Regulation of Insulin Gene Transcription by the Immediate-Early Growth Response Gene Egr-1

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Changes in extracellular glucose levels regulate the expression of the immediate-early response gene and zinc finger transcription factor early growth response-1 (Egr-1) in insulin-producing pancreatic β-cells, but key target genes of Egr-1 in the endocrine pancreas have not been identified. We found that overexpression of Egr-1 in clonal (INS-1) β-cells increased transcriptional activation of the rat insulin 1 promoter. In contrast, reductions in Egr-1 expression levels or function with the introduction of either small interfering RNA targeted to Egr-1 (siEgr-1) or a dominant-negative form of Egr-1 decreased insulin promoter activation, and siEgr-1 suppressed insulin gene expression. Egr-1 did not directly interact with insulin promoter sequences, and mutagenesis of a potential GC-rich consensus sequences within a few hundred base pairs upstream of the transcription initiation site conferred β-cell-specific expression to exogenously introduced genes (1). Insulin genes of human, rat, and mouse share a number of conserved regulatory elements within the insulin promoter sequences, and mutagenesis of a potential G box recognition sequence for Egr-1 did not impair the Egr-1 responsiveness of the insulin promoter, suggesting that regulation of insulin gene expression by Egr-1 is probably mediated through additional transcription factors. Overexpression of Egr-1 increased, and reduction of Egr-1 expression decreased, transcriptional activation of the glucose-responsive FarFlat minienhancer within the rat insulin 1 promoter despite the absence of demonstrable Egr-1-binding activity to FarFlat sequences. Notably, augmenting Egr-1 expression levels in insulin-producing cells increased the mRNA and protein expression levels of pancreas duodenum homeobox-1 (PDX-1), a major transcriptional regulator of glucose-responsive activation of the insulin gene. Increasing Egr-1 expression levels enhanced PDX-1 binding to insulin promoter sequences, whereas mutagenesis of PDX-1-binding sites reduced the capacity of Egr-1 to activate the insulin promoter. We propose that changes in Egr-1 expression levels in response to extracellular signals, including glucose, can regulate PDX-1 expression and insulin production in pancreatic β-cells. (Endocrinology 147: 2923–2935, 2006)

THE TISSUE-SPECIFIC expression of the insulin gene in pancreatic β-cells is controlled by 5′-flanking regulatory sequences within the insulin promoter. Conserved sequences within a few hundred base pairs upstream of the transcription initiation site confer β-cell-specific expression to exogenously introduced genes (1). Insulin genes of human, rat, and mouse share a number of conserved regulatory elements in the 5′-flanking sequences (2). E, A, and C1/RIPE3b elements within the insulin promoter are major determinants of β-cell-specific expression of the insulin gene (3). E boxes (E1 and E2) are recognition sites for members of the basic helix-loop-helix (bHLH) family of transcription factors that bind as heterodimeric complexes consisting of ubiquitous and tissue-specific bHLH proteins. In pancreatic β-cells, these bHLH protein complexes include alternatively spliced E2A gene products E12, E47, and HeLa E-box-binding factor (HEB) (4) as well as the β-cell-specific bHLH protein Beta-2/NeuroD1, which is known to regulate insulin gene expression and pancreas development (5, 6). A boxes are comprised of core TAAT sequence motifs that constitute binding sites for transcription factors of the homeodomain protein family, including the β-cell-specific factor pancreas duodenum homeobox-1 (PDX-1) (7–11). PDX-1 is a critical factor for the development and differentiation of β-cells and the maintenance of glucose homeostasis (reviewed in Refs. 12 and 13). Reductions in PDX-1 expression levels are associated with hyperglycemia in humans and in mouse models. In addition to the regulation of insulin gene expression, PDX-1 regulates insulin secretion and β-cell mass (14–16).

Glucose is a key nutritional regulator of insulin gene transcription via multiple proposed mechanisms. Increases in extracellular glucose concentrations stimulate nuclear translocation, DNA-binding activity, and transcriptional activation by PDX-1 (17–20). PDX-1 participates with bHLH transcription factors in the synergistic activation of glucose-responsive E and A box enhancer sequences, including the FarFlat minienhancer within the rat insulin 1 promoter (21–23). Glucose metabolism by β-cells is required for glucose-induced increases in insulin gene transcription (24). However, the key intermediary messengers that link glucose or its metabolites to the regulation of β-cell-enriched transcription factors have not been fully elucidated.

The zinc finger transcription factor Egr-1, also known as zif268, nerve growth factor I-A, Krox24, and TIS8, is a member of a group of immediate-early response genes that are induced by growth factors, hormones, and neurotransmitters (25–29). Egr-1 is a modular protein with an amino-terminal transactivation domain, a central transcriptional repression domain, and a carboxyl-terminal zinc finger DNA-binding domain that consists of three zinc finger motifs. Egr-1 binds GC-rich consensus sequences within 5′-regulatory sequences
of target genes (30). Both cAMP response element-binding protein-binding protein and p300 serve as transcriptional coactivators for Egr-1-mediated gene expression (31, 32). Egr-1 is widely expressed and regulates a range of cellular processes, including proliferation, growth, and apoptosis (33). The zinc finger domain of Egr-1, encoded by amino acids 331–427, has a dominant-negative effect on the transcriptional activity of wild-type Egr-1 (34). This dominant-negative form of Egr-1 (DN-Egr-1) blocks the actions of Egr-1 on neurite outgrowth and cerebellar granule cell apoptosis and provides a useful experimental tool for analyzing the transcriptional regulation of Egr-1 target genes (35, 36). Mice deficient in Egr-1 (Egr-1−/−) are viable (37, 38), with phenotypes that include female infertility (38, 39), reductions in cardiac allograft vasculopathy (41), and diminished expression of mediators for vascular injury (42).

Egr-1 is also expressed in pancreatic β-cells. Egr-1 expression is induced rapidly by glucose in insulin-producing MIN6 and INS-1 clonal β-cells and in primary rat islets (43–46). The induction of Egr-1 expression levels by glucose is mediated in part by recruitment of serum response factor and cAMP response element-binding protein to respective binding elements on the Egr-1 promoter (47). Among several β-cell lines, the induction of Egr-1 expression by glucose is stronger in highly glucose-responsive MIN6, βTC3, and INS-1 cells than in weakly glucose-responsive RINm5F cells and is not detected in β-cell lines that fail to secrete insulin in response to glucose (43). Thus, Egr-1 is a glucose-responsive gene, and the induction of Egr-1 expression by glucose appears to correlate with β-cell secretory function. However, a function for Egr-1 in the regulation of insulin production in pancreatic β-cells has not been identified.

In these studies we implicate Egr-1 in the regulation of insulin gene transcription. Our results suggest that Egr-1 can indirectly regulate the insulin promoter through the regulation of PDX-1 expression to converge on glucose-responsive enhancer sequences. We propose that Egr-1 contributes to the glucose responsiveness and function of pancreatic β-cells through the regulation of PDX-1 expression and the activation of insulin gene transcription.

Materials and Methods

Plasmids

Mouse Egr-1 cDNA in pBS/KS+ was obtained from American Type Culture Collection (Manassas, VA). Full-length Egr-1 cDNA, including the 5′ Kozak sequence and the 3′-polyadenylation signal, was excised with Smal/Apal and inserted into the EcoRV/Apal site of pcDNA3 (Invitrogen Life Technologies, Inc., Carlsbad, CA) to generate the Egr-1 expression vector pcDNA3/Egr-1. The plasmid pcDNA3/DN-Egr-1 that encodes the zinc finger domain (amino acids 331–427) of mouse Egr-1 was a gift from N. Perkins (University of Dundee, Dundee, UK). The pXp2–412/luciferase (luc), pA3–83/luc, pA3–118/luc, pA3–412/luc, and pA3 luc plasmids encoding the +2 to −412, +2 to −83, +2 to −118, +2 to −184, +2 to 345, and +2 to −412 bp and no sequences, respectively, from the rat insulin 1 promoter with luc reporters have been described previously (48, 49). The luc reporter pFoxFarFlat/luc, that encodes pentamerized FarFlat minimal enhancers, and the empty reporter plasmid (pFox) (50) were gifts from M. German (University of California, San Francisco, CA). The Tet-Egr-1 construct was generated by cloning full-length mouse Egr-1 cDNA downstream of heptamerized Tet operator sequences in the plasmid pUHD10-3 (51). Both pUHD10-3 and cytomegalovirus plasmid (pCMV)-rtTA (pUHHG17-1) plasmids were obtained from H. Bujard (University of Heidelberg, Heidelberg, Germany) (52).

Site-directed mutagenesis of the G box within the pA3–184/luc plasmid and of the A elements within the pA3–412/luc and pA3–83/luc plasmids was conducted with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Sense oligonucleotides and their reverse complements used for mutagenesis (with mutations underlined) were 5′-GCGCAAAACGGCCAAAGTCCAGGTTTACTAGGAGGAAGTCTTGGAGC-3′ for the G box mutation within pA3–184/luc, 5′-ATCCCCGGGTACCCCTTCGGCCAAACGGC-3′ for the A1 element mutation within pA3–83/luc, 5′-TCTAGAGCCTCTTCCTGGCCTTGAAGGCGGCCGAGGCTC-3′ for the A1 element mutation within the pA3–412/luc, 5′-CCCTGTGTAATATCGAAGCTGAGGTTGAGC-3′ for the A1 element mutation within pA3–412/luc, and 5′-CCATCTGGCCCCCTTGGTCCGAATGCTGACCCCTA-3′ for the A1 element mutation within pA3–412/A3mut/luc. All constructs generated by cloning and mutagenesis experiments were confirmed by sequence analyses.

Small interfering RNAs (siRNAs)

To generate siRNA/Egr-1, a 19-mer sequence was identified as unique to Egr-1, as determined by BLAST searches of the nonredundant National Center for Biotechnology Information nucleotide databases, and was flanked with two adenine ribonucleotides and two deoxythymidines. Double-stranded siRNA sequences were as follows: siEgr-1, 5′-AAGGUAUAACGCGCGAAAAGAGC-3′; siA1, 5′-CCCTGTGTAATATCGAAGCTGAGGTTGAGC-3′; siA2, 5′-ATCCCCGGGTACCCCTTCGGCCAAACGGC-3′; and siA3, 5′-CCATCTGGCCCCCTTGGTCCGAATGCTGACCCCTA-3′.

Cell culture and transfections

INS-1 cells (gift from C. Wollheim, University Medical Center, Geneva, Switzerland) were cultured in RPMI 1640 medium at 11.1 mm glucose supplemented with 10% heat-inactivated fetal calf serum, 10 mm HEPES, 1 mm sodium pyruvate, 100 μM β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transient transfections were conducted with Lipofectamine 2000 (Invitrogen Life Technologies, Inc.) according to the manufacturer’s instructions. Culture medium was removed and substituted with OptiMEM I (Invitrogen Life Technologies, Inc.) 2 h before transfection and was replaced 4 h after transfection. siRNAs were transfected to INS-1 cells at a final concentration of 100 nm using Lipofectamine 2000. Cells were harvested 72 h after transfection. The luc assays were conducted as previously reported (48), with relative luc activities of cell lysates normalized by cellular protein content.

CMV-rtTA/Tet-Egr-1 cell lines were generated first by transfection of INS-1 cells with CMV-rtTA (pUHHG17-1) and the neomycin resistance plasmid pSV2-neo (BD Clontech, Palo Alto, CA), followed by selection with G418 (Calbiochem, La Jolla, CA) to generate stable clones of CMV-rtTA INS-1 cells. CMV-rtTA INS-1 cells were then transfected with Tet-Egr-1 in pUHD10-3 and the hygromycin resistance pTK-Hyg plasmid (BD Clontech). Double-stable CMV-rtTA/Tet-Egr-1 INS-1 cells were grown for passage under selection with G418 and hygromycin.

Western blot analyses

Cells were washed and resuspended in TBS buffer [10 mm Tris (pH 8.0) and 150 mm NaCl], then lysed by adding an equal volume of lysate buffer [10 mm Tris (pH 8.0), 150 mm NaCl, 40% glycerol, 2 mm EDTA, 0.2% Igepal CA-630, 2 mm dithiothreitol, and 0.4 mm phenylmethylsulfonyl fluoride]. After centrifugation, supernatants were stored at −80 C. Protein concentrations were measured with a Bradford assay (Bio-Rad Laboratories, Richmond, CA) using albumin as standard. Protein samples were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Membranes were incubated with rabbit polyclonal anti-PDX-1 (gift from J. Habener, Massachusetts General Hospital and Harvard Medical School, Boston, MA), rabbit polyclonal anti-Egr-1 (C-19), or goat polyclonal anti-HNF3β (Foxa2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antisera, followed by washing and subsequent incubation with horseradish peroxidase-conjugated secondary antisera. Protein bands were detected.
were stored at 4°C in buffer C [20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride]. Cells were swollen for 15 min on ice and vortexed for 10 sec in the presence of 0.06% Igepal CA-630. After centrifugation for 30 sec, the pellet was gently incubated in ice-cold buffer A [10 mM HEPES (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride]. Cells were swollen for 15 min on ice and vortexed for 10 sec in the presence of 0.06% Igepal CA-630. After centrifugation for 30 sec, the pellet was gently incubated in ice-cold buffer A [10 mM HEPES (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride] for 15 min. Nuclear extract supernatants were subjected to nondenaturing PAGE, followed by autoradiography.

Preparation of nuclear extracts

Nuclear extracts were prepared according to the method of Schreiber (54). Cells were washed with PBS buffer and resuspended in ice-cold buffer A [10 mM HEPES (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride]. Cells were swollen for 15 min on ice and vortexed for 10 sec in the presence of 0.06% Igepal CA-630. After centrifugation for 30 sec, the pellet was gently incubated in ice-cold buffer C [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride] for 15 min. Nuclear extract supernatants were stored at −80°C.

EMSAs

EMSAs were conducted according to previously described methods (9). Oligonucleotides for probes were purified by PAGE and column chromatography. Annealed double-stranded probes were labeled with [α-32P]dATP. The sequences of the probe encompassing the rat insulin 1 promoter P1 enhancer were (sense strand) 5′-GATCCCATCCTACCTTCCTGCACCCTTAATGGGCTTAACGGCGAAAACGGCAAAGTCCAGGCCTTAATGGAGACCCAGTTCCCAACCC-3′ and its reverse complement. Overlapping probe sequences spanning the 3′-412 rat insulin 1 promoter (sense strands) were as follows: probe 1, 5′-CAAAATCCACGGGAGGGGAAGAGTAGCTTTGGAGCAAAGTATTAAAACTACAGCTCAGCCCGCT-3′; probe 2, 5′-TTCAGGCCCTCTCATGCCCCTGCTCATGTCCTACCATGCCCATGTGGGCTTCAA-3′; probe 3, 5′-TACGCTGAAATGAGGTGGAAAATGCTCAGCCAAGGGTCCACG-3′; probe 4, 5′-TTTCATACGAGCCGACATCTGGCCCTTCTATTAAATCTAATTACCCTAGGTCTAAGTAGAGTTGTTG-3′; probe 5, 5′-TCAGCCAGGAAAAAGACGCGCCCTACCCCTTCTGTCGAAACTTTCTGGGAAATGAGGTGGAAAATGCTCAGCCAAGGGTCCACG-3′; probe 6, 5′-GGGACAAAGATACCCGCCTCCCAAAAACTACTCACTTCTGCGGAATGAGGTTGAAATGCTCAGGCCAACAGAAAGAGGTTGAGTGCTGCAAGTTGAAATGAGGTTGAAATGCTCAGCCAAGGGTCCACG-3′; probe 7, 5′-AGCTGGGTTCATCTAGCTGATTCTAAAGATACCCGCCTCCCAAAAACTACTCACTTCTGCGGAATGAGGTTGAAATGCTCAGGCCAACAGAAAGAGGTTGAGTGCTGCAAGTTGAAATGAGGTTGAAATGCTCAGCCAAGGGTCCACG-3′ and their respective reverse complements. The sequence of the positive control probe for Egr-1 binding was the synthetic sequence 5′-GGGAGTCCGCGTTGCGGGGGGGGCGTTAGTCAGTCGGG-3′ and its reverse complement, with the nonameric binding site underlined, as derived from a screen of Egr-1-binding sites (30). Probes were incubated with in vitro translated proteins, nuclear extracts from INS-1 cells, or both in the presence of polydeoxyinosinic-polydeoxyctydilic acid (Sigma-Aldrich Corp., St. Louis, MO) for 20 min in a buffer composed of 12 mM HEPES (pH 7.9), 50 mM KCl, 47 mM MgCl2, 20 mM NaN3, 0.85 mM dithiothreitol, 12.5% glycerol, and 0.5 mg/ml BSA. In some instances, binding reactions included a 20-min preincubation of protein samples with anti-Egr-1 antibody or anti-PDX-1 antiserum. Reaction mixtures were subjected to nondenaturing PAGE, followed by autoradiography.

RNA expression analyses

Total RNA and cDNA were prepared, and quantitative real-time RT-PCR was conducted on an ABI PRISM 7900HT sequence detection system using reagents and methods obtained from the manufacturer (Applied Biosystems, Foster City, CA). RT-PCR was conducted in triplicate for each sample and was normalized to cyclophilin expression. Sequences of primers and minor groove binder (MGB) probes for cyclophilin, insulin, Egr-1, PDX-1, and Foxa2 are available upon request.
Chromatin immunoprecipitation (ChIP) assays

ChIP assays were conducted using enzymatic shearing with CHIP-IT kits from Active Motif (Carlsbad, CA) according to the manufacturer’s instructions, with minor modifications. INS-1 cells were fixed in 1% formaldehyde for 10 min at room temperature. Preclearing of chromatin and immunoprecipitation of antibody/chromatin mixtures were performed with protein A-Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden). Complexes were precipitated with rabbit polyclonal anti-PDX-1 antiserum (gift from J. Habener), rabbit polyclonal anti-Egr-1 (C-19) IgG antibody, or control normal rabbit IgG (Santa Cruz Biotechnology, Inc.). Immunoprecipitated complexes were washed eight times with RIPA buffer composed of 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, and 3 mM sodium fluoride with protease inhibitor mixtures. Primer pairs used for amplification of purified DNA were 5'-ATTGTGCTGTGAACGTCTCATCA-3' (261 to 238 bp upstream of the transcription initiation site) and 5'-CCTGGACCTTTGCGTTTG- GCCCAT-3' (-76 to -53 bp) for the rat insulin 1 promoter, and 5'- GGTTCCACCGGAGAGGCTAC-3' (-1766 to -1746 bp upstream of the ATG start codon) and 5'-ACGACATGTGACTTCGAGGG-3' (-1646 to -1626 bp) for the rat Nab2 promoter. Nab2 promoter sequences were obtained from Rat Genome Database 131172. PCR conditions included a melting step at 94°C for 3 min for completion of extension. PCR products were separated by agarose gel electrophoresis.

Statistical analyses

Data were analyzed by Student’s t test or one-way ANOVA for multiple group comparisons. Differences of P < 0.05 were considered significant.

Results

Overexpression of Egr-1 increases transcriptional activation of the insulin promoter

To examine whether Egr-1 regulates the insulin promoter, we transfected clonal INS-1 β cells with a wild-type Egr-1 expression vector pcDNA3/Egr-1 or with the empty expression vector control and a −412 bp rat insulin I promoter-luc reporter pXp2−412/luc. Protein expression of Egr-1 from the pcDNA3/Egr-1 expression vector was confirmed by in vitro transcription and translation experiments and by Western blot analysis of lysates from transfected INS-1 cells (Fig. 1A). No-
Overexpression of Egr-1 significantly increased transcriptional activation of the insulin promoter by 2.4-fold (Fig. 1B).

**Overexpression of DN-Egr-1 decreases transcriptional activation of the insulin promoter**

We overexpressed DN-Egr-1, which was composed only of the zinc finger DNA-binding domain of Egr-1 (amino acids 331–427), in transfections of INS-1 cells with the plasmid pcDNA3/DN-Egr-1. This DN-Egr-1 is known to antagonize the transcriptional activation of target gene promoters by wild-type Egr-1 (34). Expression of DN-Egr-1 protein was confirmed by *in vitro* transcription and translation experiments. Overexpression of DN-Egr-1 significantly decreased basal insulin promoter activity by 32% (Fig. 1C).

**Reduction of endogenous Egr-1 expression with siRNA decreases transcriptional activation of the insulin promoter**

siRNA-mediated gene silencing has been widely used to analyze the functions of specific gene products (55, 56). We designed and employed a duplex siRNA targeted at Egr-1 (siEgr-1) that reduced Egr-1 mRNA and protein levels in INS-1 cells by 25% and 31%, respectively, compared with a duplex siRNA control (siScramble; Fig. 2, A and B). The effectiveness of siEgr-1 to reduce Egr-1 expression levels in INS-1 cells by 25% and 31%, respectively, compared with a duplex siRNA control (siScramble; Fig. 2, A and B). The effectiveness of siEgr-1 to reduce Egr-1 expression levels in

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**Fig. 3.** Egr-1 does not directly bind to rat insulin I promoter sequences in EMSAs. A, EMSAs performed with *in vitro* transcribed and translated Egr-1 proteins. A synthetic positive control probe for Egr-1 binding that contains the canonical Egr-1 binding consensus sequence 5′-GCGGGGCG-3′ (positive control) and seven overlapping probes spanning the −412 to +2 bp rat insulin I promoter sequence (RIP 1–7) were used in EMSAs with *in vitro* transcribed and translated Egr-1 proteins (Egr-1 IVTT) and reticulocyte lysate controls (empty IVTT) in the presence or absence of preincubation with anti-Egr-1 antibody as indicated (+). Arrows indicate the migration positions of Egr-1, free probes (fp), or nonspecific complexes (ns). B, EMSAs performed with INS-1 nuclear extracts. EMSAs were conducted as described in A with the addition of nuclear extracts from INS-1 cells (NE) with or without *in vitro*-transcribed and *in vitro*-translated Egr-1 proteins (Egr-1 IVTT) or reticulocyte lysate controls (empty IVTT). EMSAs in A and B were conducted with saturating amounts of Egr-1 IVTT proteins to enhance the detection of Egr-1-binding activity. Shorter exposures that illustrate Egr-1 binding to positive control probes are shown in supplemental Fig. 3 (published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org), in which supershifted complexes are indicated (ss).
individual transfected cells was probably substantially higher, because the transfection efficiency of INS-1 cells with these methods rarely exceeds 30% and the Egr-1 mRNA and protein expression levels represented in both transfected and nontransfected cells.

The introduction of siEgr-1 significantly suppressed transcriptional activation of the insulin promoter by 53% compared with control cells (Fig. 2C). Moreover, the addition of siEgr-1 to the transfection cocktail containing Egr-1 expression vector pcDNA3/Egr-1 completely eliminated the effect of exogenous Egr-1 expression to further activate the insulin promoter compared with controls in which siScramble (siEgr-1; C) or pcDNA3/Egr-1 (pcDNA3; E) as indicated. Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01; ##, P < 0.01. C, siEgr-1 decreases activation of the G box mutant insulin promoter. INS-1 cells were transfected with 0.6 μg of the rat insulin I promoter-luc reporter plasmids pA3–184/luc (−184) or G box mutant pA3–184/luc (−184Gm) and with 1.0 μg of the expression vectors pcDNA3 (− Egr-1; □) or pcDNA3/Egr-1 (− Egr-1; ■) as indicated. Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01; ##, P < 0.01.

Egr-1 proteins do not directly bind to insulin promoter sequences

Next, we asked whether Egr-1 was able to directly bind sequences within the +2 to −412 bp of the rat insulin I promoter in EMSAs. For this purpose, we divided the +2 to −412 bp rat insulin I promoter sequences into seven overlapping oligonucleotide segments. We employed a synthetic positive control probe for Egr-1 binding that includes the potent consensus sequence of 5′-GCGGGGCG-3′ (30). In vitro-translated Egr-1 proteins demonstrated substantial binding activity with this probe, as confirmed by protein complex mobility changes in response to preincubation with anti-Egr-1 antibody (Fig. 3A). Incubation of each of the seven radiolabeled oligonucleotide probes with saturating amounts of in vitro-translated Egr-1 proteins or reticulocyte lysate control samples, selected to enhance the detection threshold for Egr-1-binding activity, did not alter the electrophoretic mobility patterns in EMSAs (Fig. 3A). Furthermore, incubation of the protein samples with anti-Egr-1 antibody did not alter probe mobility patterns (Fig. 3A). In an independent series of experiments, we performed EMSAs in the presence of nuclear extracts from INS-1 cells that were enriched with transcription factors important for insulin promoter regulation with the potential to stabilize or strengthen Egr-1 binding to the promoter sequences. In DNA-binding assays with a positive control probe, we observed Egr-1 DNA-binding activity within INS-1 cell nuclear extracts that was attenuated by preincubation with an anti-Egr-1 antibody (Fig. 3B, arrow). The addition of in vitro-translated Egr-1 proteins substantially increased Egr-1 binding to the positive control probe, with an increased interference pattern after preincubation with anti-Egr-1 antibody (Fig. 3B). However, we did not observe direct binding of Egr-1 to the insulin promoter probes with the addition to INS-1 nuclear extracts to the DNA-binding reactions (Fig. 3B). These results sug-

Fig. 4. Regulation of insulin promoter mutants by Egr-1. A, Activation of 5′-deletion mutants of the rat insulin I promoter by Egr-1. INS-1 cells were transfected with 0.6 μg of the rat insulin I promoter-luc reporter plasmids pA3–83/luc (−83), pA3–118/luc (−118), pA3–184/luc (−184), pA3–345/luc (−345), pA3–412/luc (−412), or empty reporter plasmid pA3/luc (vector) and with 1.0 μg of the expression vectors pcDNA3 (−Egr-1; □) or pcDNA3/Egr-1 (+Egr-1; ■) as indicated. Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01. B, Overexpression of Egr-1 activates the G box mutant insulin promoter. INS-1 cells were transfected with 0.6 μg of the rat insulin I promoter-luc reporter plasmids pA3–184/luc (−184) or G box mutant pA3–184/luc (−184Gm) and with 1.0 μg of the expression vectors pcDNA3 (−Egr-1; □) or pcDNA3/Egr-1 (+Egr-1; ■) as indicated. Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01; ##, P < 0.01. C, siEgr-1 decreases activation of the G box mutant insulin promoter. INS-1 cells were transfected with 0.6 μg of the rat insulin I promoter-luc reporter plasmids pA3–184/luc (−184) or G box mutant pA3–184/luc (−184Gm) as indicated and with 100 nM siScramble (siEgr-1; □) or siRNA/Egr-1 (+siEgr-1; ■). Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01; ##, P < 0.01.
siRNA/Egr-1 decreases FarFlat activation. INS-1 cells were transfected with 0.6 factors by Egr-1.

activation. INS-1 cells were transfected with 0.6 μg of the multimerized FarFlat minienhancer-luciferase reporter plasmid pFoxFarFlat/luc (FarFlat) or empty reporter vector pFox (vector) and with 1.0 μg of the expression vectors pcDNA3 (− Egr-1; □) or pcDNA3/Egr-1 (+ Egr-1; ■) as indicated. Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01. B, Duplex siRNA/Egr-1 decreases FarFlat activation. INS-1 cells were transfected with 0.6 μg of the multimerized FarFlat minienhancer-luciferase reporter plasmid pFoxFarFlat/luc (FarFlat) or empty vector pFox (vector) and with 100 nM duplex siScramble (− siEgr-1; □) or siEgr-1 (+ siEgr-1; ■) as indicated. Results shown are the mean ± SD of three independent transfections, each conducted in duplicate. **, P < 0.01.

Eg-1 regulates transcriptional activation of the glucose-responsive FarFlat minienhancer within the rat insulin I promoter

The FarFlat minienhancer within the rat insulin I promoter is a potent regulatory element for basal and glucose-responsive transcriptional activation. This minienhancer contains A box binding sites (A3 and A4 elements) for homeodomain transcription factors including PDX-1, and E box binding sites for bHLH transcription factors such as E12, E47, and Beta-2/NeuroD1. Although we found no evidence for direct Egr-1 binding to a radiolabeled oligonucleotide probe (probe RIP-4) that included the FarFlat sequences in EMSAs (Fig. 3), we considered the possibility that Egr-1 might indirectly activate the insulin promoter via these important enhancer sequences. Therefore, we conducted a series of transfections in INS-1 cells to determine whether Egr-1 could regulate the transcriptional activation of the multimerized FarFlat minienhancer reporter. The overexpression of Egr-1 significantly enhanced the activation of FarFlat enhancer sequences by 82%, but not that of the empty reporter vector (Fig. 5A). Furthermore, the administration of siEgr-1 to INS-1 cells substantially reduced transcriptional activation of the FarFlat reporter (Fig. 5B), suggesting that endogenous Egr-1 is required for FarFlat minienhancer activation. These results suggest that Egr-1 can regulate transcriptional activation of the insulin promoter at least in part through its action on the FarFlat minienhancer.

Eg-1 regulates PDX-1 expression and DNA-binding activity

To identify transcription factors that may mediate Egr-1-dependent regulation of the FarFlat sequences, we developed double-stable CMV-rtTA/Tet-Egr-1 INS-1 cell lines in which we could inducibly increase Egr-1 mRNA (Fig. 6A) and protein (Fig. 6B) expression levels by addition of the tetracycline analog, doxycycline. Basal Egr-1 protein expres-
tion levels in the double-stable cells were similar to that of parental INS-1 cells (data not shown). Notably, the induction of Egr-1 expression increased both the mRNA (Fig. 6C) and protein (Fig. 6D) expression levels of the homeodomain transcription factor PDX-1, but not those of the PDX-1 regulator Foxa2 (59–61) (Fig. 6, E and F). In contrast, neither Egr-1 nor PDX-1 expression levels were significantly altered by comparable treatment of control INS-1 cells with doxycycline (data not shown). The increases in PDX-1 expression levels in response to increased Egr-1 induced by doxycycline were accompanied by progressive increases in PDX-1 DNA-binding activity observed by EMSA, as demonstrated by time-dependent increased binding to a radiolabeled probe encompassing the rat insulin I promoter A1 element (P1 enhancer) after treatment with doxycycline to induce Egr-1 overexpression (Fig. 7A). Doxycycline administration to negative control INS-1 cells did not increase PDX-1 DNA-binding activity (Fig. 7B).

**Fig. 6.** Overexpression of Egr-1 increases PDX-1, but not Foxa2, expression in INS-1 cells. A–F, CMV-rtTA/Tet-Egr-1 double-stable INS-1 cells were treated with doxycycline (100, 200, 500, or 1000 ng/ml as indicated) or vehicle (0) for 24 h before isolation of total RNA or whole-cell protein extracts. A, Relative Egr-1 mRNA expression levels as determined by quantitative TaqMan RT-PCR are shown. Results shown are the mean ± SD of three RT-PCRs, each normalized to cyclophilin expression. ***, P < 0.01 compared with vehicle. B, Relative Egr-1 protein expression levels on Western blots. Western blots were conducted with whole-cell extracts and anti-Egr-1 antibody and detected by enhanced chemiluminescence. The migration position of Egr-1 on a representative film is indicated (arrow). C, Relative PDX-1 mRNA expression levels as determined by quantitative TaqMan RT-PCR are shown. Results shown are the mean ± SD of three RT-PCRs, each normalized to cyclophilin expression. ***, P < 0.01 compared with vehicle. D, Relative PDX-1 protein expression levels on Western blots. Western blots were conducted with whole-cell extracts and anti-PDX-1 antiserum and detected by enhanced chemiluminescence. The migration position of PDX-1 on a representative film is indicated (arrow). E, Relative Foxa2 mRNA expression levels as determined by quantitative TaqMan RT-PCR are shown. Results shown are the mean ± SD of three RT-PCRs, each normalized to cyclophilin expression. F, Relative Foxa2 protein expression levels on Western blots. Western blots were conducted with whole-cell extracts and anti-Foxa2 antibody and detected by enhanced chemiluminescence. The migration position of Foxa2 on a representative film is indicated (arrow).

**Mutagenesis of PDX-1-binding sites reduces responsiveness of the insulin promoter to Egr-1.**

To determine the importance of PDX-1 in mediating the Egr-1 responsiveness of the insulin promoter, we introduced point mutations in the A1 (P1) and A3/4 (FarFlat) elements in pA3–412/luc and pA3–83/luc vectors to disrupt PDX-1-binding sites. Mutagenesis of either the A1 or A3/4 element in pA3–412/luc decreased the responsiveness of the rat insulin I promoter construct to Egr-1 to a similar extent (Fig. 8A). The combination of A1 and A3/4 mutations (pA3–412A134m) further reduced the ability of Egr-1 overexpression to activate the promoter (Fig. 8A). However, the disruption of PDX-1-binding sites by mutagenesis did not completely abolish activation of the promoter by Egr-1. Similarly, mutagenesis of the PDX-1-binding site P1 element within the −83 rat insulin I promoter construct reduced Egr-1 responsiveness by approximately 40% (Fig. 8B). These data
confirmed that a significant portion of the Egr-1 responsiveness of the insulin promoter is mediated through PDX-1-binding sites. The residual Egr-1 response in the mutant promoter constructs also suggested that Egr-1 may regulate the insulin promoter through additional indirect mechanisms.

Egr-1 regulates endogenous insulin gene expression

Finally, we asked whether Egr-1 can regulate endogenous insulin gene expression levels. When siEgr-1 was transfected into INS-1 cells to decrease Egr-1 expression levels, insulin mRNA levels were reduced by 31%, as determined by quantitative TaqMan RT-PCR analyses (Fig. 9A). These data represent the total insulin mRNA expression levels in both non-transfected INS-1 cells and those cells transfected with siEgr-1. Due to the limitations of transfection efficiency in these assays (maximally estimated at 30%), this reduction in insulin mRNA expression probably reflects an underestimate of the relative importance of endogenous Egr-1 in maintaining normal levels of insulin gene expression.

To investigate the interaction of Egr-1 and PDX-1 proteins with endogenous insulin gene regulatory sequences in intact INS-1 cells, we performed ChIP assays. ChIP assays with INS-1 cell DNA immunoprecipitated with anti-Egr-1 antibody clearly demonstrated Egr-1 binding to the promoter of Nab2, a recognized direct regulatory target of Egr-1 (62, 63) (Fig. 9B, lower panel). However, we did not observe any interaction between Egr-1 and rat insulin I promoter sequences by ChIP assays, although interaction of PDX-1 with

![Image of gel electrophoresis](https://academic.oup.com/endo/article-abstract/147/6/2923/2879574/2931)
the promoter was easily detectable by immunoprecipitations with anti-PDX-1 antiserum (Fig. 9B, upper panel). In ChIP assays with doxycycline-treated and untreated double-stable CMV-rtTA/Tet-Egr-1 INS-1 cells, overexpression of Egr-1 induced by doxycycline increased in vivo binding of PDX-1 to the rat insulin I promoter by approximately 1.6-fold (Fig. 9, C and D). Doxycycline treatment of negative control INS-1 cells did not significantly change the association of PDX-1 with the insulin promoter by ChIP assays (Fig. 9E). These results for the endogenous insulin gene were consistent with the findings by EMSA (Figs. 3 and 7) and support our model of indirect regulation of insulin gene expression by Egr-1 (Fig. 10).

### Discussion

The most important physiological function of pancreatic β-cells is to produce and secrete appropriate levels of insulin in response to changes in extracellular glucose concentrations. Our understanding of the range of molecular mechanisms by which glucose levels regulate transcriptional activation of the insulin promoter is limited. Glucose is known to induce the expression of Egr-1 and to increase the expression of insulin mRNA (43). As an early response gene product, the short half-life of the Egr-1 protein may facilitate rapid regulatory control for intracellular signaling to reflect the real-time status of extracellular nutrient dynamics. However, a causal relationship between the induction of Egr-1 expression and the activation of insulin gene transcription has not been previously elucidated. In the present study we describe a function for Egr-1 in transcriptional regulation of the insulin gene. Our studies indicate that changes in Egr-1 expression levels regulate insulin promoter activity to modify the levels of insulin mRNA expression in β-cells. Based on our findings we suggest that insulin-producing cells may use the regulation of Egr-1 expression levels as a mechanism to transmit signals of changing extracellular glucose levels to the insulin promoter through varying the levels of PDX-1 expression.

The GC-rich sequence 5′-GCGT/gG/aGGCG-3′ was proposed as a DNA-binding site for Egr-1 (57, 58). Although we considered the G box cis-element (5′-CAGGGGCCA-3′) within the rat insulin I promoter as a potential Egr-1-binding site, our studies indicated that the G element does not bind Egr-1 and is not required to mediate activation of the insulin promoter by Egr-1. Nevertheless, we observed significant reductions in basal insulin promoter activity with introduction of the G box mutation, consistent with reports that this cis regulatory element is important for regulation of the insulin promoter by other transcription factors, including the myc-associated zinc finger protein (MAZ/Pur-1) (64–66).

Our data suggest that Egr-1 does not directly interact with sequences within the insulin promoter, but, rather, indirectly regulates insulin gene expression through other β-cell transcription factors. Glucose is known to signal through the conserved FarFlat minienhancer element within the insulin promoter. Our findings implicate Egr-1 in the transcriptional activation of A elements, including the FarFlat regulatory minienhancer, and suggest that the effects of Egr-1 are mediated at least in part by PDX-1. It is likely that Egr-1 regulates the expression and/or activity of additional β-cell transcription factors that contribute to the Egr-1 responsiveness of the insulin promoter, and additional studies will be needed to define the scope of Egr-1 regulatory targets in pancreatic β-cells.
Multiple lines of investigation suggest that Egr-1 can function in a broader context in metabolic regulation. Egr-1 can regulate proliferation and apoptosis in other cellular contexts (35, 36, 67, 68), and Garnett and colleagues (46) recently reported that siRNA-mediated silencing of Egr-1 expression significantly inhibits the proliferation of INS-1 cells. Our studies implicate PDX-1, with its known function in the regulation of β-cell mass, as a regulatory target for Egr-1 in insulin-producing cells. We propose that Egr-1 may finely regulate insulin gene transcription via regulation of PDX-1 expression levels in response to changes in glucose levels in order for β-cells to appropriately meet metabolic demands.
of insulin production and strategies for the treatment of diabetes, both in the restoration of the regulation of Egr-1 may be of relevance to new insights into the biology of atherosclerosis and angiogenesis (40, 41, 70, 72–74). Thus, we envision that future studies of the regulation of Egr-1 may be of relevance to new strategies for the treatment of diabetes, both in the restoration of insulin production and β-cell mass as well as in the treatment or prevention of vascular complications.

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