Interactions between Areas I and II Direct pdx-1 Expression Specifically to Islet Cell Types of the Mature and Developing Pancreas*

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PDX-1 regulates transcription of genes involved in islet β cell function and pancreas development. Islet-specific expression is controlled by 5’-flanking sequences from base pair (bp) −2917 to −1918 in transgenic experiments, which encompasses both conserved (i.e. Area I (bp −2761/−2457), Area II (bp −2153/−1923)) and non-conserved pdx-1 sequences. However, only an Area II-driven transgene is independently active in vivo, albeit in only a fraction of islet PDX-1-producing cells. Our objective was to identify the sequences within the −2917/−1918-bp region that act in conjunction with Area II to allow comprehensive expression in islet PDX-1+ cells. In cell line-based transfection assays, only Area I effectively potentiated Area II activity. Both Area I and Area II functioned in an orientation-independent manner, whereas synergistic, enhancer-like activation was uniquely found with duplicated Area II. Chimeras of Area II and the generally active SV40 enhancer or the β cell-specific insulin enhancer suggested that islet cell-enriched activators were necessary for Area I activation, because Area II-mediated stimulation was reduced by the SV40 enhancer and activated by the insulin enhancer. Several conserved sites within Area I were important in Area I/Area II activation, with binding at bp −2614/−2609 specifically controlled by Nkx2.2, an insulin gene regulator that is required for terminal β cell differentiation. The ability of Area I to modulate Area II activation was also observed in vivo, as an Area I/Area II-driven transgene recapitulated the endogenous pdx-1 expression pattern in developing and adult islet cells. These results suggest that Area II is a central pdx-1 control region, whose islet cell activity is uniquely modified by Area I regulatory factors.

The pancreatic-glandular homeobox-1 (PDX-1)3 transcription factor is essential for pancreatic exocrine and endocrine islet cell development and the maintenance of adult islet β cell function. Initially detected in dorsal gut endoderm by embryonic day (E) 8.5 in mice, PDX-1 later becomes widely expressed during development in the dorsal and ventral pancreatic buds, as well as the duodenal endoderm (1–3). Homozygous disruption of the pdx-1 gene in mice and humans results in pancreatic agenesis (1, 2, 4). The impact of PDX-1 on pancreatic development is more profound than any other characterized exocrine- or islet-enriched transcription factor, all of which act at later steps, with most affecting the formation of specific pancreatic cell types (5, 6).

PDX-1 is principally expressed within adult pancreas islet β cells and a small fraction of δ cells, where it regulates cell type-specific gene expression (e.g. insulin, glucose transporter type 2, β-glucokinase in β cells; somatostatin in δ cells) (7). Strikingly, the conditional removal of pdx-1 from islet β cells in mice results in a diabetic phenotype, presumably due (at least) to reduced insulin and glucose transporter type 2 expression (8). A PDX-1 heterozygous state causes glucose intolerance in mice, whereas humans develop maturity onset diabetes of the young (termed MODY4), a monogenic form of Type II diabetes (9, 10).

Because PDX-1 is a critical transcriptional regulator of both pancreatic organogenesis and islet β cell activity, efforts have focused on identifying the factors important for tissue-specific expression. Transgenic analysis has demonstrated that cell type-specific expression in the pancreas, stomach, and duodenum is mediated by sequences within 6.5 or 4.5 kilobase pairs upstream of the transcription start site in the rat and mouse genes, respectively (4, 11–13). This area contains several domains of significant identity between mouse, human, and chicken, and includes an orientation-independent domain, Area II (bp −2917 to −1923) (14, 15). Areas I, II, and IV can independently direct β cell-type-specific reporter gene expression in cell line-based transfection assays (14–16). Detailed analysis of conserved control elements within each of these regions has revealed that transcriptional control is mediated in part by factors involved in islet β cell development, including FoxA2 (12, 14, 15, 17, 18), MafA (19), Pax6 (18), HNF1a (17, 20), and PDX-1 (15, 20) itself.

The −2917 to −1918-bp region of mouse pdx-1, a nuclear-hypersensitive domain that includes Areas I and II, is sufficient to direct transgene expression to islet β cells in vivo (12, 13). However, neither Area I nor contiguous Area III was capable of independently directing expression to islet cells in vivo (18). Furthermore, although an Area II transgene is expressed in a β cell-specific manner, activity is only found in a fraction of the PDX-1 positive β cell population (13, 18). Collectively, these results suggested that factors binding to Area II and non-Area II sequences within bp −2917 to −1918 play a critical role in regulating pdx-1 transcription.

The primary objective of this study was to identify the sequences within bp −2917 to −1918 that cooperate with Area II to direct temporal and spatial transgenic expression in the islet. Cell line-based assays suggested that distinct properties of Area I were necessary for potentiating Area II-directed activity, in that neither non-conserved pdx-1 region sequences nor the ubiquitously active SV40 enhancer region

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3 The abbreviations used are: PDX-1, pancreatic-duodenal homeobox 1; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; hsp, heat shock protein; E, embryonic.
were able to reproduce the precise β cell-selective regulatory properties of bp −2917/−1918. In addition, the β cell-specific enhancer of the rodent insulin gene was able to stimulate Area II activity, but differently than Area I. Activation of Area I/Area II was shown to be mediated by Nkx2.2, a homeodomain protein of the NK-2 class that is essential for proper islet β cell formation and function (21). A transgene driven by Area I/Area II alone was first detected in vivo within developing insulin+ cells destined to populate the islet, and later to recapitulate the expression pattern of the endogenous gene in adult islets. The co-expression of Nkx2.2 in Area I/Area II transgene expressing cells is consistent with a role in both early developing and adult islet pdx-1 transcription. Collectively, the data indicate that islet cell-specific expression of pdx-1 is principally controlled by transcription factors controlling Area I/Area II-mediated activity.

**MATERIALS AND METHODS**

**Transfection Constructs**—The pds-1 reporter constructs were made using mouse and human sequences, which were cloned directly upstream of the herpes simplex virus thymidine kinase (TK) minimal promoter in the chloramphenicol acetyltransferase (CAT) expression vector, pTK(An) CAT. The construction of the pds-1: pTK constructs representing the −2917/−1918-bp region (mouse, PsI/−2917:Bst/−1918 bp; human, −2839/−2023 bp), Area I (mouse, −2761/−2457 bp; human, −2839/−2521 bp), Area II (mouse, −2153/−1923 bp; human, −2252/−2023 bp), or Area IV (mouse, −6529/−6010 bp) were described previously (16). The Area I block transversion mutants and bp −2613, −2612 mutation in S6 were constructed in −2917/−1918: pTK and/or Area I: pTK using the QuikChange mutagenesis kit (Stratagene). The pds-1 sequences were cloned in the forward orientation in each of the 2X Area I: pTK, 2X Area II: pTK, and 2X Area IV: pTK plasmids. The 2X:72 siiman virus 40 (SV40) enhancer fragment was obtained by HindIII digestion of pB2:72−22 (22, 23) and subcloned with the same relative 5’ to 3’ orientation and nucleotide spacing just upstream of the Area I and Area II sequences in Area I: pTK or Area II: pTK, respectively. The −374 to −46-bp region of the rat insulin II gene was used to create InsIII-AreaI:pTK, InsIII-AreaII:pTK, and InsIII:pTK (23, 24). All constructs were confirmed by restriction enzyme digest and partial sequence analysis.

**Cell Transfections**—Monolayer cultures of pancreatic islet (βTC-3 and MIN-6) and non-β (NIH-3T3) cell lines were maintained as described previously (24). Rous sarcoma virus enhancer-driven luciferase (pRSV-Luc) activity was used to normalize the CAT activity from co-transfected pds-1: pTK. All cell lines were transfected using the Lipofectamine reagent (Invitrogen) with 1 μg of pds-1: pTK and 1 μg of RSV-LUC (2 μg total). Extracts were prepared 40–48 h after transfection, and LUC and CAT enzymatic assays were performed as described (25, 26). Each experiment was carried out at least three independent times.

**Electrophoretic Mobility Shift Assays**—Nuclear extract from βTC-3 cells was prepared as described previously (27). The mouse Area I S6 double strand oligonucleotide (bp −2623/−2600) was poly nucleotide kinase-labeled with [32P]dATP. Binding reactions (20 μL, total volume) (27) were conducted with 5–10 μg of nuclear extract protein and probe (8 × 104 cpm). The conditions for the competition analyses were the same, except that a 50–100-fold excess of the unlabeled specific competitor DNA was included in the mixture prior to addition of radiolabeled probe. The TNT Coupled Reticulocyte Lysate system (Promega) was used to in vitro transcribe and translate Nkx2.2 (pCRII-Nkx2.2, Palle Serup, Hagedorn Research Institute, Gentofte, Denmark). Nkx2.2 monoclonal antibody (74.5A5; Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA) was preincubated with extract protein for 10 min prior to the addition of the DNA probe. The probe and competitor sequences were: mouse S6 (5'-ATT-ATTATCATAAAGACTGCTGTATTA-3'); mouse S6 block mutant (5'-ATTATCATAAAGACTGCTGTATTA-3'); mouse S6 −2613, −2612 mutant (5'-ATTATCATAGGGACTGCTGTATTA-3'); rat Insulin II S6−221.40−119 (5'-ACACAGGAATTGTGGAAACT-3') (28); and rat Insulin II −133, −132 mutant (5'-ACACAGGAGGCTGCTTTGGAAA-3') (28). Core-like Nkx2.2 binding sequences are underlined and mutated nucleotides are lowercase. The samples were electrophoresed on 6% nondenaturing polyacrylamide gels at 150 V for 2 h under high ionic strength polyacrylamide gel electrophoresis conditions before drying and autoradiography.

**Chromatin Immunoprecipitation Assays**—βTC-3 cells (0.5–1.0 × 10⁵) were formaldehyde cross-linked, and the sonicated protein–DNA complexes were isolated under conditions described previously (20). Nkx2.2 (25 μL, 74.5A5), normal rabbit immunoglobulin G (IgG; 10 μg; Santa Cruz Biotechnology), or no antibody was added to the sonicated chromatin, followed by incubation for 1 h at 4°C. Antibody-protein–DNA complexes were isolated by incubation with protein A/G-PLUS agarose slurry (Santa Cruz Biotechnology). PCR was performed on one-tenth of the purified, immunoprecipitated DNA using Ready-to-Go PCR beads (Amersham Biosciences) and 15 pmol of each primer. The primers used for amplification of mouse Area I were: 5'-CCACTAAGAGAAGGAAAGGCAAG-3' (2785) and 5'-CTGGAGTTCCTTTCTCTGG-3' (2435); and for phosphoenolpyruvate carboxylkinase were: 5'-GAGTGGACACCTCAACGCTGTGG-3' (434) and 5'-GGACGGCCTTTGGATCATAGCC-3' (96). Cycling parameters were one cycle of 95°C for 2 min and 28 cycles of 95°C for 30 s; 59°C (Area I) or 61°C (phosphoenolpyruvate carboxykinase) for 30 s; and 72°C for 30 s. The PCR products were confirmed by sequencing. Amplified products were electrophoresed through a 1.4% agarose gel in TAE buffer and visualized by ethidium bromide staining.

**Area I/Area II Transgene Construction and Generation of Transgenic Mice**—Mouse Area I (−2759 to −2439 bp) was fused directly upstream of Area II (−2200 to −1923 bp) and ligated just 5' to the heat shock protein (hsp) minimal promoter in hsp68lacZpa, a bacterial β-galactosidase (lacZ) expression plasmid (12, 13). DNA was NotI digested and the isolated Area I/Area I transgene injected into the Vanderbilt Transgenic Mouse/ES Cell Shared Resource into the pronuclei of one-cell embryos from B6D2 females, and embryos were transplanted into pseudopregnant ICR females. The F0 founders were used to establish transgenic lines. Area I/Area I transgenics were identified by PCR analysis using primers to lacZ with DNA from neonatal leg or adult tail tissue prepared using the Puregene DNA Purification Kit (Genta Systems, Minneapolis, MN).

**X-Gal Staining**—Neonatal digestive organs were fixed at 3 h in 4% paraformaldehyde at 4°C, then washed three times in permeabilization solution (0.2% Nonidet P-40 in phosphate-buffered saline). Activity from β-galactosidase was detected using X-gal as described (12). Macroporous pictures were taken using a Stemi 2000-C microscope (Zeiss) and Nikon 5700 digital camera.

**Immunohistochemistry**—Abdominal organs and pancreata were fixed as described above, dehydrated, embedded in paraffin, and then 6-μm serial sections were mounted on glass slides. Immunofluorescence was performed using rabbit a,β-galactosidase (1:5000; Molecular Probes), guinea pig α-insulin (1:2000; Linco Research Immunoassay, St. Charles, MO), guinea pig α-glucagon (1:2000; Linco), guinea pig α-pan...
creatinic polypeptide (1:2000; Linco), sheep α-somatostatin (1:2000; American Research Products, Belmont, MA), and mouse α-NKx2.2
(1:200; 74.5A5) primary antibodies. Secondary antibodies were Cy-2, Cy-3, or Cy-5-conjugated donkey α-guinea pig, α-rabbit, α-mouse, and α-sheep (1:500; Jackson ImmunoResearch, West Grove, PA). Fluorescent images were captured with an LSM 510 confocal microscope (Zeiss) at an optical depth of 1 μm. Nuclear counterstaining was performed using YoPro1, 1:1000 (Molecular Probes). Quantification was determined on serial adult pancreas (8 weeks of age) sections that were co-stained for β-galactosidase/insulin or β-galactosidase/somatostatin through a depth of at least 96-μm. Optical section images were captured using a Zeiss AxioPlan2 microscope and processed as TIFF images in Adobe Photoshop.

RESULTS

Area I/Area II Alone Controls the β Cell Line-specific Activation Properties of the −2917/−1918-bp Region—A comprehensive transgenic analysis of the 5′-flanking region of the mouse pdx-1 gene revealed that the −2917 to −1918 bp region was uniquely capable of directing islet cell-selective expression (13). This region is found within a larger β cell-specific nuclease-hypersensitive domain that spans blocks of conserved (i.e. Areas I (mouse bp −2761/−2457), II (bp −2153/−1923), III (bp −1879/−1600), and non-conserved sequences (12, 14). Individually, only Areas I and II effectively directed β cell-selective activation in transfection based assays, although their isolated activities were less effective than the −2917/−1918 bp-driven reporter (14, 15). The activity of these conserved domains was also compromised relative to the −2917/−1918 bp-driven transgene in vivo. Thus, whereas Area II was able to direct transgenic expression to islet cells, activity was only found in a small fraction of the PDX-1 producing cells detected with the −2917/−1918 bp-driven transgene (18). Collectively, these results indicate that functional interactions between Area II and non-Area II sequences are required to obtain −2917/−1918 bp-like regulation.

To analyze the activation properties of the non-conserved sequences within the −2917/−1918-bp region, mouse pdx-1 sequences from −2917 to −2762 and −2456 to −2145 were subcloned directly upstream of the minimal TK promoter sequences in the CAT expression plasmid, pTK(An). Their activity was compared with −2917/−1918 bp-, Area I-, and Area II:pTK constructs in transfected NIH-3T3 cells. The normalized activity of the duplicated constructs were compared with both −2917/−1918 bp-, Area I-, and Area II:pTK constructs in transfected β-3TC-3 cells (19). The activity of the duplicated constructs was compared with both the single copy and the Area I/Area II construct, with control regions maintained in the normal orientation in each multimerized pdx-1 construct. The 2×Area II-driven reporter had unique functional properties when compared with 2×Area I:pTK or 2×Area IV:pTK in β-cells (Fig. 3B). Thus, the increase in activity of 2×Area I was additive, whereas no effect was found upon duplicating Area IV. However, 2×Area II had cooperative enhancer-like stimulation characteristics, with an activity level equivalent to Area I/Area II. Multimerized human Area II also stimulated transcription in a synergistic manner (data not shown). Together, these data revealed the distinct enhancer-like properties of Area II and further supported a central role in pdx-1 expression in the pancreas.

The β Cell-enriched Activators of Area I Appear Essential in Area II Activation—The nature of the factors involved in Area I/Area II control was investigated by fusing a ubiquitously expressed (SV40) or β cell-specific (rat insulin II) enhancer upstream of Area I or Area II. The
activity of the heterologous enhancer-driven Area I and Area II constructs were compared with Area I/Area II:pTK, Area I:pTK, Area II:pTK, and enhancer alone:pTK (SV40:pTK, Ins II:pTK). The SV40 enhancer increased Area I and II activity to a much greater extent in non-β than β cells (Fig. 4A). In contrast, the insulin enhancer had quite distinct effects on the activity of these control regions. Thus, there was no difference between InsII:pTK and InsII-Area I:pTK activity in β cells, whereas Area II was activated by the insulin enhancer to a greater degree than by Area I (Fig. 4B). Notably, the results with InsII-Area II:pTK imply that −2917/−1918 bp-mediated activation involves communication between Area II and the β cell-enriched control factors of Area I.

The Nkx2.2 Transcription Factor, Which Regulates Rodent Insulin Gene Expression and β Cell Development, Binds to Area I and Activates Area I/Area II—To localize regulatory elements within Area I, segments of identity between mouse, human, and chicken were previously mutated and their effect on activation determined in transfection assays. These studies localized sequences that were involved in Area I stimulation (S2, S4, S5, S6, S7, and S8), as well as conserved segments of little or no apparent regulatory importance (S1, S3, and S9) (20). In addition, S6 at bp −2613, −2612 compromises both −2917/−1918:pTK and Area II:pTK activity in βTC-3 cells.

**FIGURE 3.** Area II has unique enhancer-like properties. Areas I, II, and IV are represented by white, black, and hatched boxes, respectively. Each pdx-1 region was: A, cloned in forward (5'-3') or reverse (3'-5') orientation, or B, duplicated in pTK(CAT) and transfected in βTC-3 cells. β Cell activity is expressed as -fold induction relative to pTK(A) or Area I/Area II(B) ± S.E. The statistical difference between both single and duplicated versions of Area I and Area II (*, p < 0.05; **, p < 0.01; Student’s t test) is shown.

**FIGURE 4.** The insulin enhancer selectively stimulates Area II activity. A, SV40 (slashed line box), and B, rat insulin II (InsII, striped box) enhancer sequences in the Area I (white box) and Area II (black box) constructs are shown schematically. The activity ± S.E. in A is presented relative to Area I in Min-6 versus NIH-3T3 cells, whereas presented in B relative to Area I/Area II in only Min-6 cells. The asterisk in B denotes the statistical significance between Area I and InsII/Area II (**, p < 0.01; Student’s t test).

**FIGURE 5.** Conserved segments 4, 6, 7, and 8 within Area I contribute to Area II activation. A, the boxes represent the conserved sequence segments of Area I. The transcription factors associated with segment control are labeled. B, wild type and mutants of Area I were analyzed in −2917/−1918:pTK-transfected βTC-3 cells. The activity is expressed relative to pTK ± S.E. (*, p < 0.05; **, p < 0.01; Student’s t test). S4, S6, S7, and S8 reduce −2917/−1918:pTK activity, whereas only S9 appears to be under negative control. C, the sequence conservation around S6 (−2614/−2609) is shown and compared with the Nkx2.2 consensus (28, 29); mutation of the two-nucleotide core (lower-case) eliminates binding to the insulin control element (28, 29). Mutation of S6 at bp −2613, −2612 compromises both −2917/−1918:pTK and Area II:pTK activity in βTC-3 cells.
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FIGURE 6. Nkx2.2 binds to the S6 of Area I. Nkx2.2 binding to the Area I S6 (bp – 2623/2600) probe in βTC-3 nuclear extracts is specifically competed by a 50-fold excess of unlabeled wild type (WT) S6 or the rat insulin II Nkx2.2 control element, but not by the S6 (–133/–132) element mutants. Nkx2.2 binding is indicated by the arrow, and the antibody super-shifted complex with a black circle shifted complex with a black circle. The in vitro translated (IVT) Nkx2.2 binding complex (lane 7) co-migrated with the one formed in βTC-3 nuclear extracts (lane 1). The C– lane contains in vitro translated insert-less pCRII vector.

FIGURE 7. Nkx2.2 binds within Area I in vivo. Formaldehyde cross-linked chromatin from βTC-3 cells was incubated with antibodies (Ab) raised to Nkx2.2 (lane 3). Immunoprecipitated DNA was analyzed by PCR with primers specific to transcriptional regulatory sequences of mouse Area I and phosphoenolpyruvate carboxykinase (PEPCK). As controls, PCR were performed with no DNA (lane 1), input DNA (1:100 dilution; lane 2), DNA precipitated by normal mouse immune serum (lane 4), and DNA that was precipitated in the absence of antiserum (lane 5).

not shown). Conversely, the conserved site mutants at S1, S3, S9, S10, or S12 do not appear to stimulate −2917/−1918:pTK (Fig. 5B) or Area I:pTK activity (i.e. S10, S11, S12, data not shown).

Upon inspection of S4, S6, S7, and S8, a potential binding site for the islet-enriched Nkx2.2 transcription factor was found in S6 (Fig. 5C) (28, 29). Nkx2.2 is a key regulator of islet β cell development and function. Thus, the β cell PDX-1 expression is lost in Nkx2.2−/− mice (21), whereas binding regulates rodent insulin transcription (28). The ability of Nkx2.2 to bind S6 was determined in gel mobility shift assays. A co-migrating S6 complex was found between the βTC-3 nuclear extract and in vitro translated Nkx2.2 that supershifted with α-Nkx2.2 antibody (Fig. 6). Competition assays also demonstrated that this complex was able to bind wild type S6, but not a 2-bp mutation that either blocks binding to the insulin II site or reduces S6-mediated activation (see the S6 mutant in Figs. 5C and 6). These data indicated that Nkx2.2 could specifically bind to the S6 control element of Area I.

The chromatin immunoprecipitation assay was used next to investigate if Nkx2.2 bound in vivo to Area I in βTC-3 cells. Area I sequences were selectively amplified by PCR from chromatin precipitated by α-Nkx2.2 antiserum, but not from chromatin treated with normal rabbit IgG or in the absence of antiserum (Fig. 7). In contrast, Nkx2.2 antiserum did not precipitate control sequences from the phosphoenolpyruvate carboxykinase gene, which is not expressed in β cells. Collectively, these results strongly suggest that Nkx2.2 binding to S6 contributes in the activation of Area I and Area I/Area II-mediated expression in β cells.

Area I/Area II Drives Transgene Expression to All PDX-1 Producing Islet Cell Types in Vivo—Our in vitro data suggested that communication between Areas I and II was involved in regulating islet pdx-1 expression. To formally test this hypothesis in vivo, two independent lines of transgenic mice were derived that express an Area I/Area II-driven lacZ transgene, termed Area I/Area IIhsp-lacZ.

Expression from Area I/Area IIhsp-lacZ was only seen in the pancreas, with the analysis of β-galactosidase expression revealing staining throughout the islet in tissues from neonates (Fig. 8; 1-day-old, P1) and adults (data not shown). Quantitation of β-galactosidase co-staining in adult islet insulin (β cells), somatostatin (α), and pancreatic polypeptide hormone-producing cells revealed that Area I/Area IIhsp-lacZ expression was in 98% of insulin+ and 12% of somatostatin+ cells (TABLE ONE). In contrast, glucagon+/β-galactosidase−/ pancreatic polypeptide+/β-galactosidase+ cells were not observed in neonate or adult transgenic islets (data not shown). The Area I/Area IIhsp-lacZ expression pattern is essentially identical to endogenous pdx-1 expression in these ages (3, 12, 13), clearly demonstrating the capacity of Areas I and II to selectively and comprehensively direct pdx-1 transcription in islet PDX-1+ cells.

The activity of Area I/Area IIhsp-lacZ was next examined at various stages of development to obtain insight into when control was implemented during pancreas formation. PDX-1 is essential at E8.5 for organogenesis (1), when it is widely expressed in proliferating exocrine and endocrine pancreatic precursors, and subsequently in forming islet cells (3). Area I/Area IIhsp-lacZ activity was first detected at E14.5, and restricted to most, but not all, insulin+ cells (Fig. 9). Because transgenic expression was found in most insulin− PDX-1+ cells at E14.5 and essentially all at E15.5, the absence of Area I/Area IIhsp-lacZ expression at E13.5 indicates that Area I/Area II likely controls pdx-1 transcription in the secondary and principal wave of insulin+ cell production initiated at this time (30). Importantly, Nkx2.2 was also found in the Area I/Area IIhsp-lacZ expressing cells (Fig. 9), consistent with a role in regulating pdx-1 transcription during development and in islet β cells.

DISCUSSION

These studies have defined the sequences within the pdx-1 transcription unit that are capable of directing expression uniquely to pancreatic
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islet cells. Through a systematic analysis of the nuclease-hypersensitive −2917 to −1918-bp region, we have demonstrated that Area I sequences augment Area II activity in both cell-based transfection and transgenic assays. An Area I/II-driven transgenic targeted expression throughout the PDX-1+ islet cell population in mice, a property distinct from Area II alone, which was only active in a fraction of producing cells. Mutational studies of Area I showed that Area I/Area II activation was regulated in part by binding of the islet-enriched Nkx2.2 transcription factor to conserved S6 at bp −2614/−2609. Our data further suggest that Area I and II act as the principal regulators of pdx-1 transcription during the start of the massive wave of islet β cell differentiation during organogenesis.

Our deletional analysis of the −2917 to −1918 bp region demonstrated that non-conserved sequences play little, if any, role in regulation. Thus, −2917/−2762pTK and −2456/−2145pTK were not selectively activated in transfected β cells (Fig. 1). Furthermore, Area I/Area II alone retained the regulatory properties of the larger transgene in in vitro and in vivo based pdx-1-driven reporter assays, consistent with non-conserved sequences having little, if any, influence on bp −2917/−1918 control. To begin to address the unique activation properties of Area I and II, we constructed pdx-1 control domain chimeric reporter constructs with either a general or β cell-specific enhancer. In other studies, activation by individual β cell-enriched activators of the insulin gene was amplified by broadly expressed SV40 enhancer control factors (22, 23, 31). The SV40 enhancer also increased Area I- or Area II-directed stimulation in β cells. However, the -fold increase in activity was much greater in non-β cells, resulting in an overall decrease in β cell-specific activation (Fig. 4A). These results suggested that communication between β cell-enriched regulatory factors was key to Area I/Area II activation. As a consequence, it was surprising that the rat insulin II enhancer was only able to stimulate Area II activity. The level of rat insulin-Area II activity was ~3-fold greater than Area I/Area II,

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epithelium or adult islets also indicates that this factor does not play a defining role in cell-specific \textit{pdx-1} activation. Presumably, efforts focused on identifying the regulators of conserved S4, S7, and S8 will help clarify the nature of the factors that contribute to the ability of Area I to drive comprehensive expression of the Area II transgene throughout the endogenous islet \textit{PDX-1} cell population.

Strikingly, Area I/Area II transgene activity was only first detected in insulin \textit{\textsuperscript{\textit{PDX-1}}} cells of E14.5 and E15.5, and not those at E13.5 (Fig. 9). In addition, expression from this transgene was not found in exocrine acinar cells or other islet hormone-producing cells, even though glucagon, amylase, somatostatin, and pancreatic polypeptide are first produced at around E10.5, E13.5, E15.5, and E18.5, respectively (30, 40). The presence of Area I/Area II transgene activity in most insulin \textit{\textsuperscript{\textit{PDX-1}}} cells during development essentially recapitulates its endogenous penetrance within the mature islet \(\beta\) cell population. A few insulin \textit{\textsuperscript{\textit{PDX-1}}} cells are detected as early as E10.5 in mice, but the majority of islet \(\beta\) cells evolve from a distinct insulin-positive cell population that develops around E14 (41). As MaFA is the only known islet-enriched transcription factor expressed at late in islet \(\beta\) cell development or in such a cell restricted manner, \textit{pdx-1} expression in developing and adult \(\beta\) cells may principally result from functional interactions between MaFA and other islet-enriched Area I/Area II activators, which are expressed earlier and in a broader set of cell types (e.g. FoxA2 (42), BETa2 (35, 43), and Pax6 (39)). The ability of MafA to coordinate the assembly of the insulin gene transcription complex by directly binding to BETa2 and PDX-1 provides support for such a hypothesis (44).

Collectively, our transfection and transgenic data strongly indicate that Areas I and II represent the islet cell-specific control domain of the \textit{pdx-1} transcription unit. However, the functional significance of Areas III and IV are less clear. Transfection analysis has shown that Area IV is selectively active in \(\beta\) cell lines and capable of potentiating e.g. FoxA2 (42), BETa2 (35, 43), and other islet-enriched Area I/Area II activators, which are expressed earlier and in a broader set of cell types (42). However, in Areas I–III-deficient mice, perhaps indicating a need for Area IV in these conserved control domains in \textit{pdx-1} expression. Thus, when Areas I–III were deleted from the endogenous murine \textit{pdx-1} gene at the one-cell stage of embryogenesis using a Cre-\textit{LoxP} strategy, homozygous mice displayed highly aberrant pancreas development with a profound reduction in islet cell numbers.\(^4\) In contrast to the global \textit{pdx-1} knock-out, duodenal and antral stomach development were relatively normal in Areas I–III-deficient mice, perhaps indicating a need for Area IV in expression of \textit{pdx-1} in non-pancreatic cell types. We believe that efforts focused on characterizing the regulators of \textit{pdx-1} transcription will provide valuable information on how islet expression is affected under normal and diabetic conditions.

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