Regulation of Pancreas Duodenum Homeobox-1 Expression by Early Growth Response-1*

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The homeodomain transcription factor pancreas duodenum homeobox-1 (PDX-1) is a key regulator of pancreatic β-cell development, function, and survival. Deficits in PDX-1 expression result in insulin deficiency and hyperglycemia. We previously found that the glucose-responsive transcription factor early growth response-1 (Egr-1) activates the insulin promoter in part by increasing expression levels of PDX-1. We now report that Egr-1 binds and activates multiple regulatory sites within the pdx-1 promoter. We identified consensus Egr-1 recognition sequences within proximal and distal regions of the mouse pdx-1 promoter and demonstrated specific binding of Egr-1 by chromatim immunoprecipitation and electrophoretic mobility shift assays. Overexpression of Egr-1 increased transcriptional activation of the −4500 proximal pdx-1 promoter and of the highly conserved regulatory Areas I, II, and III. Mutagenesis of a specific Egr-1 binding site within Area III substantially decreased Egr-1-mediated activation. Egr-1 increased the transcriptional activation of Areas I and II, despite the absence of Egr-1 recognition sequences within this promoter segment, suggesting that Egr-1 also can regulate the pdx-1 promoter indirectly. Egr-1 increased, and a dominant-negative Egr-1 mutant repressed, the transcriptional activation of distal pdx-1 promoter sequences. Mutagenesis of a specific Egr-1 binding site within regulatory Area IV reduced basal and Egr-1-mediated transcriptional activation. Our data indicate that Egr-1 regulates expression of PDX-1 in pancreatic β-cells by both direct and indirect activation of the pdx-1 promoter. We propose that Egr-1 expression levels may act as a sensor in pancreatic β-cells to translate extracellular signals into changes in PDX-1 expression levels and pancreatic β-cell function.

The homeodomain transcription factor pancreas duodenum homeobox-1 (PDX-1)2 is an essential regulator of the development of the pancreas in the embryo and of insulin production and glucose homeostasis in the adult. Homozygous disruption of the pdx-1/ipf-1 gene in mice or humans prevents normal pancreas development and leads to a phenotype of pancreatic agenesis (1–4). PDX-1 functions in later developmental stages to regulate the generation, function, and survival of insulin-producing pancreatic β-cells (5–10). PDX-1 mediates the adaptive responsiveness of pancreatic β-cells to changes in extracellular glucose concentrations through the regulation of expression levels of glucose sensors, such as glucose transporter-2 and glucokinase, and through the activation of glucose-responsive enhancers within the insulin promoter, in conjunction with other transcriptional regulators (11). PDX-1 also functions downstream of the insulin signaling pathway in the regulation of pancreatic β-cell mass (12, 13).

Deficits in PDX-1 expression levels in mice result in reduced insulin secretion, accelerated β-cell apoptosis, and insulin-deficient diabetes (5, 7–10). The appropriate regulation of PDX-1 expression is of clinical importance, as illustrated by the findings that different mutations in the pdx-1/ipf-1 gene increase susceptibility to the development of hyperglycemia in humans, with a range of phenotypes that include maturity-onset diabetes of the young, Type 2 diabetes, and gestational diabetes (11, 14–19). Therefore, intracellular mechanisms that govern the dynamic regulation of PDX-1 expression are potential determinants of physiologic and pathologic glucose levels.

Several regions within the 5’-flanking sequences of the pdx-1 gene are conserved across multiple species, including mouse, rat, chick, and humans (20–22). Within the proximal −4.5 to −4.6 kb segment of the mouse pdx-1 promoter, three highly conserved and distinct regulatory areas (Areas I, II, and III) were identified within a nuclease-hypersensitive region that confers selective expression in β-cells in culture and in vivo (20, 21, 23). Regulatory Areas I and II confer β-cell selectivity and are active regulatory regions during embryonic pancreas development and in the adult mouse islet (20, 24). Multiple transcription factors converge on regulatory Areas I and/or II within the pdx-1 promoter, including HNF-1α, Foxa2, Maf, Nkx2.2, Pax6, SP1, SP3, and even PDX-1 (20, 21, 23–28). Key transcriptional regulators of Area III of the pdx-1 promoter are yet to be identified. Regulatory Area III sequences confer some β-cell selectivity, albeit less so than sequences derived from regulatory Areas I and II (20, 21). A distal segment of the pdx-1 promoter, designated regulatory Area IV, is conserved in the human, mouse, rat, and chick and confers β-cell selectivity (22, 29, 30). Area IV sequences are activated by Foxa2, NeuroD1/Beta-2, Nkx 2.2, and PDX-1 (22, 30).

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2 The abbreviations used are: PDX-1, pancreas duodenum homeobox-1; Egr-1, early growth response-1; WT, wild type; nt, nucleotide(s); mut, mutant; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; IRS, insulin receptor substrate.
Regulation of PDX-1 by Egr-1

We recently demonstrated that early growth response-1 (Egr-1), a zinc finger transcription factor that also is known as zif268, NGFI-A, Krox24, and TIS8, regulates PDX-1 expression levels in clonal pancreatic β-cells (31). As an immediate early response gene, pulses of increased Egr-1 expression levels can be rapidly induced by a variety of hormones, growth factors, and nutrients (32). In pancreatic β-cells Egr-1 expression levels are known to be regulated by changes in extracellular glucose, membrane depolarization, and intracellular cAMP and calcium levels (33–35). Reduction of Egr-1 expression in insulin-producing pancreatic β-cells decreases insulin mRNA expression (31) and cellular proliferation (36).

In our studies of Egr-1-mediated regulation of insulin production in β-cells, we found that Egr-1 positively regulated the transcriptional activation of the rat insulin 1 promoter. Egr-1 activated the insulin promoter in part via the glucose-responsive A boxes that serve as PDX-1 recognition sites. Interestingly, the overexpression of Egr-1 in INS-1 cells increased the mRNA and protein expression levels of PDX-1. Thus, we proposed that Egr-1 regulates insulin gene transcription through the activation of PDX-1 expression (31).

In these studies, we have examined mechanisms by which Egr-1 regulates the expression of PDX-1 in pancreatic β-cells. Our results indicate that changes in Egr-1 expression levels influence the transcriptional activation of both proximal and distal regulatory segments of the mouse pdx-1 promoter. We have identified Egr-1 recognition sites within the pdx-1 promoter that contribute to its responsiveness to regulation by Egr-1. We propose that Egr-1 provides a route by which extracellular signals modify the transcriptional activation of the pdx-1 promoter to regulate PDX-1 expression levels and pancreatic β-cell function.

EXPERIMENTAL PROCEDURES

Screening Analysis of Mouse pdx-1 Promoter Sequences for Egr-1 Consensus Binding Sites—Approximately 10 kb of mouse pdx-1 promoter sequences available in GenBankTM public databases were analyzed with GENETYX-MAC (10.1) software (Genetyx, Tokyo, Japan) and screened for consensus nonameric Egr-1 recognition sites as defined by Swirnoff and Milbrandt (37). Sequences of potential Egr-1 recognition sites were confirmed by automated sequencing analysis of sense and antisense strands of corresponding regions of mouse genomic DNA amplified by PCR.

Plasmids—The plasmids pcDNA3/Egr-1 and pcDNA3/DN-Egr-1 were constructed as previously reported (31, 38). The 4500 WT/luc plasmid consists of −4531 bp of the proximal mouse pdx-1 promoter cloned upstream of a luciferase reporter in the vector pGL3 (Promega, Madison, WI) and was previously designated −4.6 IDX-pGL3 (39). The plasmid Area 123 WT/luc spans nucleotides −2821 to −1563 to include regulatory Areas I, II, and III of the mouse pdx-1 promoter. Area 123 WT/luc was constructed by cloning the product of PCR amplification from the 4500 WT/luc plasmid template generated with the primers 5′-AGATCTAGTCCAAAAACGCCTTTGG-3′ and 5′-AAGCTTGGAGATCCAGGGAACTCT-3′ into the luciferase reporter vector pGL3/basic. The plasmid Area 12 WT/luc spans nucleotides −2821 to −1885 to include regulatory Areas I and II of the mouse pdx-1 promoter. Area 12 WT/luc was constructed by cloning a product of PCR amplification from the 4500 WT/luc plasmid template generated with the primers 5′-AGATCTTTTGTCCCAAATCTGCTGTA-3′ and 5′-AAGCTTGGAGATCCAGGGAACTCT-3′ into the luciferase reporter vector pGL3/basic. The plasmid Area 4 WT/luc spans nucleotides −6529 to −6047 to include regulatory Area IV of the mouse pdx-1 promoter. Area 4 WT/luc was constructed by first cloning a product of PCR amplification from FVB mouse genomic DNA template generated with the primers 5′-CTCAGGTCTAGCTAGTTGCTATGAAAGG-3′ and 5′-AAGCTTCAGCTCCTTTAAAGGTTAATGAATT-3′ into pcBluntII-TOPO (Invitrogen), subsequent excision of the promoter segment with XhoI and Hind III, and cloning into the luciferase reporter vector pGL3/basic.

The plasmids 4500 mut/luc, Area 123 mut/luc, Area 3 mut/luc, and Area 4 mut/luc were generated by site-directed mutagenesis of Egr-1 recognition sites within 4500 WT/luc, Area 123 WT/luc, Area 3 WT/luc, and Area 4 WT/luc constructs, respectively, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Oligonucleotides used for mutagenesis were as follows: 4500 mut/luc, Area 123 mut/luc, and Area 3 mut/luc, 5′-CTTGGCCGTGATCCAAAACCCCTCGGGCCCTTT-3′ and 5′-AAGGGCCGAGGGTTGGTTGA-TACGCGCAAG-3′; and Area 4 mut/luc, 5′-TGCCCTTTACTCAGGAGCCCAGAAGTAAATAAG-3′ and 5′-CTTATTTTACTTCTGTTCTGGGGCTCTGAGTAAGCAG-3′ (mutations underlined). All constructs generated by cloning and mutagenesis experiments were confirmed by automated sequencing analyses.

Cell Culture and Transfections—Rat INS-1 cells (gift of C. Wollheim, University Medical Center, Geneva, Switzerland) and mouse MIN6 cells (gift of J. Miyazaki, Osaka University Graduate School of Medicine, Osaka, Japan) were cultured as previously reported (31, 40). CMV-rTA/Tet-Egr-1 INS-1 stable cell lines were generated and maintained under selection with G418 and hygromycin (31). For induction of Egr-1 expression, CMV-rTA/Tet-Egr-1 INS-1 cells were grown in vehicle or 1000 ng/ml doxycycline for 24 h prior to harvest. Transfections were conducted with Lipofectamine 2000 (Invitrogen). Transfected cells were harvested 48 or 72 h after transfection, and luciferase activities within cell lysates were measured in replicate samples and normalized by cellular protein content (31). Data from transfections were analyzed by Student’s t tests or by one-way analysis of variance for multiple group comparisons. Differences between samples of p < 0.05 were considered significant.

Electrophoretic Mobility Shift Assays—EMSAs were conducted according to previously described methods (31). Double-stranded 32P-radiolabeled oligonucleotide probes were
either incubated with nuclear extracts prepared according to the method of Schreiber (41) from CMV-rtTA/Tet-Egr-1 INS-1 stable cells pretreated with doxycycline or vehicle or incubated with in vitro translated proteins prepared from pcDNA3 or pcDNA3/Egr-1. Binding reactions were conducted in a buffer composed of polyoxyethylene-sodium polyoxyethylene(10) tyloprolamid acid (Sigma), 12 mM HEPES (pH 7.9), 50 mM KCl, 4.7 mM MgCl₂, 20 μM ZnSO₄, 0.85 mM dithiothreitol, 12.5% glycerol, and 0.5 mg/ml bovine serum albumin for 20 min. In some reactions, proteins were preincubated with anti-Egr-1 antibody (Ab1, rabbit polyclonal anti-Egr-1 (sc110 (588)); Ab2, rabbit polyclonal anti-Egr-1 (C-19), Santa Cruz Biotechnology), or positive control rabbit polyclonal anti-ChIP-IT kits from Active Motif (Carlsbad, CA). Cells were fixed in 1% formaldehyde for 10 min at room temperature, and chromatin was immunoprecipitated with Protein A-Sepharose (mutations underlined).

Chromatin Immunoprecipitation Assays—ChIP assays were conducted with previously reported methods (31) using CHIP-IT kits from Active Motif (Carlsbad, CA). Cells were fixed in 1% formaldehyde for 10 min at room temperature, and genomic DNA was purified and sheared by sonication. Chromatin was immunoprecipitated with Protein A-Sepharose CL-4B (Amersham Biosciences), and complexes were precipitated with rabbit polyclonal anti-Egr-1 (C-19) antibody (Santa Cruz Biotechnology), negative control normal rabbit IgG (Santa Cruz Biotechnology), or positive control rabbit polyclonal anti-PDX-1 antisemur (gift from J. Habener, Massachusetts General Hospital, Boston, MA). Primer pairs used for PCR amplification of immunoprecipitated DNA were as follows: mouse Area I, 5'-CCAGTATCAGGGAGGACTAT-3' (nt = 2712 to 2692) and 5'-CCTAGGGTCTATCCCT-3' (nt 2558); mouse Area II, 5'-TGGCCACTAGGTAGATTATCTGTTG-3' (nt = 1851 to 1828) and 5'-TGCTCCAATGGTCCATGTTCACG-3' (nt = 1720 to 1697); mouse Area IV, 5'-GTCGGCTAGCTGCTGTCAG-3' (nt = 6331 to 6308) and 5'-ACTGGTAAACACATGGGGCTAAC-3' (nt = 5826 to 5807). Products were generated by PCR with a melting step of 94 °C for 3 min followed by 30–35 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), terminal extension at 72 °C for 3 min and subsequent separation by agarose gel electrophoresis.

RNA Expression Analyses—mRNA expression levels for selected transcriptional regulators of the pdx-1 promoter were determined by quantitative real-time TaqMan reverse transcription-PCR on an ABI Prism 7900 HT sequence detection system, using total RNA derived from CMV-rtTA/Tet-Egr-1 INS-1 stable cell lines treated with 0–1000 ng/ml doxycycline for 24 h prior to harvest. Replicate samples were analyzed, and expression levels were normalized to cyclophilin expression. Student’s t tests were employed to detect significant differences in expression levels.

RESULTS

Identification of Egr-1 Recognition Sequences within Proximal and Distal Regions of the Mouse pdx-1 Promoter—We previously discovered that PDX-1 mRNA expression levels were increased in clonal pancreatic β (INS-1)-cells in response to increasing Egr-1 expression levels (31), raising the possibility that Egr-1 regulates the pdx-1 promoter. We analyzed ~10 kb of upstream mouse pdx-1 promoter sequences (GenBank™ accession numbers AC127549, AC115847, AF192495, and AF334615) to identify sequence elements similar to nonanemic consensus Egr-1 recognition sites or their reverse complements as defined by Swirnoff and Milbrandt (Fig. 1A) (37). From this analysis we identified three potential Egr-1 binding sites within highly conserved regulatory regions of the mouse pdx-1 promoter, one in the distal region within regulatory Area IV (~6529 to ~6047 bp) and two within the proximal region encompassing regulatory Areas I, II, and III (~2761 to ~1600 bp) (Fig. 1B).

Egr-1 Binds a Consensus Sequence within Regulatory Area III of the Mouse pdx-1 Promoter—To evaluate whether any of these potential Egr-1 recognition sites were of functional significance, we first examined the sites identified within the proximal region of the pdx-1 promoter. In DNA binding assays, in vitro translated Egr-1 bound to a radiolabeled oligonucleotide probe (Probe 2) that spanned nucleotides ~1817 to ~1782 within regulatory Area III of the mouse pdx-1 promoter and included the
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FIGURE 2. Egr-1 binds to Area III, but not Area II, sequences within the mouse pdx-1 promoter. A, specific binding of in vitro translated Egr-1 to Area III sequences. EMSAs were conducted with radiolabeled wild-type (WT) or mutant (mut) oligonucleotide probe 2 and in vitro transcribed and translated Egr-1 (IVTT +) or empty vector in vitro transcription control reactions (IVTT -), as indicated (+). In competition assays, DNA-binding reactions were preincubated with 100-fold (×100) or 400-fold (×400) excess unlabeled WT or mut probe 2 as indicated (+). Specific protein-DNA complexes containing Egr-1 (arrow) were identified by interference from preincubation with either of two anti-Egr-1 antibodies (Ab1 and Ab2). B, specific binding of Egr-1 derived from INS-1 nuclear extracts to Area III sequences. EMSAs were conducted with nuclear extracts (ne) from stable CMV-rtTA/Tet-Egr-1 INS-1 cells treated with vehicle (ne 0) or doxycycline (ne 1000) for 24 h and radiolabeled WT or mut oligonucleotide probe. DNA-binding reactions were preincubated with 100-fold (×100) or 400-fold (×400) excess unlabeled WT or mut probe 2 as indicated (+). Specific protein-DNA complexes containing Egr-1 (arrow) were identified by interference from preincubation with either of two anti-Egr-1 antibodies (Ab1 and Ab2). C, in vitro translated Egr-1 does not bind to a candidate recognition site in Area II sequences. EMSAs were conducted with radiolabeled WT or mut oligonucleotide probe 3 and in vitro transcribed and translated Egr-1 (IVTT +) or empty vector in vitro transcription control reactions (IVTT -). Competition assays were conducted with excess unlabeled WT or mut probe 3 as indicated (+). Specific protein-DNA complexes containing Egr-1 (arrow) were identified following preincubation with either of two anti-Egr-1 antibodies (Ab1 and Ab2). D, Egr-1 binds to a proximal segment of the pdx-1 gene that includes regulatory Area III but not Area I sequences in ChIP assays. ChIP assays were performed with chromatin derived from MIN6 cells, and immunoprecipitated DNA was analyzed by PCR for detection of proximal pdx-1 promoter segments spanning nucleotides −1851 to −1697 from regulatory Area III (upper panel) or nucleotides −2712 to −2558 from regulatory Area I (lower panel) with control template (input, positive control DNA 1/10 dilution) or water (no DNA negative control). Immunoprecipitations were conducted with no antibody (no Ab), control IgG (IgG), anti-Egr-1 antibody (anti-Egr-1), or anti-PDX-1 antisemur (anti-PDX-1). Representative images are shown of agarose gel electrophoresis of PCR products.

sequence 5′-CACCCCCAC-3′ (Fig. 2A, lane 3 as compared with lane 2). Preincubation of in vitro translated Egr-1 with anti-Egr-1 antibodies attenuated specific Egr-1 binding to this site (Fig. 2A, lanes 8 and 9 as compared with lane 3). The specificity of the Egr-1 recognition sequence was demonstrated by the failure of Egr-1 to bind to a radiolabeled mutant probe 2 with the nonameric sequence 5′-CAACCCAC-3′ (mutations underlined, Fig. 2A, lane 12 as compared with lane 3). The addition of excess unlabeled wild-type (Fig. 2A, lane 5 as compared with lane 3), but not mutant (Fig. 2A, lane 7 as compared with lane 3), probe reduced binding of Egr-1 to the radiolabeled probe 2 in competition assays. We employed a cellular model for the inducible overexpression of Egr-1 that we previously established, stable CMV-rtTA/Tet-Egr-1 INS-1 cells in which the administration of doxycycline increases Egr-1 expression (31), to prepare nuclear extracts with basal or enhanced Egr-1 expression levels. Nuclear proteins derived from CMV-rtTA/Tet-Egr-1 INS-1 cells bound radiolabeled probe 2 in DNA binding assays in a doxycycline-dependent manner (Fig. 2B, lane 3 as compared with lane 2), and specific Egr-1 protein-DNA complexes were identified by attenuation of binding by preincubation with anti-Egr-1 antibodies (Fig. 2B, lanes 8 and 9 as compared with lane 3). These complexes were not observed in DNA binding assays conducted with radiolabeled mutant probe 2 (Fig. 2B, lane 12 as compared with lane 3).

In contrast, we did not observe evidence for direct binding of in vitro translated Egr-1 to radiolabeled oligonucleotide probe 3, spanning nucleotides −1941 to −1907 at the 3′-end of regulatory Area II of the mouse pdx-1 promoter that includes the sequence 5′-AAGGG-GGTG-3′ in a similar series of studies (Fig. 2C). In these assays, the single nucleotide mismatch within the potential nonameric Egr-1 binding site in probe 3 as compared with the consensus Egr-1 recognition
FIGURE 3. Egr-1 increases transcriptional activation of proximal segments of the pdx-1 promoter. A, schematic diagram of mouse pdx-1 enhancer-luciferase reporter constructs. Wild-type (WT) or mutant (mut) constructs containing −4500 (4500), Areas I−III (Area 123), Areas I and II (Area 12), Area III (Area 3), or Area IV (Area 4) promoter/enhancer sequences are depicted with sequence locations relative to the transcription start site indicated on the upper horizontal scale. Approximate locations of Egr-1 recognition site mutations are indicated (asterisk). B, Egr-1 increases transcriptional activation of the −4500-bp pdx-1 promoter. INS-1 cells were transfected with 1.0 μg of empty vector pcDNA3 (open bar) or pcDNA3/Egr-1 (Egr-1, solid bar) and 0.6 μg of 4500 WT/luc reporter construct. Data shown represent the average ± S.D. fold activation of four independent transfections (n = 4), each conducted in duplicate (**, p < 0.01). C, DN-Egr-1 decreases transcriptional activation of the −4500-bp pdx-1 promoter. INS-1 cells were transfected with 1.0 μg of empty vector pcDNA3 (open bar) or pcDNA3/DN-Egr-1 (DN-Egr-1, solid bar) and 0.6 μg of 4500 WT/luc reporter construct. Data shown represent the average ± S.D. fold activation of four independent transfections (n = 4), each conducted in duplicate (**, p < 0.001). D, Egr-1 increases transcriptional activation of a segment encompassing Areas I−III within the pdx-1 promoter. INS-1 cells were transfected with 1.0 μg of empty vector pcDNA3 (open bar) or pcDNA3/Egr-1 (Egr-1, solid bar) and 0.6 μg of Area 123 WT/luc reporter construct. Data shown represent the average ± S.D. fold activation of four independent transfections (n = 4), each conducted in duplicate (**, p < 0.01). E, Egr-1 increases transcriptional activation of a segment encompassing Area III within the pdx-1 promoter. INS-1 cells were transfected with 1.0 μg of empty vector pcDNA3 (open bar) or pcDNA3/Egr-1 (Egr-1, solid bar) and 0.6 μg of Area 3 WT/luc reporter construct. Data shown represent the average ± S.D. fold activation of three independent transfections (n = 3), each conducted in duplicate (**, p < 0.001). F, DN-Egr-1 decreases transcriptional activation of a segment encompassing Area III within the pdx-1 promoter. INS-1 cells were transfected with 1.0 μg of empty vector pcDNA3 (open bar) or pcDNA3/DN-Egr-1 (DN-Egr-1, solid bar) and 0.6 μg of Area 12 WT/luc reporter construct. Data shown represent the average ± S.D. fold activation of three independent transfections (n = 3), each conducted in duplicate (**, p < 0.001).
sequence (Fig. 1A) may have interfered with interactions between Egr-1 and Area II sequences.

Egr-1 Is Associated with Proximal pdx-1 Promoter Segments in Vivo—We also performed ChIP assays in MIN6 cells to assess whether Egr-1 proteins associate with proximal segments of the mouse pdx-1 promoter in insulin-producing cells in vivo. In ChIP assays, we immunoprecipitated proximal pdx-1 promoter regulatory segments encompassing Area III within mouse genomic DNA derived from MIN6 cells with anti-Egr1 antibodies but not with negative control immunoglobulin IgG (Fig. 2D, upper panel). These segments of the mouse pdx-1 promoter also were immunoprecipitated by anti-PDX-1 antiserum, consistent with previous reports of PDX-1 binding to Area III sequences in vivo (26). Anti-Egr1 antibody did not immunoprecipitate negative control Area I sequences within mouse genomic DNA (Fig. 2D, lower panel).

Activation of Proximal Regions of the Mouse pdx-1 Promoter by Egr-1—In INS-1 cells transfected with a −4500 mouse pdx-1 promoter–luciferase reporter construct designated 4500 WT (Fig. 3A), the overexpression of Egr-1 increased proximal pdx-1 promoter activity by ∼50% (Fig. 3B). In contrast, the introduction of a dominant-negative mutant Egr-1 protein comprised of only the zinc finger DNA-binding domain (38) significantly decreased the basal activity of the promoter both in INS-1 cells (Fig. 3C) and in MIN6 cells (data not shown). Similarly, Egr-1 overexpression significantly increased the transcriptional activation of a −2821 to −1563 bp Area I–III enhancer construct in transfected INS-1 cells (Fig. 3D). Consistent with our identification of an Egr-1 binding site within regulatory Area III of the mouse pdx-1 promoter, the overexpression of Egr-1 significantly increased, and expression of dominant-negative mutant Egr-1 significantly decreased, the transcriptional activation of an isolated Area III enhancer-reporter construct (Fig. 3, E and F). Although we did not identify a functional Egr-1 binding site within the sequences of regulatory Areas I and II, the enhancer region of the mouse pdx-1 promoter encompassing Areas I and II also was activated by Egr-1 and inhibited by dominant-negative mutant Egr-1 in transfection assays (Fig. 3, G and H), raising the possibility that Egr-1 regulates the pdx-1 promoter through a combination of direct and indirect mechanisms.

To determine the functional impact of the Egr-1 binding site identified within regulatory Area III of the pdx-1 promoter, we introduced the mutation 5\'-CAAACCAAC-3\' within the −4500, Area I–III, and Area III enhancer-reporter constructs. In transfected INS-1 (Fig. 4) or MIN6 cells mutagenesis of the Egr-1 binding site within Area III significantly reduced the activation of pdx-1 promoter sequences by Egr-1. Mutagenesis of the Area III Egr-1 binding site attenuated the activation of the −4500 pdx-1 promoter-reporter construct by 22% (Fig. 4A). Activation of Area I–III and Area III enhancer constructs by Egr-1 was highly dependent on the Egr-1 binding site, with mutagenesis reducing Egr-1-dependent activation by 43% (Fig. 4B) and 55% (Fig. 4C), respectively. Notably, the mutagenesis of the Area III Egr-1 binding site significantly reduced but did not eliminate the Egr-1 responsiveness of the proximal segment of the pdx-1 promoter or of the Area I–III regulatory regions in INS-1 cells, suggesting that additional regulatory options exist for the activation of pdx-1 expression by Egr-1.

To consider the possibility that Egr-1 modifies the expression levels of other transcriptional regulators of the pdx-1 promoter, we employed the CMV-rtTA/Tet-Egr1-INS1 cell model of inducible Egr-1 expression in which the administration of doxycycline to CMV-rtTA/Tet-Egr1-INS1 cells significantly increased Egr-1 mRNA levels (31). The expression levels of several transcription factors that regulate PDX-1 expression, including Foxa2 (31), HNF-1α, HNF-1β, MafA, Pax6, USF, and SP1, did not significantly change in response to increasing levels of Egr-1 in CMV-rtTA/Tet-Egr1-INS1 cells as determined by quantitative real-time reverse transcription-PCR (data not shown).

Egr-1 Binds a Consensus Sequence within Regulatory Area IV of the Mouse pdx-1 Promoter—Next we considered whether Egr-1 binds the recognition site identified within the distal pdx-1 promoter sequences. We conducted EMSAs with probe 1, corresponding to nucleotides −6277 to −6255 of the distal
unlabeled wild-type (Fig. 5A, lane 5 as compared with lane 3) but not unlabeled mutant (Fig. 5A, lane 7 as compared with lane 3) probe. We conducted similar experiments with nuclear extracts prepared from CMV-rtTA/Tet-Egr-1 INS-1 cell lines in which Egr-1 protein expression was increased by the addition of doxycycline. We observed doxycycline-dependent increases in a protein-DNA complex (Fig. 5B, lane 3 as compared with lane 2) that was attenuated by preincubation with anti-Egr-1 antibodies (Fig. 5B, lanes 8 and 9 as compared with lane 3). Competition experiments with excess unlabeled wild-type (Fig. 5B, lane 5) and mutant (Fig. 5B, lane 7) probes confirmed the specificity of the Egr-1 recognition site.

**Egr-1 Is Associated with Distal pdx-1 Promoter Segments in Vivo—** To determine whether Egr-1 proteins also associate with distal segments of the mouse pdx-1 promoter in intact insulin-producing cells, we conducted a second series of ChIP assays with genomic DNA derived from MIN6 cells. Egr-1 antibodies immunoprecipitated a distal pdx-1 promoter regulatory segment that encompassed the Egr-1 recognition sequence identified within regulatory Area IV in a specific manner (Fig. 5C, upper panel). As a positive control in these studies we also confirmed a previous report that PDX-1 associates with regulatory Area IV sequences in vivo (22). Because the Egr-1 recognition site sequences in Area IV are conserved in the mouse and rat pdx-1 promoters (22, 29, 30), we were able to conduct additional ChIP assays with genomic DNA derived from INS-1 cells. In these studies we observed a specific association of Egr-1 with rat genomic Area IV sequences that encompass the conserved Egr-1 recognition site (Fig. 5C, lower panel) as compared with negative control rat insulin 1 promoter sequences (31).

**Activation of a Distal Segment of the Mouse pdx-1 Promoter by Egr-1—** To assess the potential function of Egr-1 in the regulation of the distal mouse pdx-1 promoter segment, we generated an Area IV pdx-1 enhancer (~6529 to ~6047 bp) reporter construct. Overexpression of Egr-1 significantly increased transcriptional activation of the Area IV enhancer-reporter construct by 84% in transfected INS-1 cells (Fig. 6A, left panel).
Regulation of PDX-1 by Egr-1

Mutagenesis of the Egr-1 binding site significantly decreased the basal activity of the Area IV enhancer by 28%, suggesting that endogenous Egr-1 likely functions to maintain the activity of distal pdx-1 enhancer sequences through this site. The mutant Area IV enhancer construct was substantially less responsive to Egr-1, although a small component of Egr-1 responsiveness was retained (Fig. 6A, right panel), as further demonstrated by the dose-dependent response to increasing expression levels of Egr-1 (Fig. 6B). In transfected MIN6 cells the expression of Egr-1 significantly increased the transcriptional activation of wild-type but not mutant Area IV enhancer-reporter constructs (data not shown). Expression of the dominant-negative mutant Egr-1 significantly reduced the activation of the Area IV enhancer in INS-1 cells (Fig. 6C), consistent with a function for native Egr-1 in maintaining distal pdx-1 enhancer activation. These results indicate that Egr-1 can activate the distal pdx-1 promoter segment through the Egr-1 binding site within Area IV and suggest that additional indirect mechanisms contribute to the activation of this promoter segment by Egr-1.

**FIGURE 6.** Egr-1 increases transcriptional activation of distal segments of the mouse pdx-1 promoter. A, Egr-1 increases activation of a segment of the pdx-1 promoter encompassing regulatory Area IV, and mutagenesis of the Area IV Egr-1 binding site disrupts that activation. INS-1 cells were transfected with 1.0 µg of empty vector pcDNA3 (open bars) or pcDNA3/Egr-1 (Egr-1, solid bars) and 0.6 µg of Area 4 mut/luc (Area 4 mut) or Area 4 WT/luc (Area 4 WT) reporter constructs. Data shown represent the average ± S.D. fold activation for each reporter construct from four independent transfections (n = 4), each conducted in duplicate (***, p < 0.01). B, Egr-1 activates Area IV sequences in a dose-dependent manner. INS-1 cells were transfected with 0, 0.4, 0.7, or 1.0 µg of pcDNA3/Egr-1 (Egr-1) and 0, 0.6, 0.3, or 0 µg of control empty vector pcDNA3, respectively, and 0.6 µg of Area 4 mut/luc (Area 4 mut, solid bars) or Area 4 WT/luc (Area 4 WT, open bars) reporter constructs. Data shown represent the average ± S.D. fold activation for each reporter construct from four independent transfections (n = 4), each conducted in duplicate (***, p < 0.01). C, DN-Egr-1 decreases transcriptional activation of Area IV sequences. INS-1 cells were transfected with 1.0 µg of empty vector pcDNA3 (solid bar) or pcDNA3/DN-Egr-1 (DN-Egr-1, open bar) and 0.6 µg of Area 4 WT/luc (Area 4 WT) reporter construct. Data shown represent the average ± S.D. fold activation from four independent transfections (n = 4), each conducted in duplicate (**+, p < 0.01).

**DISCUSSION**

In these studies we report that the early response gene and zinc finger transcription factor Egr-1 activates the expression of the pancreatic regulator PDX-1. We have identified two specific Egr-1 recognition sites that are located within highly conserved regulatory areas within proximal (Area III) and distal (Area IV) regions of the mouse pdx-1 promoter. These Egr-1 binding sites reside in regulatory areas that serve as convergence points for multiple transcription factors and collectively contribute to the specificity of the transcriptional expression of the pdx-1 gene in embryonic and adult pancreatic β-cells (20, 22). The functional importance of each of the two Egr-1 recognition sites is illustrated in our mutagenesis experiments, in which modifications of the nonamer Egr-1 consensus sequence disrupt both Egr-1 binding in EMSAs as well as basal and Egr-1-mediated transcriptional activation of pdx-1 promoter segments in transfected cells. Multiple consensus sites for other transcription factors have been identified within regulatory Areas I and II within the proximal 5’-flanking sequences of the mouse pdx-1 promoter, but current knowledge is limited regarding regulatory mechanisms that converge on Area III. In this respect the proximal Egr-1 recognition site is unique.

In transfected clonal pancreatic β-cells, we demonstrate that the overexpression of Egr-1 enhances the transcriptional activation of both proximal and distal segments of the pdx-1 promoter. Furthermore, interference with activation by Egr-1, through expression of a dominant-negative mutant Egr-1 protein, suggests that endogenous Egr-1 expression is required for maintaining basal promoter activation. However, not all of the Egr-1-responsive transcriptional activation of the pdx-1 promoter appears to be mediated through the identified Egr-1 recognition sites. Mutagenesis of Egr-1 binding sites significantly reduces but does not completely eliminate activation by Egr-1 of either proximal or distal promoter regions. We attribute this residual activation to an indirect effect of Egr-1 to activate other regulatory elements within the pdx-1 promoter, possibly mediated by other transcription factors. We have found no evidence for Egr-1-dependent increases in mRNA expression levels of multiple known regulators of the pdx-1 promoter, with the exception of PDX-1. PDX-1 binds to both proximal and distal regions of the pdx-1 pro-
Regulation of PDX-1 by Egr-1

The regulation of target genes by Egr-1 also offers the advantage of temporal flexibility in the dynamic regulation of PDX-1 expression. Negative feedback loops have been well characterized for the regulation of Egr-1 expression levels (47). Induction of Egr-1 expression increases expression levels of the corepressor Nab2. Both Nab2 and Egr-1 can subsequently down-regulate and modify Egr-1-mediated regulation of the promoter through PDX-1 binding sites within proximal and distal promoter segments.

It is possible that the Egr-1 recognition sites we identified within the PDX-1 promoter may provide loci for more complex control of transcriptional regulation. In several instances, other transcription factors have been identified in overlapping interactions with Egr-1 recognition sites in selected promoters (47). Many zinc finger transcription factors demonstrate some binding affinity for GC-rich sequences, and other Egr-1 family members, including WT-1, often bind Egr-1 recognition sites in vitro. Precedents also exist for complex regulation of promoters by both Egr-1 and SP1. Notably the human pdx-1 promoter can be activated by both SP1 and SP3 (25). SP1 consensus recognition sequences partially overlap with Egr-1 consensus sequences in some cases, providing the opportunity for Egr-1 to compete with SP1 for promoter occupancy (47). In addition to competition between Egr-1 and SP1 proteins for occupancy of some recognition sites, the potential also exists for phosphorylated Egr-1 to directly interact with and sequester SP1 in selected experimental contexts (47). Despite this potential regulatory complexity, in our ChIP studies we observed in vivo occupancy of regions spanning both proximal and distal Egr-1 recognition sites within the mouse pdx-1 promoter by Egr-1 proteins.

It is important to note that pancreatic β-cell function appears to be exquisitely sensitive to small changes in PDX-1 expression levels within a 2-fold range in both humans and mice. Decreased insulin synthesis and secretion, hyperglycemia, reduced β-cell mass, and increased β-cell apoptosis result from heterozygous inheritance of mutant pdx-1 or ipf-1 genes (6, 7, 10, 14, 15). Furthermore, β-cell phenotypes, associated with reductions in PDX-1 expression levels of 50% or less, increase in severity with aging (7, 8, 14, 15).

The activation of the pdx-1 promoter by increasing levels of Egr-1 expression may have broad physiologic relevance. Egr-1 expression is rapidly and transiently increased by an extensive range of extracellular signals, including nutrients, hormones, and growth factors. In pancreatic β-cells, extracellular glucose levels regulate Egr-1 expression (33–35, 48, 49). Interestingly in many other cell types, but not in β-cells under the experimental conditions tested to date (34, 35), increases in extracellular insulin levels augment Egr-1 expression (50, 51). Insulin-mediated activation of Egr-1 expression has been reported to work through multiple components of insulin signaling pathways, including insulin receptor substrate (IRS) proteins IRS-1, IRS-2, and IRS-3; kinases p38K, Erk (extracellular signal-regulated kinase), phosphatidylinositol 3-kinase, and protein kinase Ca; and the adaptor protein Gab1 (51–55). Egr-1 may be required for insulin-dependent increases in DNA synthesis (56), and increases in Egr-1 expression occur in response to insulin even in selected cellular models of insulin resistance (57). Egr-1 expression also increases in many cell types in response to hypoxia as well as in post-injury-regenerative responses (58, 59). Thus, the identification of Egr-1 recognition sites within the pdx-1 promoter raises multiple possibilities for the regulation of PDX-1 expression levels by a range of extracellular signals that converge on Egr-1.

The regulation of target genes by Egr-1 also offers the advantage of temporal flexibility in the dynamic regulation of PDX-1 expression. Negative feedback loops have been well characterized for the regulation of Egr-1 expression levels (47). Induction of Egr-1 expression increases expression levels of the corepressor Nab2. Both Nab2 and Egr-1 can subsequently down-regu-
late egr-1 gene expression to restrict the duration of increases in Egr-1 levels in response to activating signals.

In addition to the regulation of PDX-1 and insulin gene expression, broader functions for Egr-1 likely exist within the endocrine pancreas. Reductions in Egr-1 expression levels diminish the proliferation rates of clonal insulin-producing cells (36). Furthermore, exendin-4 augments the expression levels of the cell cycle regulator cyclin D1 by increasing the binding of Egr-1 to the cyclin D1 promoter (60). These findings are concordant with the identification of multiple cell cycle regulators and growth factors as targets of Egr-1-mediated transcription in other cell types (61, 62). Egr-1 also is likely to be important in the regulation of glucagon expression in pancreatic α-cells in response to extracellular hormones, including gastrin (63). Egr-1 regulates cellular functions in multiple endocrine cell types, including pancreatic α- and β-cells, pituitary somatotropes and gonadotropes, and ovarian follicular cells (64).

Future studies will be needed to better define the transcriptional targets and functions of Egr-1 in pancreatic β-cells. We suggest that regulators of Egr-1 expression and/or function may provide effective mechanisms to modify PDX-1 expression levels, insulin production, and pancreatic β-cell function.

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REFERENCES
1. 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
2. Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994) Nature 371, 606–609
3. Stoffers, D. A., Zinkin, N. T., Stanoejivc, V., Clarke, W. L., and Habener, J. F. (1997) Nat. Genet. 15, 106–110
4. Schweitzgebel, V. M., Mamin, A., Brun, T., Ritz-Laser, B., Zaiko, M., Maret, A., Jornayvaz, F. R., Theintz, G. E., Michelini, O., Melloul, D., and Philippe, J. (2003) J. Clin. Endocrinol. Metab. 88, 4398–4406
5. Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., and Edlund, H. (1998) Genes Dev. 12, 1763–1768
6. Dutta, S., Bonner-Weir, S., Montminy, M., and Wright, C. (1998) Nature 392, 560
7. Johnson, J. D., Ahmed, N. T., Luciani, D. S., Han, Z., Tran, H., Fujita, J., MISLER, S., Edlund, H., and Polonsky, K. S. (2003) J. Clin. Invest. 111, 1147–1160
8. Thomas, M. K., Devon, O. N., Lee, J. H., Peter, A., Schlosser, D. A., Tenser, M. S., and Habener, J. F. (2001) J. Clin. Invest. 108, 319–329
9. Holland, A. M., Hale, M. A., Kagaami, H., Hammer, R. E., and MacDonald, R. I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12236–12241
10. Brissova, M., Shiota, M., Nicholson, W. E., Gannon, M., Knobel, S. M., Piston, D. W., Wright, C. V., and Powers, A. C. (2002) J. Biol. Chem. 277, 11225–11232
11. Thomas, M. K., and Habener, J. F. (2004) in Molecular Basis of Inborn Errors of Development (Epstein, C. J., Erikson, R. P., and A. W. -B., eds) pp. 552–556, Oxford University Press, New York
12. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W. H., 3rd, Wright, C. V., White, M. F., Arden, K. C., and Accili, D. (2002) J. Clin. Invest. 110, 1839–1847
47. Silverman, E. S., and Collins, T. (1999) Am. J. Pathol. 154, 665–670
48. Susini, S., Roche, E., Prentki, M., and Schlegel, W. (1998) FASEB J. 12, 1173–1182
49. Bernal-Mizrachi, E., Wice, B., Inoue, H., and Permutt, M. A. (2000) J. Biol. Chem. 275, 25681–25689
50. Jhun, B. H., Haruta, T., Meinkoth, J. L., Leitner, W., Draznin, B., Saltiel, A. R., Pang, L., Sasaoka, T., and Olefsky, J. M. (1995) Biochemistry 34, 7996–8004
51. Bruning, J. C., Winnay, J., Cheatham, B., and Kahn, C. R. (1997) Mol. Cell Biol. 17, 1513–1521
52. Tsuruzoe, K., Emkey, R., Kriauciunas, K. M., Ueki, K., and Kahn, C. R. (2001) Mol. Cell Biol. 21, 26–38
53. Harada, S., Esch, G. L., Holgado-Madruga, M., and Wong, A. J. (2001) DNA Cell Biol. 20, 223–229
54. Lin, Y. L., Chen, H. C., Yeh, S. F., and Chou, C. K. (1995) Endocrinology 136, 2922–2927
55. Keeton, A. B., Bortoff, K. D., Bennett, W. L., Franklin, J. L., Venable, D. Y., and Messina, J. L. (2003) Endocrinology 144, 5402–5410
56. Gousseva, N., Kugathasan, K., Chesterman, C. N., and Khachigian, L. M. (2001) J. Cell Biochem. 81, 523–534
57. Sartipy, P., and Loskutoff, D. J. (2003) J. Biol. Chem. 278, 52298–52306
58. Bae, S. K., Bae, M. H., Ahn, M. Y., Son, M. J., Lee, Y. M., Bae, M. K., Lee, O. H., Park, B. C., and Kim, K. W. (1999) Cancer Res. 59, 5989–5994
59. Taub, R., Greenbaum, L. E., and Peng, Y. (1999) Semin. Liver Dis. 19, 117–127
60. Kang, J. H., Kim, M. J., Ko, S. H., Jeong, I. K., Koh, K. H., Rhie, D. J., Yoon, S. H., Hahn, S. J., Kim, M. S., and Jo, Y. H. (2006) Diabetologia 49, 969–979
61. Svaren, J., Ehrig, T., Abdulkadir, S. A., Ehrengruber, M. U., Watson, M. A., and Milbrandt, J. (2000) J. Biol. Chem. 275, 38524–38531
62. Fu, M., Zhu, X., Zhang, J., Liang, J., Lin, Y., Zhao, L., Ehrengruber, M. U., and Chen, Y. E. (2003) Gene (Amst.) 315, 33–41
63. Leung-Theung-Long, S., Roulet, E., Clerc, P., Escrieu, C., Marchal-Victorion, S., Ritz-Laser, B., Philippe, J., Pradayrol, L., Seva, C., Fourmy, D., and Dufresne, M. (2005) J. Biol. Chem. 280, 7976–7984
64. Topilko, P., Schneider-Maunoury, S., Levi, G., Trembleau, A., Gourdi, D., Driancourt, M. A., Rao, C. V., and Charnay, P. (1998) Mol. Endocrinol. 12, 107–122