells, β-TC3 cells, and the in vitro binding assay, we normalized the fold differences observed for each promoter to the fold difference observed for the albumin promoter in the same experiment (a promoter not believed to be bound or regulated by Pdx1). These normalized data are presented in Table II. Three striking findings arise upon analysis of these data. First, binding to promoters with TAAT sequences in vitro displays an overall uniformity, with fold differences ranging from 2.3 to 5.9 (with the exception of the IAPP promoter). When these normalized values are corrected for the number of Pdx1 binding sites within each promoter fragment, the variations in promoter binding are virtually eliminated (Table II, values in parentheses). This finding emphasizes that the number of TAAT sequences is the primary factor in the binding of Pdx1 to targets in vitro. Second, the insulin, IAPP, and Pdx1 promoters show between 2- and 4-fold greater association with Pdx1 in β-TC3 cells than in NIH3T3/Pdx1 cells. This result suggests that the nuclear environment of the β-cell (primarily the local structure of chromatin but also the prevail-
ing protein makeup of β-cells) favors the binding of Pdx1 to genes that are expressed in β-cells.

Last, the data in Table II demonstrate that Pdx1 binds significantly less to the glucokinase promoter in β-TC3 cells than in NIH3T3/Pdx1 cells (2.8-versus 17.8-fold over controls), suggesting that the β-cell nuclear environment may also function to limit binding of Pdx1 to certain β-cell-specific genes. To determine whether nuclear proteins in β-TC3 nuclear extract might limit or prevent accessibility of Pdx1 to key glucokinase regulatory regions, we performed EMSAs using nuclear extract from β-TC3 cells, α-TC1.6 cells, and NIH3T3 cells and an oligonucleotide probe corresponding to a regulatory upstream promoter element at −206 to −226 of the glucokinase promoter (see Ref. 31). As shown in Fig. 7, Pdx1 from β-TC3 nuclear extract binds to the glucokinase promoter element to at least the same extent as observed in NIH3T3/Pdx1 nuclear extract (expression levels of Pdx1 in these extracts did not differ significantly, see Fig. 2b). This result suggests that the structure of chromatin in β-TC3 cells rather than the presence of inhibitory factors likely accounts for the lower association of Pdx1 with the glucokinase promoter in this cell type.

**DISCUSSION**

The first step in transcription factor action is the binding of the factor to upstream regulatory elements within the promoter of target genes. For Pdx1, both this initial binding step and subsequent gene regulatory events have been shown to be significantly influenced by the presence of interacting proteins within the nucleus, such as E47, NeuroD/BETA2, Pbx, and P300 (12, 13, 42–44). However, an additional factor that can affect DNA-protein interactions within the nucleus is the structure of chromatin. The nature of chromatin has been well studied, and it is clear that although histone packing around DNA, methylation of DNA at CpG dinucleotides, and secondary structural features of DNA can lead to well ordered chromatin compaction, the higher ordered nature of compacted segments cannot be predicted for any given segment of DNA nor for any given cell type (for review, see Ref. 45). That this nuclear chromatin environment can unpredictably affect promoter binding by transcription factors has been well recognized. For example, several *Drosophila* homeodomain transcription factors (Eve, Ftz, Prd, Bed) are known to bind to a set of target genes *in vivo* that cannot be ascertained from their *in vitro* DNA binding specificities (46, 47). By comparing *in vivo* and *in vitro* binding by use of a quantitative ChIP assay, we were able to determine how Pdx1 binding might be influenced by chromatin structure. Because our *in vitro* experiments used nuclear extracts (and thus contained a similar nuclear protein environment to the cell lines), we feel that our studies emphasize more of a role for chromatin structure (rather than interacting proteins) in promoter binding by Pdx1.

Several reports have established the utility of the ChIP assay in studying the mammalian transcription factors c-Myc, p53, and Hepatocyte Nuclear Factor 1-α (48–50). In addition, these and other (34) studies emphasize the quantitative nature of the assay in discriminating between different degrees of protein-DNA interactions. The data presented here are to our knowledge the first quantitative study of mammalian transcription factor binding distributions *in vitro* and *in vivo* using the ChIP assay coupled to real-time PCR. To understand our findings with regard to the functional studies on Pdx1 promoter regulation, we will separately discuss each of the
promoters analyzed in this study below.

**Albumin Promoter**—In our studies, the albumin promoter was used as an internal control for a random genomic element that contained Pdx1 binding sites but was not believed to be regulated by Pdx1. The albumin gene is regulated at the transcriptional level primarily in liver (51, 52), a tissue that does not express Pdx1 (6). Our finding that Pdx1 is not associated with the albumin promoter in either NIH3T3/Pdx1 fibroblasts or β-TC3 cells (despite its ability to bind to this promoter in *in vitro* assays) is important, since it implies that binding by Pdx1 to TAAT elements within the nucleus is not a stochastic event. Furthermore, our finding also suggests that high level overexpression of Pdx1 mediated by viral promoters in cell lines does not lead to promiscuous binding to random TAAT-containing DNA sequences.

**Insulin Promoter**—Several studies suggest that the insulin promoter is a target gene for Pdx1 action. Both germ-line (8, 9) and β-cell-targeted (10) mouse knockouts of the *pdx1* gene result in either absent or dramatically reduced expression of insulin, whereas ectopic expression of Pdx1 in non-β-cell tissue results in heterologous insulin expression (53, 54). These animal studies are supported by transfection experiments in various cell lines that point to specific A-box elements (sequences that contain the TAAT motif) in the insulin promoter as sites for Pdx1 interaction (6, 12, 13, 43, 55). Our studies now provide the first evidence that the insulin promoter is in fact a genomic target for Pdx1 binding in both β-cells and non-β-cells. Importantly, they also emphasize that the relative extent of association of Pdx1 with the insulin promoter is significantly greater in β-cells (by about 4-fold), implying that the chromatin structure and protein make-up of β-cells may make them more amenable to insulin gene regulation by Pdx1. In this regard, previous studies have linked enhanced Pdx1 binding (through interaction with basic helix-loop-helix factors and structural changes in DNA induced by High Mobility Group proteins) to cooperative transactivation of reporter genes (12, 13, 43).

**Glut2 Promoter**—Several studies suggest that Pdx1 activates the promoter for the *glut2* gene (10, 11, 14, 15). On the one hand, our findings confirm those of others that demonstrate that Pdx1 strongly interacts with Glut2 regulatory elements *in vitro*; on the other hand, our *in vivo* findings suggest that this level of interaction is not recapitulated within the nucleus, since no significant Pdx1 binding was observed to this promoter in NIH3T3/Pdx1 and β-TC3 cell lines. To reconcile our data with the functional observation that Pdx1 activates Glut2 expression, it would appear that either (a) Pdx1 indirectly activates the Glut2 promoter (through its effect on some other unidentified gene), or (b) Pdx1 is only a minor (i.e. weakly associated) component of a transcriptional complex that activates the Glut2 promoter. In support of the latter possibility, recent studies employing quantitative Northern blots show that overexpression of Pdx1 in a β-cell-derived INS-1 cell line leads to only a modest increase in Glut2 expression, whereas expression of a dominant negative form of Pdx1 reduced but did not eliminate Glut2 expression (15).

**IAPP Promoter**—Although the specific role of IAPP in normal β-cell function has remained unclear, the highly β-cell-specific nature of IAPP expression has led to studies examining its potential regulation by Pdx1 (10, 15−17). Our studies demonstrate an interaction of Pdx1 with the IAPP promoter *in vitro* and an even more prominent interaction *in vivo* in NIH3T3/Pdx1 and β-TC3 cells. These findings taken together with functional studies (16, 17, 29) suggest not only that IAPP is a direct target gene for Pdx1 but that the interaction of Pdx1 with the IAPP promoter is significantly enhanced by the chromatin environment of the cell.

**Glucagon and Pax4 Promoters**—During embryogenesis, the insulin (β) and glucagon-secreting (α) cells of the islet differentiate from a common (Neurogenin3-expressing) precursor as a result of variations in both the levels and types of transcription factors expressed (56−58). Pdx1 expression, although present in α-cells during embryogenesis, is most prominent in β-cells (5). Thus, although Pdx1 might be responsible for activating a subset of genes unique to β-cells, it is also conceivable that, through an alternative mechanism, it may be responsible for repressing another subset of genes in β-cells that are otherwise expressed in α-cells. Several studies suggest a direct and/or indirect role for Pdx1 in the repression of glucagon gene expression in the β-cell (10, 11, 15). As part of a direct role, Pdx1 may be involved in a larger transcriptional complex on the glucagon promoter that leads to its repression, notwithstanding that Pdx1 is an activator of transcription. In this regard, it has been demonstrated that the same basic helix-loop-helix factors (E47, BETA2) that interact with Pdx1 to activate the insulin promoter also serve to repress the glucagon promoter (59). Importantly, our results show that Pdx1 binds directly to the glucagon promoter *in vivo* in β-cells and non-β-cells, thereby suggesting a role for Pdx1 in directly regulating glucagon expression.

As part of an indirect role in glucagon gene regulation, Pdx1 might be involved in the activation of another gene, which itself is a direct repressor of the glucagon gene. Pax4 is a potent transcriptional repressor of the glucagon gene and is believed to suppress the α-cell gene expression program and commit endocrine cells to develop along β- and δ-cell lineages (60−62). Functional data in NIH3T3 cells show that Pdx1, in combination with Hepatocyte Nuclear Factor (HNF) 1-α, HNF4-α, and basic helix-loop-helix transcription factors, can dramatically activate the Pax4 promoter (21). Our study confirms the interaction *in vitro* of Pdx1 with the Pax4 promoter and demonstrates an even more prominent interaction *in vivo*. Taken together, our data with the glucagon and Pax4 promoters suggest a model in which Pdx1 may both directly and indirectly lead to the repression of glucagon gene expression in β-cells.

**Pdx1 Promoter**—Autoregulation is a prominent feature of biological systems that allows for amplification or attenuation of signals. It was recently suggested that Pdx1 might play a role in the activation of its own gene through interaction with the conserved Area 1 sequence within its promoter (20). In the same study, it was shown by qualitative ChIP that Pdx1 interacts with the Area 1 region in β-TC3 cells. Our ChIP studies confirm these findings but extend upon them by demonstrating that this interaction (similar to the insulin promoter interaction) is more robust in β-cells than non-β-cells by a factor of nearly 4-fold (Table II). This enhanced selectivity in β-cells might explain how the broad expression of Pdx1 in the early developing pancreas (5) becomes progressively amplified in β-cells as differentiation of cell types proceeds.

**Glucokinase Promoter**—Although cell line studies suggest a role for Pdx1 in the activation of the glucokinase gene (18, 19), recent studies involving inactivation of the Pdx1 gene in both animal and cell line models report contradictory findings on this issue (10, 15). These disparate results might be explained by the differences in cell line models used to study this regulatory phenomenon. In this regard, our findings demonstrate a significant diversity in the ability of Pdx1 to bind to the glucokinase promoter *in vivo*, since we observed robust binding in NIH3T3/Pdx1 fibroblasts and almost no binding in β-TC3 cells (Figs. 5 and 6 and Table II). Because we and others (19) demonstrate that no protein factors exist in β-cell nuclear extract to prevent the binding of Pdx1 to glucokinase promoter elements (see Fig. 7), it is likely that the absence of this binding inter-
action in β-TC3 cells reflects an inherently restrictive chromatin environment in the region of the glucokinase gene in this cell line. Indeed, our β-TC3 cell line may represent a more physiologically relevant cell model, since the β-cell specific inactivation of the pdx1 gene in mice shows no alteration in glucokinase expression (10).

Conclusions—Taken together, our findings form a framework upon which to merge functional and biochemical data on Pdx1 action. Although functional data may point to the regulation of specific genes by Pdx1, it is necessary to know which genes the factor actually binds to within the cell, since in vitro binding data may be misleading. Depending upon the cell type, it is clear that the structure of chromatin and the presence of interacting proteins have the potential to dramatically affect the genomic binding distributions of transcription factors. In this context, it may be very difficult to know precisely which cell line models and culture conditions faithfully recapitulate a developing or mature β-cell. However, the studies presented here introduce a new quantitative tool (ChIP/real-time PCR) that should allow us to look directly within the developing or mature islet in future experiments to see exactly how the dynamics of Pdx1-promoter associations may be correlated to phenotypic and morphologic changes during β-cell development and aging.

Acknowledgments—We are grateful to Dr. Richard Day for critically reading this manuscript. We also acknowledge Dr. Michael German for generously providing the Pdx1 antisera used in these studies.

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