The Role of Hepatic Nuclear Factor 1α and PDX-1 in Transcriptional Regulation of the pdx-1 Gene*

Kevin Gerrish, Michelle A. Cissell, and Roland Stein:

From the Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee 37215

The PDX-1 homeodomain transcription factor regulates pancreatic development and adult islet β cell function. Expression of the pdx-1 gene is almost exclusively localized to β cells within the adult endocrine pancreas. Islet β cell-selective transcription is controlled by evolutionarily conserved subdomain sequences (termed Areas I (−2839 to −2520 base pairs (bp)), II (−2252 to −2023 bp), and III (−1939 to −1664 bp)) found within the 5′-flanking region of the pdx-1 gene. Areas I and II are independently capable of directing β cell-selective reporter gene activity in transfection assays, with Area I-mediated stimulation dependent upon binding of hepatic nuclear factor 3β (HNF3β), a key regulator of islet β cell function. To identify other transactivators of Area I, highly conserved sequence segments within this subdomain were mutagenized, and their effect on activation was determined. Several of the sensitive sites were found by transcription factor data base analysis to potentially bind endodermally expressed transcription factors, including HNF1α (−2758 to −2746 bp, Segment 2), HNF4 (−2742 to −2730 bp, Segment 4; −2683 to −2671 bp, Segment 7–8), and HNF6 (−2727 to −2715 bp, Segment 5). HNF1α, but not HNF4 and HNF6, binds specifically to Area I sequences in vitro. HNF1α was also shown to specifically activate Area I-driven transcription through Segment 2. In addition, PDX-1 itself was found to stimulate Area I activation. The chromatin immunoprecipitation assay performed with PDX-1 antisera also demonstrated that this factor bound to Area I within the endogenous pdx-1 gene in β cells. Our results indicate that regulatory factors binding to Area I conserved sequences contribute to the selective transcription pattern of the pdx-1 gene and that control is mediated by endodermal regulators like HNF1α, HNF3β, and PDX-1.

Several transcription factors that are enriched within pancreatic islet cells mediate differentiation during embryogenesis and the maintenance of specialized cellular functions in the adult (1–3). Some of these proteins are also dysfunctional in patients with diabetes, a disease caused in part by the failure of β cells to produce sufficient insulin to meet the needs of the body (4–6).

* This work was supported by National Institutes of Health Grant RO1 DK50203 (to R. S.) and in part by Vanderbilt University Diabetes Research and Training Center Molecular Biology Core Laboratory Public Health Service Grant P60 DK05953 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 615-322-7026; Fax: 615-322-7236; E-mail: roland.stein@mcmail.vanderbilt.edu.

The PDX-1 (pancreas duodenum homeobox-1; also known as STF-1, IDX-1, IPF-1, and IUF-1) transcription factor is an essential regulator of pancreatic development and adult islet β cell function (7–10). In the pancreas, PDX-1 is expressed almost exclusively in islet β cells (11) and regulates transcription of the genes associated with β cell identity, including insulin (12–16), glucokinase (17), islet amyloid polypeptide (18–21), and glucose transporter type 2 (GLUT2) (22). Selectively removing PDX-1 from islet β cells in mice compromised their ability to maintain glucose homeostasis and resulted in the development of diabetes, at least partially because of reduced β cell expression of insulin and GLUT2 (23). In addition, mice that are heterozygous mutant carriers of PDX-1 exhibit signs of glucose intolerance, suggesting that pdx-1 gene dosage affects insulin expression and β cell function (23, 24). Humans with this condition are susceptible to different forms of non-insulin-dependent diabetes, including mature onset diabetes of the young (25–27).

Pancreatic development and islet β cell function also are influenced by hepatic nuclear factors (HNFs)1 1α (28–30), 1β (31), 3β, 4α (32–34), and 6 (35). For instance, HNF1α−/− (30) and HNF6−/− (35) mice exhibit a non-insulin-dependent diabetic phenotype that is associated with β cell dysfunction. Heterogeneous nonfunctional mutations in HNF1α (29), HNF1β (31), and HNF4α (34) are also found in patients with human mature onset diabetes of the young. Each distinct HNF transcription factor appears to be required for controlling key differentiation and metabolic programs in the pancreas and liver (30, 35–38).

The sequences that control appropriate developmental and adult specific expression of the pdx-1 gene were demonstrated in transgenic studies to be located in the 5′-flanking region of the mouse (39, 41) and rat (40) genes. Three nuclease hypersensitive sites, termed HSS1 (−2560 to −1880 bp), 2 (−1330 to −880 bp), and 3 (−260 to +180 bp), were identified within this region of the endogenous mouse gene by DNase I and micrococcal nuclease analysis (39). However, only HSS1 sequences were capable of directing pancreatic β cell-selective expression in transgenic and transfection studies (39). Sequence analysis of the mouse, chicken, and human pdx-1 genes revealed that the HSS1 region also represented the only area of significant identity within 4.5 kilobases of the transcription start site (41). Sequence conservation and function allowed the HSS1 region to be divided into the Area I (−2839 to −2520 bp), Area II (−2252 to −2023 bp), and Area III (−1939 to −1664 bp) subdomains (41). Areas I and II, but not Area III, were capable of independently directing β cell-selective reporter gene activity

1 The abbreviations used are: HNF, hepatic nuclear factor; bp, base pairs; PCR, polymerase chain reaction; Tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; β-FIB, β-fibrinogen; PEPCK, phosphoenolpyruvate carboxykinase.
in transfection assays, with Area I activation mediated by HNF3β (41). This forkhead transcription factor is also necessary for stimulation by Area II, although only in the context of both Area I and II sequences (39, 41). The importance of HNF3β in pdx-1 transcription was also supported by results showing that pdx-1 mRNA levels were reduced in homozygous null HNF3β embryo bodies (41).

Collectively, the data obtained from analysis of transgenic and transfected pdx-1-driven reporter constructs strongly suggested that the HSS1 region played an essential role in directing transcription during development and in the adult. We proposed that conserved subdomains of HSS1 (i.e. Areas I, II, and III) contained transcription factor binding sites that were critical in this process, like the HNF3β element (41). A comprehensive series of block mutants within the conserved sequences of Area I were prepared to test this hypothesis. Our results indicate that HNF1α and pdx-1 itself are key positive regulators of Area I activation in β cells. These studies suggest that pdx-1 transcription is regulated by factors associated with controlling both the developmental and metabolic states of the β cell and that an inactivating mutation in one of its critical regulators (e.g. PDX-1, HNF1α, and HNF3β) could affect its expression and contribute to β cell dysfunction and disease in patients.

**EXPERIMENTAL PROCEDURES**

**Transfection Constructs—**Human pdx-1 sequences spanning Area I (–2639 to –2550 bp) were generated by polymerase chain reaction (PCR) and cloned directly upstream of the herpes simplex virus thymidine kinase (Tk) promoter region in the chloramphenicol acetyltransferase (CAT) expression vector, pTkAn (42). Pst-BstpTk contains mouse pdx-1 sequences from –2917 (PstI) to –1918 (BstEII) bp (39). Noncomplementary transversional block and point mutations (G to T; C to A) within the conserved sequences of human Area IpTk and Pst-BstpTk were generated using the Quik Change mutagenesis kit (Stratagene); the human Area I oligonucleotides used were: S1 mutant, CAGTATCGGAGAACGACTGCTGCTTGAATA (–2788 to –2752); S2 mutant, AGGACTTACAGGCGAGGATCGGGGACCAGACCTTTACTGTT (–2777 to –2755); S3 mutant, TTTCAGCTAATAACGCAAGGGGGCACTGTCCACACTTT (–2788 to –2762); S4 mutant, ACTACGTACGTACAGTAAAATATTGACAGTT (–2765 to –2752); S5 mutant, TATCAGGACTAAGACTCTAATTACGACTTTTTCCCTTTTTTCACTGTCCACACTGATATTGATTTTTACCTTTTTTGTTTAT (–2758 to –2741); human S2 7 μM mutant, AGGACT- AATCAGGACGGAAGTAGCCGCCACGA- CTTTACTGTTATTACATGTTAAATGGCTCTTTCCTT (–2765 to –2741); human S4, TTTTCACGCCTCACACT (–2745 to –2728); human S5, ACTACCTTAATTGCATT (–2732 to –2725); human S8 mutant, GCTGTTAAATGGCTCTTTCCTT (–2768 to –2741). The mutated nucleotides are underlined.

**Cell Transfections—**Monolayer cultures of pancreatic islet β (βTC3, HIT T-15, MIN6) and non-β (NIH3T3) cells were maintained as described previously (41). The LipofectAMINE reagent (MolecularBio) was used to transfect 1 μg each of pdx-1pTk and the Rous sarcoma virus enhancer-driven luciferase expression plasmid, pRSVLUC (44). Cell transfection studies in NIH3T3 cells were performed with 1 μg of each pdx-1pTk, pRSVLUC, and pB5 or pB5-HNF1α. Extracts were prepared 48–48 h after transfection and luciferase (44) and CAT (45) enzymatic assays performed. pdx-1CAT activity was normalized to that of pRSVLUC. Each experiment was carried out at least three independent times.

**Phoretic Mobility Shift Assays—**Gel shift conditions to detect HNF1α (46), HNF4α (47), and HNF6 (48) binding were carried out as described. Nuclear extracts were prepared using methods described previously (49). The TNT-coupled reticulocyte lysate system (Promega) was used to in vitro transcribe and translate HNF1α (pGEM7-HNF1α (Richard O’Brien, Vanderbilt University)), HNF4α (pMT7-HNF4α (50)), HNF6 (pGEM1-HNF6 (51)), pDI1 (SKI900 (52), Fax6 (pKW10-Fax6 (53)), Fax4 (pBluescript SKII+)), Fax4, Beatrix Sosa-Pineda, St. Jude’s Children’s Research Hospital, Memphis, TN). Nkk 6.1 (pBluescript SKII+), Nkk 6.2 (PCRII-Nkk 2.2, Pelle Serup, Hagedorn Research Institute, Gentofte, Denmark), and Hb9x (HB9C (55)). Approximately 5 μg of extract protein or 0.5 μl of in vitro translated protein was used in gel mobility shift reaction. Anti-PDX-1 antisera was provided by Dr. Linda Wright, Vanderbilt University) and anti-C-terminal (amino acids 271–283 (10)) antisera to pDI1 was used in these assays. The same conditions were used with anti HNF1α (Santa Cruz Biotechnology) and anti HNF6 (Santa Cruz Biotechnology) antisera. The double-stranded oligonucleotides used to detect binding were end-labeled with polynucleotide kinase and [-32P]ATP. The samples were electrophoresed on 8% non-denaturing polyacrylamide gels at 150 V for 2 h under high ionic strength polyacrylamide gel electrophoresis conditions (15) before drying and autoradiography. The probe and competitor sequences were: human S2, GTGCTGCTACTTCACCAAAATTTTGTCTTTCTTCT (–2763 to –2741); human S2 block mutant, GGGTTAATTCTTCTTCTTCTC (–2762 to –2741); S2 mutant, AGGACTTACAGGCGAGGATCGGGGACCAGACTTTACTGTTATTACATTACGACTTTTTCCCTTTTTTCACTGTCCACACTGATATTGATTTTTACCTTTTTTGTTTAT (–2758 to –2741); human S4, TTTTCACGCCTCACACT (–2745 to –2728); human S5, ACTACCTTAATTGCATT (–2732 to –2725); human E5 block mutant, ACACCGGCGGTGGGGCCACC (–2732 to –2715); human S8 mutant, GCTGTTAAATGGCTCTTTCCTT (–2768 to –2741); human S2 to β-FIR, TTTTCACGCCTCACACT (–2765 to –2741). The mutated nucleotides are underlined.

**Optimization Protocol—**

**Chromatin Immunoprecipitation (ChIP) Assays—**Monolayer cultures of mouse βTC-3 cells (0.5–1.0×10^6) were exposed to 1% formaldehyde in Dulbecco’s modified Eagle’s medium for 5 min at 23 °C; glycine was added to 0.125 M, and the cultures were incubated for 2 min. The cells were collected in cold phosphate-buffered saline, pelleted by centrifugation, and incubated for 10 min on ice in 0.6 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride). Lyzed samples were transferred to prechilled microcentrifuge tubes containing 250 mg of glass beads (<106 μm diameter, 160 μm), and the chromatin was sonicated at setting power 4 with a Vib sonic 100 sonicator (Virtis Company, Inc.) for 10 s at 4 °C. The reactions were centrifuged for 10 min at 4 °C to remove debris and stored at –70 °C. A 100-μl aliquot was diluted with 0.9 ml of buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% protease inhibitor mixture for mammalian cells (Sigma)) and preclarified with 0.5 μl of bovine serum albumin-blocked protein A-Sepharose for 1 h at 4 °C. After removal of the Sepharose beads by centrifugation, 1 μl of anti-PDX-1 antisera, 10 μg of normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and 1 μl of antibody was added to the supernatant. The supernatant was incubated for 1 h at 4 °C. Antiserum raised to the N-terminal (amino acids 1–75) and C-terminal (amino acids 271–283 (10)) regions of PDX-1 were used. Antibody-protein-DNA complexes were isolated by incubation with 60 μl of blocked protein A-Sepharose for 3 h at 4 °C. After extensive washing, bound DNA fragments were eluted and analyzed by PCR using Ready-to-Go PCR beads (Amersham Pharmacia Biotech), 15
amplification of mouse Area I were 5'H11032 bromide staining.

by sequencing. Amplified products were electrophoresed through a 1.4%

CCTTTGGATCATAGCC-3

CAG-3

/H11002

and MIN6 were obtained between these

/H9252

° 95

° 95

were: 5' and 72

° 95

° 95

(41). To determine whether the conserved sequences between

/H11002

2435). Cycling parameters for amplification of mouse PEPCK were:

/H11002

2520 bp display β cell-specific activation (41). To determine whether the conserved sequences between

/H11002

2785), not determined.

/H11002

2520 bp) display

/H11002

2785 to

/H11002

fig. 3 8h a v ea9o f1 3 nucleotide match to the consensus binding

/H11005

/K

pyrimidine, C or T) is shown. Nonmatching nucleotides are in

/H11005

G

A, C, G, or T; K

pyrimidine, C or T) is shown. Nonmatching nucleotides are in

/H11005

A

C

HNF1

/H9251

PDX-1 in Transcriptional Regulation of pdx-1

Apoptosis and PDX-1 in Transcriptional Regulation of pdx-1

Fig. 1. Localization of transcriptional control segments within conserved Area I sequences. A, conserved sequences within Area I sequences are shaded (41). The nucleotides are numbered relative to the human pdx-1 gene. Each bar spans the mutated sequences; the HNF3β control element is labeled. B, wild type and mutant Area I ϕTK CAT were transfected into the HIT-T15, βTC3, and MIN6 cell lines. The percentage of mutant Area I ϕTK activity ± S.D. from at least three to five independent experiments is presented relative to wild type activity. ND, not determined.

RESULTS

Conserved Area I Sequences Are Essential for β Cell Activation—Transfected pdx-1 driven reporter constructs spanning Area I (∼−2839 to −2520 bp) display β cell-specific activation (41). To determine whether the conserved sequences between −2773 and −2643 bp are involved in regulation, each segment (S) of identity was independently mutated, and the effect on Area I activation was assayed in transfected HIT-T15, βTC3, and MIN6 β cell lines (Fig. 1A). Essentially equivalent results were obtained between these β cell lines (Fig. 1B).

Mutations in S4, S5, S6, S7, and S8 all reduced Area I-driven activity (Fig. 1B), and their effect was comparable with mutat-

ing the HNF3β site (41). The sequences found within S4 and S7–8 have a 9 of 13 nucleotide match to the consensus binding site for the HNF4 family of proteins (Refs. 59 and 60 and Fig. 2A). Gel mobility shift binding assays were performed with a protein corresponding to the HNF4 binding site in the apoliprotein CIII gene (apoCIII) (47), in vitro translated HNF4α, and S4, S7–8, and apoCIII competitors. In contrast to apoCIII, neither S4 nor S7–8 competed effectively for HNF4α binding (Fig. 2B). There was also no detectable binding between S4 or S7–8 probes and HNF4α (data not shown). These results demonstrated that HNF4α can not bind to S4 or S7–8, and as a consequence, neither HNF4α nor HNF4γ appear to directly regulate Area I activation.

HNF1α Binds to S2—The HNF1α and HNF1β homeoproteins bind with identical specificity as homodimers or heterodimers (61). S2 has a 10 of 13 nucleotide homology with the consensus HNF1 binding site (Fig. 3A). Binding experiments were performed with the S2 probe and in vitro translated HNF1α, and nuclear extracts were prepared from β (HIT-T15, βTC3), islet α (aTC6), and pancreatic acinar (AR42J) cell lines and rat liver. The in vitro translated HNF1α-bound complex co-migrated with one formed in liver, acinar, and β extracts (Fig. 3B). The β cell complex was supershifted with an antisemur raised to HNF1α but not to HNF1β (Fig. 3C). However, HNF1β was also found in the HNF1 complex from kidney extracts (data not shown). These results indicated that HNF1α and HNF1β could bind to S2. In addition, two other S2 complexes were identified, with the faster migrating βEF1 (β-enriched factor 1) complex only detected in β cell extracts and the other in all (see βEF-1 and U (i.e. for ubiquitous) labeled complexes in Fig. 3B).

The binding properties of the S2 complexes formed in β extracts were determined by competition analyses. All three appeared to be specific, because their formation was decreased by unlabeled wild type probe sequences and not by the S2 block mutant (Fig. 3D). In addition, mutating four base pairs within the HNF1α/β binding core sequence also prevented competition (i.e. S2 7–11 MUT). In contrast, HNF1α binding was selectively eliminated using either the HNF1 binding site from the β-fi-
brinogen (β-FIB) gene or an S2 mutant that contained the HNF1 binding consensus sequences of the β-FIB competitor (S2 to β-FIB, 12 of 13 nucleotide homology; Fig. 3D).

HNF1α has also been shown to bind in vitro to a distinct control element of the rat I (28) and human (57) insulin genes. The homology between the human insulin and the consensus HNF1 binding site is poorer than to S2 (Fig. 3A), and competition analysis demonstrated that HNF1α has a 5-fold higher affinity for S2 than the human site. These results suggest that the diabetic condition found in mature onset diabetes of the young patients may not only be due to effects on insulin transcription but also to effects on pdx-1 expression.

HNF1α Potentiates Area I-driven Activation—To test whether HNF1α regulated Area I activation in β cells, S2 sequences were changed to resemble the HNF1 binding site of the β-FIB gene. The S2 to β-FIB site serves as an effective competitor and probe for HNF1α binding, although it has little or no effect on the other S2 sequences (Fig. 3D; data not shown). This altered specificity mutant was made in Area I alone or Area I and Area II combined. The larger mouse pdx-1 gene promoter construct, termed Pst-Bst, directs β cell-selective expression in transplanted β cell lines and transgenic animals (39).

The activity of the S2 to β-FIB mutant in Area I pTk and Pst-Bst-pTk was compared with the wild type and S2 7–11 mutant. We believed that the S2 7–11 core mutant would be less active than the wild type, because this mutation prevents protein complex formation (Fig. 3). Each of these plasmids was introduced into HIT-T15, βTC-3, and MIN6 cells. As expected, the S2 7–11 mutant reduced both Area I and Pst-Bst-mediated activation (Fig. 4, A and B); the effect was more pronounced in Pst-Bst-pTk (Fig. 4B). The altered specificity mutant was consistently more active than the S2 7–11 mutant in Pst-Bst, although not in Area I alone. Similarly, the HNF3β binding site mutant in Area II only decreased β cell stimulation within a Pst-Bst context, whereas both Area I and Pst-Bst activation were decreased by the HNF3β binding mutant in Area I (41).

To directly determine whether HNF1α regulated Area I expression, an HNF1α expression plasmid was co-transfected with either the wild type or S2 block mutant Area I pTk construct into NIH 3T3 cells, which lack endogenous HNF1α. Overexpression of HNF1α activated Area I pTk but had little effect on the S2 block mutant (Fig. 4C). Collectively, these results suggest that HNF1α regulates pdx-1 activation, but like HNF3β stimulation of Area II, this process involves functional interactions with a β cell activator(s) residing in a neighboring control region.

HNF6 Binds Inefficiently to S5—Although S5 is highly homologous to the consensus HNF6 binding site, in vitro translated HNF6 bound very poorly when compared with the HNF6 site of the HNF3β promoter (Fig. 5A and B). Similarly, S5 was only a slightly more effective HNF6 binding competitor than the S5 block mutant (Fig. 5C). In addition, HNF6 binding to S5 was not detected in β or non-β (BHK and liver) nuclear extract before initiation of the S2 DNA-binding reaction. The positions of the HNF1α and supershifted (SS) HNF1α complexes are shown. The left lane represents the binding reaction conducted in the absence of antisera (−). D, S2 binding reactions were conducted with HIT-T15 nuclear extract either alone (lane 1) or in the presence of a 200-fold molar excess of S2 (lane 2), S2 Block MUT (lane 3), S2 7–11 MUT (lane 4), S2 to β-FIB (lane 5), or β-FIB (lane 6) competitor to the S2 probe.
tracts, although binding was readily observed with the HNF3β site probe (Fig. 5D).

The prominent S5 binding complex was unique to β cell lines and was termed βEF2 (Fig. 5D). Competition analysis demonstrated that βEF2 binding was reduced by the S5 wild type competitor but not the S5 block mutant or HNF3β promoter competitor (Fig. 5E). These results suggested that the protein(s) that forms the βEF2 complex is the S5 activator and not HNF6. The inability to see a change in PDX-1 protein levels in HNF6 null mice also indicates that this factor does not regulate pdx-1 transcription (35).

**FIG. 4.** HNF1α stimulates Area I-driven expression in β cells. A and B, the pdx-1 Area I (A) and Pst-Bst (B) region reporter plasmids are shown diagrammatically. Area I is shaded in the S2 core block mutant (S2 7–11) and hatched in the S2 altered specificity mutant (S2 to β-FIB). The wild type and mutant Area I pTk (A) and Pst-Bst-pTk (B) plasmids were transfected into HIT-T15, βTC3, and MIN6 cell lines. The normalized activity ± S.D. of each Area I mutant construct is presented as the percentage of activity of the corresponding wild type plasmid. C, NIH 3T3 cells were co-transfected with an HNF1α expression vector (pBJ5-HNF1α or vector alone (pBJ5) and the wild type or S2 block mutant Area I pTk. Area I is shaded in the S2 block mutant (S2 BLOCK MUT). The normalized activity ± S.D. of each construct is presented as fold pBJ5-HNF1α activation relative to pBJ5.
Hlxb9 homeodomain proteins were analyzed for binding to S2 (Fig. 6A) and S5 (Fig. 6B). Binding was only observed with in vitro translated PDX-1, and the complex formed co-migrated with H9252 EF1 and H9252 EF2 (Fig. 6A and B). Preincubating the β extract with anti-PDX-1 antisera also specifically eliminated or supershifted the βEF-1 (Fig. 6C) and βEF-2 (Fig. 6D) complexes. As a final test for PDX-1 binding, a competition experiment was performed with the PDX-1 binding A3 element of the insulin gene. As expected, this competitor prevented formation of βEF-1 (Fig. 6C) and βEF-2 (Fig. 6D), although it did not affect HNF1α binding to S2 (Fig. 6C). S5 and A3 were found to bind with similar efficacy to PDX-1 in these assays and 5-fold higher than S2 (data not shown). These data demonstrated that PDX-1 can bind in vitro to two distinct cis-acting regulatory elements of Area I.

**PDX-1 Stimulates Area I Activation**—To determine whether PDX-1 binding to Area I mediated stimulation of Area I pTk and Pst-Bst pTk, S2 and S5 sequences were changed to more closely resemble the insulin A3 element. The S2 mutation specifically eliminated HNF1 binding (Fig. 6C and data not shown). The activity of the A3 conversion mutants was compared in β cells with the wild type and the core element block mutant.

There was little or no difference on S2 activation of Area I.
HNF1α and PDX-1 in Transcriptional Regulation of pdx-1

PDX-1 plays a critical role in both pancreatic development and adult islet β cell function. The nuclease HSS1 region (−2560 to −1880 bp) of the pdx-1 gene contains crucial cis-acting elements involved in expression. The basis for this proposal was supported by data demonstrating that: 1) only HSS1 was independently capable of directing islet β cell selectively expression in transfection assays performed with pdx-1 driven constructs spanning the 4.5-kilobase promoter region (39); 2) the only significant sequence conservation within the promoter region was found within HSS1, as defined by the distinct functional subdomains (i.e. Areas I, II, and III) (41); and 3) a pdx-1-driven transgenic construct spanning Areas I and II was selectively expressed in islet β cells in mice (39). Collectively, these results strongly suggested that HSS1 was regulated by transcription factors critical for β cell formation and function. To localize regulatory elements shared among the mammalian pdx-1 genes, the segments of identity within Area I were specifically mutated, and the effect on activation in β cells was determined in transfection assays. The analyses performed in HIT-T15, βTC-3, and MIN6 cells localized sequences that were involved in stimulation by this HSS1 subdomain (S2, S4, S5, S6, S7, and S8), as well as conserved segments of little or no regulatory importance (S1, S3, and S9). In addition, HNF1-α and PDX-1 itself were shown to mediate activation from Area I.

Strikingly, a transcription factor data base analysis of the mutational sensitive sequences in Area I suggested that proteins critical in endocrine cell development (HNF6, S5) and function (HNF1, S2; HNF4, S4 and S7) were the only significant sequence conservation within the promoter region. The basis for this proposal was supported by data demonstrating that: 1) only HSS1 was independently capable of directing islet β cell selectively expression in transfection assays performed with pdx-1 driven constructs spanning the 4.5-kilobase promoter region (39); 2) the only significant sequence conservation within the promoter region was found within HSS1, as defined by the distinct functional subdomains (i.e. Areas I, II, and III) (41); and 3) a pdx-1-driven transgenic construct spanning Areas I and II was selectively expressed in islet β cells in mice (39). Collectively, these results strongly suggested that HSS1 was regulated by transcription factors critical for β cell formation and function.

To localize regulatory elements shared among the mammalian pdx-1 genes, the segments of identity within Area I were specifically mutated, and the effect on activation in β cells was determined in transfection assays. The analyses performed in HIT-T15, βTC-3, and MIN6 cells localized sequences that were involved in stimulation by this HSS1 subdomain (S2, S4, S5, S6, S7, and S8), as well as conserved segments of little or no regulatory importance (S1, S3, and S9). In addition, HNF1-α and PDX-1 itself were shown to mediate activation from Area I.

Strikingly, a transcription factor data base analysis of the mutational sensitive sequences in Area I suggested that proteins critical in endocrine cell development (HNF6, S5) and function (HNF1, S2; HNF4, S4 and S7–8) contribute to activation. However, only HNF1-α was found to bind segment-specific (βNE) are shown. The HNF1α and βEF binding complexes described in the text are labeled, as are the nonspecific complexes (NS). C and D, HIT-T15 nuclear extract binding to the S2 (C) and S5 (D) probes was conducted in absence (−) or presence (+) of N- or C-terminal PDX-1 antisera and a 200-fold molar excess of the S2, S5, or insulin A3 competitor.

**DISCUSSION**

The ChIP assay is a powerful tool for analyzing the occupancy of transcription factors on their cognate binding elements in vivo (62, 63). To more fully address the possibility of PDX-1-mediated regulation of pdx-1 gene expression, we used the ChIP assay to determine whether PDX-1 binding to Area I could be observed within the context of the endogenous gene. Immunoprecipitation of formaldehyde cross-linked chromatin from βTC3 cells with antibodies specific to the N- or C-terminal region of PDX-1 precipitated Area I sequences, whereas rabbit IgG, CDX-4-specific antisera, or the no antibody controls did not (Fig. 8, top panel). In contrast, anti-PDX-1 antisera did not immunoprecipitate promoter sequences from the PEPCK gene, which is not transcribed in βTC3 cells (Fig. 8, bottom panel). Similar results were observed with MIN6 cells (data not shown). Together with the gel shift and transfection data described above, these results demonstrate that PDX-1 regulates its own transcription.

**Fig. 6. PDX-1 is present in the βEF1 and βEF2 complexes.** A and B, gel mobility shift assays with the S2 (A) and S5 (B) probes were performed in absence (−) and presence of in vitro translated PDX-1, Pax4, Pax6, Nkx2.2, Nkx6.1, or Hlx5/9 homeodomain protein. Translation reactions conducted with [35S]methionine demonstrated that a similar amount of protein was present in each reaction (data not shown). The binding complexes formed with HIT-T15 nuclear extract alone in the A3 conversion mutant versus the 7–11 mutant in HIT-T15 and βTC3 cells, although stimulation was observed in MIN6 cells (Fig. 7A). In contrast, Pst-Bst activity was stimulated in all of the β cell lines by the S2 to A3 mutation (Fig. 7B). The A3 conversion mutant in S5 more profoundly influenced activation, because both Area I and Pst-Bst activities were increased. Depending upon the cell line, Area I alone activation was potentiated 6–13-fold over the core mutant and 1.5–2.5-fold over the wild type. The S5 to A3 conversion mutation in Pst-Bst was essentially the same as wild type Pst-Bst. These results suggested that binding of PDX-1 to S5 and/or S2 was involved in regulating pdx-1 transcription.

**PDX-1 Binds to Area I in Vivo**—The ChIP assay is a powerful tool for analyzing the occupancy of transcription factors on their cognate binding elements in vivo (62, 63). To more fully address the possibility of PDX-1-mediated regulation of pdx-1 gene expression, we used the ChIP assay to determine whether PDX-1 binding to Area I could be observed within the context of the endogenous gene. Immunoprecipitation of formaldehyde cross-linked chromatin from βTC3 cells with antibodies specific to the N- or C-terminal region of PDX-1 precipitated Area I sequences, whereas rabbit IgG, CDX-4-specific antisera, or the no antibody controls did not (Fig. 8, top panel). In contrast, anti-PDX-1 antisera did not immunoprecipitate promoter sequences from the PEPCK gene, which is not transcribed in βTC3 cells (Fig. 8, bottom panel). Similar results were observed with MIN6 cells (data not shown). Together with the gel shift and transfection data described above, these results demonstrate that PDX-1 regulates its own transcription.
probes in gel shift experiments performed with in vitro translated HNF1α, HNF4α, and HNF6 or nuclear extracts prepared from factor-producing cells. Our inability to detect HNF4α or HNF6 binding strongly suggests that these key regulatory proteins do not directly control Area I activation. The absence of a change in PDX-1 protein levels in HNF6 null mice also indicates that this factor does not play any role in regulating pdx-1 transcription (35). Endocrine islet cell development was severely impaired in these mice, apparently because of the requirement for HNF6 in neurogenin 3 expression (35), a transcription factor that is critical in determination of endocrine islet precursors (64).

To test whether HNF1α regulated Area I activation in β cells, S2 sequences were changed to select for HNF1α binding (S2 to β-FIB) within the context of pdx-1 reporter constructs driven by human Area I alone or mouse Pst-Bst, which spans Areas I and II. The activity of the altered specificity mutants were compared with the wild type as well as the HNF1α binding defective mutant construct, S2 7–11 (Fig. 4). The S2 to β-FIB mutant only effectively activated the pdx-1 construct spanning Areas I and II, suggesting that stimulation by HNF1α involves functional interactions with an Area II activator, a situation that parallels the requirement of Area I for HNF3β activation of Area II (41). HNF1α was also shown to specifically activate S2-directed expression in co-transfection assays in NIH 3T3 cells. In contrast to S2, the HNF1α binding site in the human pdx-1 gene recently described may not be of general regulatory significance, because it is not conserved.

![Fig. 7. PDX-1 stimulates Area I-driven expression in β cells. Area I (A and C) and Pst-Bst (B and D) region reporter plasmids are shown diagrammatically. Area I is shaded in the core block mutant in S2 (S2 7–11) and S5 (S5 8–11) and hatched in the A3 conversion mutation. The wild type and mutant Area I pTk and Pst-Bst/pTk plasmids were transfected into HIT-T15, βTC3, and MIN6 cell lines. The normalized activity ± S.D. of each Area I mutant construct is presented as the percentage of activity of the corresponding wild type plasmid. A and B, S2; C and D, S5.](image-url)
within the mouse or chicken genes (65).

The HNF1 family of transcription factors bind DNA as homo- or heterodimers (61). Interestingly, HNF1β is co-expressed with pdx-1 in the ventral and dorsal walls of the primitive foregut during early pancreas development (66). In contrast, HNF1α is expressed at a later developmental stage and at much higher levels than HNF1β in adult β cells (66). We were only able to detect HNF1α in the S2-specific HNF1 complex from HIT-T15, βTC-3, and MIN6 nuclear extracts, although HNF1β was independently shown to be capable of binding to S2 (data not shown). Thus, these results support the possibility that HNF1α and/or HNF1β function as activators of pdx-1 expression during pancreas specification and in the adult islet β cell.

The decrease in insulin synthesis and secretion found in mice lacking HNF1α also provides support for a role in pdx-1 transcription (30, 67), because decreased expression of PDX-1 would be expected to have a debilitating effect upon β cell function through its actions on GLUT2 and insulin transcription. Two distinct lines of HNF1α null mice have been independently derived by Lee et al. (30) and Pontoglio et al. (68). The same HNF1α sequences were targeted for removal (i.e. amino acids 1–108), although each used a different strategy to eliminate them. Both lines result in decreased insulin mRNA expression. However, the absence of HNF1α expression had a distinct effect on islet pdx-1 expression as well as the overall physiology of these animals. The decrease in pdx-1 mRNA expression in HNF1α null mice from Pontoglio et al. was 2.4-fold in the newborn pancreas islets and 2.9-fold in the adult (Fig. 1A of Ref. 69), whereas little or no effect was found in those from Lee et al. in islets from 2-week-old mice (Fig. 3 of Ref. 70). It is unclear which of these mouse models more closely resembles regulation in the human.

In addition to HNF1α, PDX-1 was found to specifically bind to S2 in gel shift assays performed with β cell nuclear extracts (Fig. 6, A and C) and was the principal S5 binding activity detected in these extracts (Fig. 6, B and D). Furthermore, Area I- and Pet-B-mediated activation were compromised in the S5 8–11 mutant (Fig. 7, C and D), demonstrating the critical nature of this site in transcriptional regulation. PDX-1 activated the altered specificity mutants that replaced S2 or S5 with insulin A3. Importantly, PDX-1 was shown to specifically bind to Area I of the endogenous pdx-1 gene using the ChIP assay. In contrast, we were unable to demonstrate HNF1α binding to Area I in vivo (data not shown), which may either reflect occupancy of S2 by PDX-1 or simply a technical inability to recover HNF1α-bound complexes in our ChIP assay.

These data strongly suggest that pdx-1 transcription is autoregulated. However, PDX-1 is not absolutely required for transcription because expression is detected during embryogenesis in pdx-1−/− mice (9) and in the presence of a dominant negative PDX-1 mutant in βTC-3 cells (71). Autoregulation is utilized by other mammalian homeodomain-encoding genes, apparently to prevent extreme fluctuations in expression (72–77). Because PDX-1 is the major S5 binding factor, it is likely to bind and regulate through this site. Support for this proposal is also provided by quantitative competition assays showing that PDX-1 binds with greater affinity to S5 than S2 in vitro and transfection data indicating that PDX-1 can transactivate through S5 (78). We are currently working toward developing model systems to address the importance of PDX-1 autoregulation in vivo.

Islet β cell-specific expression clearly relies on the activity of many cell-enriched transcription factors, which function cooperatively to impart control (1, 3). The data presented have defined conserved cis-acting regulatory elements that are necessary for pdx-1 expression in the β cell. Furthermore, it is possible that the Area I sites that were insensitive to mutation in our assays reflect the limitations of our tumor cell lines rather than their importance in regulation in vivo. Identifying the regulatory factors that control the expression of genes such as pdx-1 may provide insight into the inherited defects that cause insulin deficiency and diabetes. For example, defects in β cell function in mature onset diabetes of the young patients expressing a dysfunctional HNF1α or PDX-1 protein may in part result from defects in pdx-1 expression, because reduced PDX-1 levels would, in turn, likely have profound consequences on expression on many of the target genes involved in glucose sensing, including insulin, glucokinase, and GLUT2.

Acknowledgments—We are grateful to Drs. Chris Wright and Maureen Gannon for assistance in designing and interpreting many of the experiments described here.

REFERENCES

1. Sander, M., and German, M. S. (1997) J. Mol. Med. 75, 327–340
2. St-Onge, L., Wehr, R., and Gruss, P. (1999) Curr. Opin. Genet. Dev. 9, 295–300
3. Stein, R., and Montminy, M. R. (2001) in Handbook of Physiology Section 7: The Endocrine System (Jefferson, L. S., Catt, K. D., and Goodman, H. M., eds) Vol. 2, pp. 25–47, Oxford University Press
4. Poitout, V., and Robertson, R. P. (1996) Annu. Rev. Med. 47, 69–83
5. Hattersley, A. T. (1996) Diabetes Med. 15, 15–24
6. Velho, G., and Frohling, P. (1998) Eur. J. Endocrinol. 138, 233–239
7. Jonsson, J., Carlson, L., Edlund, T., and Edlund, H. (1994) Nature 371, 606–609
8. Ahlgren, U., Jonsson, J., and Edlund, H. (1996) Development 122, 1409–1416
9. Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L., and Wright, C. V. (1996) Development 122, 983–995
10. Stoffers, D. A., Zinkin, N. T., Stanoevic, V., Clarke, W. L., and Habener, J. F. (1997) Nat. Genet. 15, 106–110
11. Guz, Y., Montminy, M. H., Stein, R., Leonard, J., Gamer, L. W., Wright, C. V., and Teitelman, G. (1995) Development 121, 11–18
12. Ohlsson, H., Karlson, K., and Edlund, T. (1993) EMBO J. 12, 4251–4259
13. Olson, L. K., Sharma, A. Peshavaria, M., Wright, C. V. E., Towl, H. C., Robertson, R. P., and Stein, R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9127–9131
14. Peers, B., Leonard, J., Sharma, S., Teitelman, G., and Montminy, M. R. (1995) Mol. Endocrinol. 9, 1798–1806
15. Peshavaria, M., Gamer, L., Hattenberg, E., Teitelman, G., Wright, C. V. E., and Stein, R. (1994) Mol. Endocrinol. 8, 806–816
16. Petersen, H. V., Serup, P., Leonard, J., Michelsen, B. K., and Madsen, O. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10465–10469
17. Wada, H., Kaijomo, Y., Miyagawa, J., Hanafusa, T., Matsuoka, K., Matsuoka, T., Yamamoto, K., Matsuura, H., Itakura, K., and Yamaki, Y. (1996) Diabetes 45, 1826–1831
18. Brehlert-Watt, D., Gore, N., and Boam, D. S. W. (1996) Biochem. J. 331, 495–502
19. Carty, M. D., Lilliquist, J. S., Peshavaria, M., Stein, R., and Soeller, W. C. (1997) J. Biol. Chem. 272, 11899–11903
20. Serup, S., Petersen, H. V., Pedersen, E. E., Edlund, H., Leonard, J., Petersen, J. S., Larsson, L. I., and Madsen, O. D. (1995) Biochem. J. 310, 997–1003
21. Wada, H., Kaijomo, Y., Kaneto, H., Matsuoka, T., Fujitani, Y., Miyazaki, J., and Yamaki, Y. (1996) Biochem. Biophys. Res. Commun. 229, 746–751
