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The Pancreatic Islet Beta Cell-Enriched Transcription Factor Pdx-1 Regulates *Slc30a8* Gene Transcription Through an Intronic Enhancer

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Synopsis

The *SLC30A8* gene encodes the zinc transporter ZnT-8, which provides zinc for insulin-hexamer formation. Genome-wide association studies have shown that a polymorphic variant in *SLC30A8* is associated with altered susceptibility to type 2 diabetes and we recently reported that glucose-stimulated insulin secretion is decreased in islets isolated from *Slc30a8* knockout mice. The present study examines the molecular basis for the islet-specific expression of *Slc30a8*. VISTA analyses identified two conserved regions in *Slc30a8* introns 2 and 3, designated enhancers A and B, respectively. Transfection experiments demonstrated that enhancer B confers elevated fusion gene expression in both β TC-3 cells and α TC-6 cells. In contrast, enhancer A confers elevated fusion gene expression selectively in β TC-3 and not α TC-6 cells. These data suggest that enhancer A is an islet beta cell-specific enhancer and that the mechanisms controlling *Slc30a8* expression in alpha and beta cells are overlapping but distinct. Gel retardation and chromatin immunoprecipitation (ChIP) assays revealed that the islet-enriched transcription factor Pdx-1 binds enhancer A *in vitro* and *in situ*, respectively. Mutation of two Pdx-1 binding sites in enhancer A markedly reduces fusion gene expression suggesting that this factor contributes to

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Author Contributions

L. P. performed most of the gel retardation and fusion gene studies and wrote parts of the manuscript.

Y. H. performed the ChIP assay studies and wrote parts of the manuscript.

S. S. designed the developmental islet gene expression studies and wrote parts of the manuscript

Y. W. assisted with the fusion gene expression studies.

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Slc30a8 expression in beta cells, a conclusion consistent with developmental studies showing that restriction of Pdx-1 to pancreatic islet beta cells correlates with the induction of *Slc30a8* gene and ZnT-8 protein expression *in vivo*.

Keywords

pancreas; transcription; enhancer; diabetes; zinc

Introduction

Since the derivation of insulin-secreting beta cells from stem cells represents a potential cure for type 1 diabetes, significant efforts have been made to understand the mechanisms controlling islet beta cell differentiation. Growth factors have been identified that drive the conversion of stem cells towards a beta cell fate [1] and multiple transcription factors, including Pdx-1, Isl-1, Pax-4, Pax-6, Nkx2.2, Nkx6.1, BETA2/NeuroD1 and MafA/B [2], have been shown to be important for islet-specific gene expression. Many of these transcription factors were identified through the analysis of key *cis*-acting elements in promoters and enhancers of genes whose expression are islet-specific or islet-enriched including those encoding insulin, glucagon, islet amyloid polypeptide, glucokinase, somatostatin and GLUT2 [2]. None of these transcription factors are islet-specific, rather islet-specific expression appears to be conferred by the particular combination of transcription factors bound to a given promoter or enhancer, with islet-enriched transcription factors playing a major role [2]. Several of these islet-enriched factors have been shown to be not only important for islet-specific gene expression in the adult but also for pancreas and islet development [2], underscoring how the study of islet-specific gene transcription can provide insight into islet physiology and development, in addition to the mechanisms of tissue-specific gene expression.

One of the most important islet-enriched transcription factors is Pdx-1, a homeodomain protein that, in adults, is primarily expressed in pancreatic beta cells and at low levels in pancreatic exocrine cells, islet delta cells and the duodenum [2]. It has been shown to regulate expression of a number of genes in islets including those encoding insulin [3], and *G6pc2*, also known as *IGRP* [4]. Although not sufficient by itself to mediate transcriptional activation, Pdx-1 is critical for *insulin* and *G6pc2* promoter activity and most likely functions in a higher order complex containing other islet-enriched factors [4, 5].

The studies described here were initiated with the goal of using the *Slc30a8* gene, which encodes zinc transporter-8 (ZnT-8), to supplement previous studies on islet-specific gene transcription. ZnT-8 belongs to a group of zinc transporters that, along with metallothioneins, are involved in intracellular zinc trafficking and storage so as to tightly maintain intracellular zinc homeostasis [6]. ZnT-8 is predominantly expressed in pancreatic alpha and beta cells [7-9], with much lower levels of expression in testis and submaxillary glands [10]. ZnT-8 localizes to insulin secretory granules within beta cells [11] and it is thought to be important for providing zinc to allow for proper maturation, storage and secretion of insulin [6].

Consistent with an important role for ZnT-8 in the beta cell, recent genome wide association studies have linked a single nucleotide polymorphism in amino acid 325 of human ZnT-8 to increased susceptibility to type 2 diabetes [12-15], gestational diabetes [16], impaired proinsulin to insulin conversion [17] and reduced first phase insulin secretion [17]. Interestingly, this same variant is also associated with autoantibody epitope specificity changes in human type 1 diabetes [18]. Also consistent with an important role for ZnT-8 in insulin secretion, we have recently shown that, in mice lacking ZnT-8, pancreatic islet zinc content and fasting plasma insulin concentrations are markedly reduced [9]. In addition, glucose-stimulated insulin secretion is impaired in islets isolated from ZnT-8 knockout mice, though glucose metabolism is surprisingly unaffected [9]. Related data from other groups suggest that the phenotype may vary depending on the genetic background [19, 20] and whether mice lack ZnT-8 globally or only in beta cells [21].

The studies described here identify key regulatory sequences driving expression of the *Slc30a8* gene. We identify two conserved intronic enhancers in *Slc30a8*, designated enhancer A and B, each of which contains multiple *cis*-acting elements that are critical for enhancer activity. Enhancer B is active in both alpha and beta cells whereas enhancer A is an islet beta cell-specific enhancer. We also show that two elements in enhancer A bind Pdx-1 *in vitro* and that Pdx-1 binds enhancer A in β TC-3 cells *in situ*, indicating that Pdx-1 plays an important role in the regulation of *Slc30a8* gene expression in beta cells, as it does with other genes whose expression are islet beta cell-enriched.

Experimental

Fusion Gene Plasmid Construction

For details on the generation of fusion gene plasmids please see Supplemental Data.

Cell Culture, Transfection and Luciferase Assays

Mouse islet β cell-derived β TC-3 cells and α cell-derived α TC-6 cells were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum whereas human cervix-derived HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) bovine serum.

For transient transfections, cells were transfected with 0.5 μ g of an expression vector encoding SV40-*Renilla* luciferase (Promega) and 2 μ g of a firefly luciferase pGL3 or pGL4 fusion gene plasmid using the lipofectamine reagent (GibcoBRL) as previously described [22]. Following overnight incubation in serum containing medium cells were harvested by trypsin digestion and then solubilized in passive lysis buffer (Promega). After two cycles of freeze/thawing, firefly and *Renilla* luciferase activity were assayed using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. To correct for variations in transfection efficiency, the results are expressed as the ratio of firefly:*Renilla* luciferase activity. In addition, three independent preparations of each fusion gene plasmid construct were analyzed in triplicate.

For stable transfections, cells were transfected with 2 μ g of a firefly luciferase pGL4 fusion gene plasmid using the lipofectamine reagent as previously described [22]. Following

overnight culture at 37°C, cells were incubated with serum containing medium supplemented with hygromycin (Sigma-Aldrich, St. Louis, MO) at a final concentration of 500 µg/ml. Every 2-3 days, hygromycin-containing media was replaced. After 3-4 weeks, or until individual colonies could be visualized, pooled colonies were harvested, luciferase activity assessed and the Pierce BCA protein assay used to correct for variations in protein content. In addition, three independent preparations of each fusion gene plasmid construct were analyzed in triplicate.

Gel Retardation Assay

A) Labeled Probes—Complementary sense and anti-sense oligonucleotides with overhanging GATC ends were synthesized, annealed and labeled with [α - 32 P]dATP using the Klenow fragment of Escherichia coli DNA Polymerase I to a specific activity of approximately 2.5 µCi/pmol [23]. [α - 32 P]dATP (>3000 Ci mmol⁻¹) was obtained from Perkin Elmer, Inc.

B) Low Salt Nuclear Extract Preparation—Low salt β TC-3, α TC-6, HeLa, and H4IIE nuclear extracts were prepared as previously described [23], except that the nuclear pellet was extracted with 20 mM HEPES (pH 7.8), 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2mM EGTA, 2 mM DTT, 25% glycerol containing 200 mM NaCl, instead of 0.4 M ammonium sulfate, and the supernatant was used directly in gel retardation assays. The protein concentration of the nuclear extracts was determined using the Bio-Rad assay and was typically ~1 µg µl⁻¹.

C) Binding Assays—~14 fmol of radiolabeled probe (~30,000 cpm) was incubated with 4 µg of β TC-3, α TC-6, HeLa or H4IIE nuclear extract in a 20 µl reaction volume containing 20 mM Hepes pH 7.9, 0.1 mM EDTA, 1 mM EGTA, 0.375 mM spermidine, 0.075 mM spermine, 12.5% glycerol (v/v), 1 mM dithiothreitol, 1 µg poly(dI-dC)-poly(dI-dC), and 50 mM NaCl. After incubation at room temperature for 10 min the reactions were loaded on to a 6% polyacrylamide gel containing 1X TGE (25 mM Tris Base, 190 mM glycine, 1 mM EDTA) and 2.5% (v/v) glycerol. Samples were electrophoresed for 1.5 hrs at 150V in 1× TGE buffer before the gel was dried and exposed to Kodak XB film with intensifying screens.

E) Competition Experiments and Gel Supershifts—For competition experiments, unlabeled competitor DNA was mixed with the radiolabeled oligomer at the indicated molar excess prior to addition of nuclear extract. For supershift experiments, specific antisera (1 µl) were pre-incubated with β TC-3 nuclear extract for 10 min at room temperature prior to the addition of the labeled oligonucleotide probe and incubation for an additional 10 min at room temperature. All subsequent steps were carried out as described above. An antiserum specific to Pdx-1 was a generous gift from Chris Wright (Vanderbilt University; Ref. [24]) whereas an antiserum specific to USF-1 (sc-229) was purchased from Santa Cruz Biotechnology, Inc.

Chromatin Immunoprecipitation (ChIP) Assays

β TC-3 cells ($0.5\text{--}1.0 \times 10^8$) were formaldehyde cross-linked and sonicated chromatin-DNA complexes were prepared as previously described [25]. The size of DNA fragments that were subjected to ChIP was ~ 500 bp. 20 μg aliquots of sheared chromatin were immunoprecipitated with either 5 μl rabbit α -MafA (Bethyl Laboratory, Inc.), 2 μl of rabbit anti-Pdx-1 (a generous gift from Chris Wright, Vanderbilt University) or normal rabbit IgG for 16 hours at 4 C. The resulting chromatin-antibody complexes were isolated with A/G-agarose (Upstate Biotechnology, Inc.). PCR was performed on 1/10 of the purified immunoprecipitated DNA using Ready-to-Go PCR beads (GE Health, Inc.) using 15 pmol of each of the following mouse primer pairs: *Pck1*, -434 (5'-GAG TGA CAC CTC ACA GCT GTG G-3') to -96 (5'-GGC AGG CCT TTG GAT CAT AGC C-3'); *Slc30a8*, +20153 (5'-CCC CAT CAT TCA TGG CTA AA-3') to +20467 (5'-TCA TTG CAA TAA TCC CCA CA-3'). The amplified PCR products were resolved by electrophoresis on 1.4% agarose gels.

Animal Care

The animal housing and surgical facilities used for the mice in these studies meet the American Association for the Accreditation of Laboratory Animal Care standards. All animal protocols were approved by the UC Denver Animal Care and Use Committee. Mice were maintained on standard rodent chow with food and water provided *ad libitum*. CD1 mice were procured from The Jackson Laboratories (Bar Harbor, Maine). Embryonic pancreata were harvested from time-mated mice at e12.5, e15.5 and e17.5 and immediately fixed for histological studies or pooled and stored in RNAlater (Ambion) at 4°C for subsequent expression analyses.

Immunohistochemistry

Mouse pancreata fixed in 4% paraformaldehyde were sectioned (6 μm) after embedding in OCT (Tissue Tek, Sakura Finetek, Inc, Torrance, CA). For immunofluorescence microscopy, sections were incubated for 1h at room temperature with blocking buffer (TSA system; Zymed, Invitrogen Corporation, Carlsbad, CA USA) and then overnight in a humid chamber with four primary antibodies: Guinea pig anti-insulin (1:50) (Sigma, St Louis, MO), rabbit anti- β -catenin (1:200) (Neomarkers, Fremont, CA), mouse anti-ZnT-8 (1:20) (John Hutton, UC Denver) and goat anti-Pdx-1 (1:10,000) (Chris Wright, Vanderbilt University). Sections were washed 3 times for 5 min in PBS before secondary antibodies (1:250) conjugated to AMCA, Cyanine 2, Cyanine 3 and Cyanine 5 fluorophores (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were applied and incubated at room temperature for 60 min. The sections were rinsed in PBS and mounted in a glycerol-based media. Image panels were acquired using Intelligent Imaging System software in conjunction with a Nikon Microphot FXA inverted microscope equipped with a Photometrics CoolSnap cooled monochromatic CCD camera. The images were later pseudo colored for illustration: red (Pdx-1), white (β -catenin) and green (insulin and ZnT-8).

RNA extraction and Quantitative RT-PCR

Total RNA was extracted from pooled fetal pancreata using TRIzol reagent® (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy columns (Qiagen, Valencia, CA, USA) with

RNA quality verified by capillary electrophoresis (Agilent-2100 Bioanalyzer; Agilent, Palo Alto CA, USA). cDNA was prepared from total RNA (1 µg) using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). 5 ng of cDNA samples served as the templates for amplification in a 5' nuclease assay based system using FAM dye labeled Taqman MGB probes (Applied Biosystems, Foster City, CA, USA) and a 96-well ABI 7000 PCR instrument. The glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) gene was used for sample normalization. The Cycle Threshold (CT) values were determined in triplicate and the samples were normalized relative to the CT values obtained using embryonic pancreatic samples from e12.5.

Statistical Analyses

The transfection data were analyzed for differences from the control values, as specified in the Figure Legends. Statistical comparisons were calculated using an unpaired Student's t test. The level of significance was $p < 0.05$ (two-sided test).

Results

A Conserved Islet Beta Cell-Specific Enhancer is Located in the Second Intron of the *Slc30a8* Gene

Intronic enhancers have previously been identified in several genes whose expression are enriched in islets including those encoding glucagon [26], islet amyloid polypeptide [27] and G6pc2 [28]. To determine whether intronic enhancers may also exist in the human *SLC30A8* and mouse *Slc30a8* genes a sequence alignment was performed using the VISTA program [29] focusing on the region between the translation start site and the last exon [7]. This analysis identified two conserved intronic regions, located between (+20125 and +20745) in intron 2 and between (+25228 and +25704) in intron 3 in the mouse *Slc30a8* gene (Fig. 1). These regions are found in similar locations in the human *SLC30A8* gene (data not shown).

We hypothesized these regions might represent transcriptional enhancers. To address this hypothesis these regions, designated intronic enhancers A and B, respectively, were isolated using PCR and ligated 5' of a heterologous *thymidine kinase* (*TK*)-luciferase fusion gene containing *TK* genomic sequence between -105 and +51, relative to the transcription start site [30]. Luciferase expression directed by these fusion genes was then analyzed by transient transfection of βTC-3 cells, an islet beta cell-derived line [31], αTC-6 cells, an islet alpha cell-derived line [32, 33] and HeLa cells, a cervix-derived cell line [34]. Figure 2 shows that in βTC-3 cells, but not αTC-6 or HeLa cells, intronic enhancer A elevated reporter gene expression beyond that driven by the *TK*-luciferase fusion gene alone, indicating that this region is an islet beta cell-specific enhancer. In contrast, intronic enhancer B had no effect on fusion gene expression in any of the cell lines (Fig. 2).

To address the possibility that intronic enhancer B is an enhancer but that this activity is not manifest in the context of the *TK* promoter, intronic enhancer B, as well as enhancer A, were ligated 5' of a heterologous *G6PC2*-luciferase fusion gene containing the proximal human *G6PC2* promoter sequence between -150 and +3, relative to the transcription start site. Luciferase expression directed by these fusion genes was then analyzed by transient

transfection of β TC-3 and α TC-6 cells. Figure 3A shows that, in β TC-3 cells, in the context of the *G6PC2* promoter, intronic enhancer B elevated reporter gene expression beyond that driven by the $-150/+3$ *G6PC2*-luciferase fusion gene alone. Figure 3A also shows that, in β TC-3 cells, intronic enhancer A markedly elevated reporter gene expression beyond that driven by the $-150/+3$ *G6PC2*-luciferase fusion gene alone, and to a much greater degree than enhancer B. In addition, this effect was independent of orientation (Fig. 3A), consistent with the strict definition of an enhancer [35], and much greater in magnitude than seen in the context of the *TK* promoter (Fig. 2). Strikingly, Figure 3A shows that, in α TC-6 cells, intronic enhancer B but not enhancer A elevated reporter gene expression beyond that driven by the $-150/+3$ *G6PC2*-luciferase fusion gene alone. These data demonstrate that enhancer B is active in both alpha and beta cells and, in contrast, again indicate that enhancer A is an islet beta cell-specific enhancer. Overall these data suggest that the mechanisms controlling *Slc30a8* expression in alpha and beta cells are overlapping but distinct. Because intronic enhancer B only enhanced reporter gene expression driven by the islet-specific *G6PC2* promoter and not the ubiquitously active *TK* promoter it is not possible to determine whether this enhancer is active in other cell lines or instead is an islet-specific enhancer.

Since enhancer A is a much stronger enhancer than enhancer B, subsequent studies focused mainly on enhancer A. To delineate the precise region of enhancer A that was primarily responsible for increasing fusion gene expression, enhancer A (+20125 to +20745) was arbitrarily divided into three regions, (+20125 to +20324), (+20325 to +20524) and (+20525 to +20745), designated regions 1-3. Each region was then ligated 5' of the $-150/+3$ *G6PC2*-luciferase fusion gene described above and reporter gene expression was again assessed following transient transfection of β TC-3 cells. Figure 3B shows that enhancer A region 2 markedly elevated reporter gene expression beyond that driven by the $-150/+3$ *G6PC2*-luciferase fusion gene alone and to a greater extent than regions 1 or 3. The effect of region 2 was also independent of orientation (Fig. 3B). This result suggests that region 2 represents the key region within enhancer A.

Alignment of mouse enhancer A region 2 using MacVector7.0 with the human, rat, dog and chicken sequences indicated that this region is highly conserved between species with 87%, 96%, 89%, and 70% identity to the human, rat, dog and chicken sequences, respectively (Fig. 4). Interestingly, this conservation exceeds the conservation observed with the -350 to -90 region of the mouse and human insulin promoters [36]. The conservation of enhancer B sequence across species was much lower (Suppl. Fig. 1). Due to the highly conserved nature of enhancer A region 2 and the presence of many putative transcription factor binding sites (Suppl. Fig. 2), a scanning mutation approach was employed to identify functionally important sites. Site directed mutagenesis was used to introduce 5 base pair block mutations across region 2 (Fig. 4) in the context of the enhancer A region 2 $-150/+3$ *G6PC2*-luciferase fusion gene described above. Reporter gene expression directed by the resulting fusion genes was again assessed following transient transfection of β TC-3 cells. The results show that multiple sites within region 2 were sensitive to mutation (Fig. 5), particularly those targeted by mutations 1, 7 and 8, 16, and 18 (Fig. 4). A similar functional analysis was performed with enhancer B. The data show that mutation of two conserved elements in enhancer B, that sequence analyses suggest have the potential to bind Pdx-1 and another

islet-enriched transcription factor, namely Foxa2 (Suppl. Fig. 1), reduce the activity of this enhancer (Suppl. Fig. 3).

Pdx-1 Binds to *Slc30a8* Enhancer A *in vitro*

Mutations 1 and 8 in enhancer A region 2 disrupt TAAT containing motifs that are conserved across multiple species including humans (Fig. 4). This motif represents the core binding site recognized not only by Pdx-1 but also many other homeodomain proteins [37]. Given the numerous reports demonstrating a role for Pdx-1 in the expression of beta cell-specific or -enriched proteins we next sought to determine if Pdx-1 interacts with these motifs.

The potential of these motifs to interact with Pdx-1 was first examined using gel retardation assays. When a labeled oligonucleotide, representing the *Slc30a8* region between +20325 and +20339 that encompasses the TAAT motif disrupted by mutation 1 (Fig. 6A), was incubated with nuclear extract prepared from β TC-3 cells a single, major protein-DNA complex was formed (Fig. 6B; see arrow). The specificity of this protein-DNA interaction was investigated by including various cold competitors in the gel retardation assay. Figure 6B shows that the wild-type (WT) unlabeled +20325/+20339 oligonucleotide competed for formation of the major protein-DNA complex whereas an oligonucleotide containing just two point mutations in the TAAT motif (Fig. 6A), rather than the more extensive block mutation used in the fusion gene experiments (Fig. 4), did not compete, suggesting that the complex represents a specific protein-DNA interaction whose formation is dependent on the TAAT motif and correlates with the gene expression data (Fig. 5).

Figure 6C shows that this complex is only detected using nuclear extract prepared from β TC-3 cells and not α TC-6, H4IIE or HeLa cells, indicating that the factor present in the complex is β -cell-specific or -enriched. To assess the presence of Pdx-1 in the major protein-DNA complex, nuclear extract was pre-incubated with antisera raised to the amino terminus of Pdx-1 [24] or, as a negative control, to USF-1. As can be seen in Figure 6D, the USF-1 antiserum had no appreciable effect on formation of the TAAT-specific complex. In contrast, pre-incubation with the Pdx-1 antisera resulted in the disappearance of the TAAT-specific complex and the appearance of a lower mobility, or supershifted complex, suggesting that the major complex detected represents Pdx-1 binding. Finally, gel retardation analyses using Pdx-1 synthesized by *in vitro* transcription and translation directly demonstrated that Pdx-1 can bind to the +20325/+20339 region of enhancer A (Fig. 6E).

Similar analyses were performed using a labeled oligonucleotide, representing the *Slc30a8* region between +20380 and +20409 that encompasses the TAAT motif disrupted by mutation 8 (Suppl. Fig. 4A). The results show specific protein binding to this region (Suppl. Fig. 4B) by a beta cell-specific factor (Suppl. Fig. 4C) that cross-reacts with Pdx-1 antisera (Suppl. Fig. 4D) and also that Pdx-1 produced by *in vitro* transcription and translation can directly bind to this element (Suppl. Fig. 4E). Interestingly, in contrast to the +20325/+20339 element, the +20380/+20409 element forms two complexes with β TC-3 nuclear extract that both cross-react with the Pdx-1 antiserum (Suppl. Fig. 4B & D). Previous studies have shown that Pdx-1 can bind certain elements either alone or in a complex with other factors [38].

Pdx-1 Binds to *Slc30a8* Enhancer A *in situ*

To complement the results of the *in vitro* gel retardation analyses, chromatin immunoprecipitation (ChIP) assays were performed to assess Pdx-1 binding to *Slc30a8* enhancer A region 2 within intact cells. Fragmented chromatin from formaldehyde cross-linked β TC-3 cells was subjected to immunoprecipitation with antibodies to either MafA or Pdx-1. The presence of region 2 in the immunoprecipitates was then analyzed by PCR using primers that recognize the *Slc30a8* gene sequence between +20153 and +20467. As can be seen in Figure 7, region 2 was enriched in the Pdx-1 immunoprecipitates compared to the MafA immunoprecipitates and IgG control. To test the specificity of the antibody-protein interactions, these immunoprecipitates were also analyzed for the presence of the *Pck1* gene, which is not expressed in these cells [25] using PCR primers that recognize the *Pck1* promoter sequence between -434 and -96. As expected no enrichment of the *Pck1* promoter was detected in the Pdx-1 immunoprecipitate compared to the IgG control (Fig. 7). The low signal in the experimental lanes cannot be explained by the lack of *Pck1* promoter in the starting material as a signal of the expected size was obtained when the PCR was performed using the chromatin input prior to immunoprecipitation. These results demonstrate that Pdx-1 binds to *Slc30a8* enhancer A region 2 within intact cells.

The Restriction of Pdx-1 to Pancreatic Islet Beta Cells Correlates with the Induction of *Slc30a8* Gene and ZnT-8 Protein Expression

Pdx-1 is expressed throughout the pancreatic epithelium in the multipotent pancreatic progenitor cells beginning as early as e8.5 [39]. It becomes down-regulated in acinar and duct cells around e15.5 and greatly up-regulated in insulin positive cells beginning around e16.5-17.5 [40, 41]. To determine whether Pdx-1 might contribute to the induction of *Slc30a8* gene expression during pancreatic islet development, we compared the time course for the appearance of *Slc30a8* mRNA and ZnT-8 protein in embryonic mouse pancreas relative to Pdx-1. Figure 8A confirms that between e12.5 and e15.5 Pdx-1 becomes restricted to pancreatic islet beta cells, as previously reported [40, 41]. This correlates with the initial induction of *Slc30a8* gene expression (Fig. 8B), while ZnT-8 protein levels were still below the limit of detection (Fig. 8A). A marked increase in *Slc30a8* gene expression and the appearance of ZnT-8 protein was observed between e15.5 and e17.5 (Fig. 8B), which correlates with the previously reported up-regulation of Pdx-1 in insulin positive cells (Fig. 8A; Ref. [40]). Little change in pancreatic *Pdx-1* gene expression was observed in whole pancreata between e12.5 and e17.5 (Fig. 8B), consistent with the reciprocal increase in beta cell expression and decrease in acinar and ductal cell expression (Fig. 8A).

Discussion

Many islet-specific promoters and enhancers utilize a similar mechanism to achieve tissue-specific expression, specifically a complex interaction between islet-enriched and ubiquitous factors [2]. We have been interested in expanding the current understanding of the molecular mechanisms driving islet-specific expression using *Slc30a8* as a model. By analyzing uncharacterized islet-specific promoters and enhancers, it may be possible to identify novel factors that are important for selective gene expression within islets. In this study we have identified two conserved intronic regions, designated enhancers A and B, that are capable of

enhancing fusion gene expression in transient transfections (Figs. 1 - 3). Enhancer A is an islet beta cell-specific enhancer whereas enhancer B is active in both alpha and beta cells. Additional studies showed that Pdx-1 regulates *Slc30a8* gene expression through binding two sites in intronic enhancer A (Figs. 4-7), though gel retardation and ChIP assays cannot distinguish whether one or both of these two sites binds Pdx-1 *in vivo*.

The *insulin* gene promoter has been extensively characterized and contains multiple cis-acting elements that are required for high promoter activity, most importantly elements designated as the A, C and E boxes, which have been shown to bind Pdx-1 [3], MafA [42], and NeuroD/BETA2 [43], respectively. Several other genes whose expression are islet-specific or enriched are regulated by the same three factors [2]. Since we have demonstrated that *Slc30a8* gene expression is regulated by Pdx-1 this raises the question as to whether MafA and NeuroD are also required for islet-specific *Slc30a8* gene expression. Future experiments will address this question. Interestingly, the induction of both *Slc30a8* and *G6pc2* gene expression between e13.5 and e17.5 parallels the induction of *MafA* expression (Fig. 8B; Refs. [44, 45]) and the restriction of Pdx-1 to beta cells (Fig. 8A). Previous promoter analyses have shown that *G6pc2* expression is regulated by both Pdx-1 and MafA [4], while recent microarray analyses demonstrate that *Slc30a8* and *G6pc2* gene expression are markedly reduced in islets lacking MafA [46], suggesting that MafA might also contribute to the induction of *Slc30a8* and *G6pc2* gene expression.

Pdx-1, MafA and NeuroD have also been shown to play major roles in the glucose responsiveness of the *insulin* gene [47]. Pdx-1 is the primary regulator of glucose-stimulated *insulin* gene expression and it is thought to modulate transcription via phosphorylation-dependent changes in subcellular localization and interactions with co-regulators [2]. Although our data show that Pdx-1 also regulates *Slc30a8* gene expression, studies in INS-1E cells surprisingly demonstrated that glucose decreases *Slc30a8* gene expression [48]. Whether this negative effect is also mediated through Pdx-1 remains to be determined.

Pdx-1 is not only critical for proper pancreas development (Pdx-1 null mice are apancreatic and die soon after birth [49]) but it is also important for proper islet function in the adult. Heterozygous mutations in the *Pdx-1* gene in humans have been shown to result in Maturity Onset Diabetes of the Young type 4 (MODY-4), a rare monogenic form of diabetes characterized by a loss of insulin production [50]. Our data suggest that a reduction in Pdx-1 levels will result in a reduction in *Slc30a8* gene expression, which may contribute to the phenotype observed in individuals with MODY-4.

It has been suggested that ZnT-8 is responsible for providing zinc to allow for proper insulin maturation, storage and secretion [6]. This concept is supported by the observed reduction in fasting plasma insulin in mice lacking ZnT-8 as well as glucose-stimulated insulin secretion in islets isolated from those mice [9]. Surprisingly, however, mice in which the *Slc30a8* gene was globally deleted have normal glucose tolerance [9, 19, 20] and mice with a beta cell-specific *Slc30a8* gene deletion have only mildly impaired glucose tolerance [21]. The observations made in mice in which the *Slc30a8* gene was globally deleted raise two key questions. Firstly, how do these knockout mice maintain normal glucose tolerance despite impaired insulin secretion? And secondly, how do polymorphisms in the *SLC30A8* gene

confer increased susceptibility to the development of type 2 diabetes if glucose tolerance is not affected by the absence of ZnT-8? Future studies with these ZnT-8 knockout mice will no doubt address both these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

ZnT-8	zinc transporter-8
ChIP	chromatin immunoprecipitation
MODY	Maturity Onset Diabetes of the Young

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Figure 1. Identification of two highly conserved regions within introns 2 and 3 of the human *SLC30A8* and mouse *Slc30a8* genes

A VISTA browser plot [29] comparing the mouse *Slc30a8* and human *SLC30A8* gene sequences between exons 2 and 4. The level of conservation is shown on the vertical axis. Conserved regions above 70%/100 bp are highlighted in red and the putative intron 2 and 3 enhancers are designated A and B, respectively.

		βTC-3 Cells	αTC-6 Cells	HeLa Cells
		Basal Expression Firefly/Renilla Luciferase (% Control)	Basal Expression Firefly/Renilla Luciferase (% Control)	Basal Expression Firefly/Renilla Luciferase (% Control)
100	100	100
Enhancer A →		179 +/- 39	56 +/- 10 *	38 +/- 1 *
Enhancer A ←		270 +/- 45 *	79 +/- 3 *	45 +/- 3 *
Enhancer B →		94 +/- 17	115 +/- 6	121 +/- 12
Enhancer B ←		74 +/- 9 *	105 +/- 7	85 +/- 11

Figure 2. Analysis of the transcriptional enhancer activity of the highly conserved regions within introns 2 and 3 of the mouse *Slc30a8* gene in the context of the *TK* promoter
βTC-3, αTC-6 and HeLa cells were transiently transfected, as described in Experimental, with the indicated firefly luciferase fusion genes (2 μg) and an expression vector encoding *Renilla* luciferase (0.5 μg). Enhancers A and B represent *Slc30a8* intron 2 and 3 sequence between (+20125 and +20745) and (+25228 and +25704), respectively. Following transfection, cells were incubated for 18-20 h in serum-containing medium and then harvested and luciferase activity assayed. Results are presented as the ratio of firefly:Renilla luciferase activity, expressed relative to the ratio obtained with the enhancerless *TK* heterologous vector, and represent the mean of three experiments ± S.E.M. each using an independent preparation of all fusion gene plasmids with each plasmid assayed in triplicate.
*P< 0.05 except for enhancer B inverted where P= 0.05.

A

	β TC-3 Cells	α TC-6 Cells
	Basal Expression Firefly/Renilla Luciferase (% Control)	Basal Expression Firefly/Renilla Luciferase (% Control)
100	100
326 +/- 37 *	87 +/- 1 *
1266 +/- 144 *	80 +/- 6 *
136 +/- 21	221 +/- 23 *
175 +/- 12 *	128 +/- 4 *

B

Figure 3. Analysis of the transcriptional enhancer activity of the highly conserved regions within introns 2 and 3 of the mouse *Slc30a8* gene in the context of the *G6PC2* promoter βTC-3 (**Panels A & B**) and αTC-6 (**Panel A**) cells were transiently transfected, as described in Experimental, with the indicated firefly luciferase fusion genes (2 μg) and an expression vector encoding *Renilla* luciferase (0.5 μg). Regions 1, 2 and 3 represent *Slc30a8* enhancer A intron 2 sequence between (+20125 to +20324) and (+20325 to +20524) and (+20525 to +20745), respectively. Following transfection, cells were incubated for 18-20 h in serum-containing medium and then harvested and luciferase activity assayed. Results are presented as the ratio of firefly:Renilla luciferase activity, expressed relative to the ratio obtained with the enhancerless *G6PC2* heterologous vector, and represent the mean of three experiments ± S.E.M. each using an independent preparation of all fusion gene plasmids with each plasmid assayed in triplicate. *P< 0.05.

Figure 4. Identification of conserved sequences within *Slc30a8* enhancer A

Mouse, human, rat, dog and chicken enhancer A region 2 sequences were aligned using the MacVector7.0 program. Base pairs targeted by the scanning block mutagenesis strategy are indicated.

Figure 5. Scanning mutagenesis of *Slc30a8* enhancer A region 2

β TC-3 cells were transiently transfected, as described in Experimental, with the indicated firefly luciferase fusion genes (2 μ g) and an expression vector encoding *Renilla* luciferase (0.5 μ g). Luciferase expression directed by fusion genes containing the 20 site directed mutants shown in Figure 6 was compared with that directed by the enhancerless *G6PC2*-luciferase heterologous vector (V) or the same vector containing the wild type region 2 (W). Following transfection, cells were incubated for 18-20 h in serum-containing medium and then harvested and luciferase activity assayed. Results are presented as the ratio of firefly:Renilla luciferase activity, expressed relative to the ratio obtained with the *G6PC2* heterologous vector containing wild type region 2, and represent the mean of three experiments $\pm$ S.E.M. each using an independent preparation of all fusion gene plasmids with each plasmid assayed in triplicate. * $P < 0.05$.

A)

Slc30a8 +20325/+20339 WT TTTTGGCTAATGAAT
Slc30a8 +20325/+20339 MUT TTTTGGCaAAaGAAT

B)

C

D

E

Figure 6. Pdx-1 binds, *in vitro*, the area of enhancer A region 2 that is disrupted by the MUT1 mutation

Panel A: Oligonucleotide probes used in Pdx-1 gel retardation assays. A TAAT motif is boxed and the sequences are labeled relative to the translation start site at +1. Mutated nucleotides are in lowercase letters. The location of site-directed mutation 1 is shown in Fig. 6.

Panel B: The labeled wild-type (WT) oligonucleotide probe (Panel A) was incubated in the absence (–) or presence of the indicated molar excess of the unlabeled WT or mutated (MUT) oligonucleotide competitors prior to the addition of β TC-3 cell nuclear extract. Protein binding was then analyzed as described in Experimental. The arrow points to the major specific protein-DNA complex that selectively forms with the WT oligonucleotide.

Panel C: The labeled WT oligonucleotide probe was incubated in the presence of β TC-3, α TC-6, H4 or HeLa cell nuclear extract. Protein binding was then analyzed as described in Experimental. The arrow points to the major specific protein-DNA complex that selectively forms with β TC-3 cell nuclear extract.

Panel D: β TC-3 cell nuclear extract was incubated in the absence (–) or presence of the indicated antisera for 10 minutes at room temperature prior to the addition of the labeled WT oligonucleotide probe and incubation for an additional 10 min at room temperature. Protein binding was then analyzed as described in Experimental. The arrows point to the β TC-3

specific protein-DNA complex whose migration is selectively supershifted by the Pdx-1 antiserum.

Panel E: The labeled wild-type (WT) oligonucleotide probe (Panel A) was incubated in the absence (–) or presence of a 500-fold molar excess of the unlabeled WT or MUT oligonucleotide competitors prior to the addition of programmed or unprogrammed reticulocyte lysate as indicated. Alternatively, programmed reticulocyte lysate was incubated in the absence (–) or presence of the Pdx-1 antisera for 10 minutes at room temperature prior to the addition of the labeled WT oligonucleotide probe and incubation for an additional 10 min at room temperature. Protein binding was then analyzed as described in Experimental. The arrow points to the specific protein-DNA complex that selectively forms with the WT oligonucleotide and whose formation is affected by the Pdx-1 antiserum.

In the representative autoradiographs shown, only the retarded complexes are visible and not the free probe, which was present in excess.

Figure 7. *Slc30a8* enhancer A region 2 binds Pdx-1 *in situ*

Pdx-1 and MafA binding to *Slc30a8* enhancer A region 2 were analyzed *in situ* using the chromatin immunoprecipitation (ChIP) assay. Chromatin from formaldehyde-treated β TC-3 cells was immunoprecipitated using anti-Pdx-1 or anti-MafA antibodies or, as a control, using IgG. The presence of *Slc30a8* enhancer A region 2 and *Pck1* promoter in the chromatin preparation prior to immunoprecipitation and in the immunoprecipitates was then assayed using PCR as described in Experimental. The results of a representative experiment are shown.

A

B

Figure 8. The restriction of Pdx-1 to pancreatic islet beta cells correlates with the induction of *Slc30a8* gene and ZnT-8 protein expression

Panel A: Immunofluorescent detection of Pdx-1, β -catenin, insulin and ZnT-8. Pancreata isolated from CD1 mice at e12.5 (Panels 1 and 2), e15.5 (Panels 3 and 4) and e17.5 (Panels 5 and 6) were incubated with goat anti-Pdx-1, rabbit anti- β -catenin, guinea pig anti-insulin and mouse anti-ZnT-8 antibodies and then stained with secondary antibodies linked to AMCA (for insulin), pseudo colored green, Cy2 (for Pdx-1), pseudo colored red, Cy3 (for ZnT-8), pseudo colored green and Cy5 (for β -catenin), pseudo colored white. β -catenin was used to mark the cellular boundaries of differentiating epithelial cells in the developing pancreas. Restriction of Pdx-1 expression to beta cells occurs between e12.5 and e15.5 and coincides with expression of insulin (Panel 3) but precedes expression of ZnT-8 (Panel 4) in e15.5 pancreas. Panel 3 shows co-expression of insulin and Pdx-1 at e15.5 whereas ZnT-8 expression is not detected at e15.5 (Panel 4). Co-localization of insulin (Panel 5) and ZnT-8 (Panel 6) is seen with Pdx-1 expressing cells in islet like structures at e17.5. Scale bar = 10 μ M.

Panel B: Analysis of *Pdx-1*, *MafA*, *G6pc2* and *Slc30a8* mRNA expression by quantitative PCR. Quantitative RT-PCR was performed on cDNA synthesized using RNA isolated from pancreata harvested from e12.5, e13.5, e15.5 and e17.5 CD1 mice as described in

Experimental. Endogenous *Gapdh* expression was used for normalization. Data are the mean $\pm$ S.E.M. of 3 experiments and are expressed relative to expression at e12.5. *P< 0.05.