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Pdx-1 Is Not Sufficient for Repression of Proglucagon Gene Transcription in Islet or Enteroendocrine Cells

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Pdx-1 plays a key role in the development of the pancreas and the control of islet gene transcription and has also been proposed as a dominant regulator of the α- *vs.* β-cell phenotype via extinction of proglucagon expression. To ascertain the relationship between Pdx-1 and proglucagon gene expression, we examined the effect of enhanced *pdx-1* expression on proglucagon gene expression in murine islet αTC-1 and GLUTag enteroendocrine cells. Although adenoviral transduction increased the levels of *pdx-1* mRNA transcripts and nuclear Pdx-1 protein, overexpression of *pdx-1* did not repress endogenous proglucagon gene expression in αTC-1 or GLUTag cells or murine islets. Immunohistochemical analysis of cells transduced with Ad-*pdx-1* demonstrated multiple individual islet or enteroendocrine cells exhibiting both nuclear Pdx-1 and cytoplasmic glucagon-like peptide-1 immunopositivity. The failure of *pdx-1* to inhibit endogenous proglucagon gene expression was not attributable to defects in Pdx-1 nuclear translocation or DNA binding as demonstrated using Western blotting and EMSA analyses. Furthermore, Ad-*pdx-1* transduction did not repress proglucagon promoter activity in αTC-1 or GLUTag cells. Taken together, these findings demonstrate that *pdx-1* alone is not sufficient for specification of the hormonal phenotype or extinction of proglucagon gene expression in islet or enteroendocrine cells. (*Endocrinology* 146: 441–449, 2005)

THE CONTROL OF insulin and glucagon secretion from islet β- and α-cells, respectively, is a subject of intense interest with direct relevance for understanding control of glucose homeostasis and the pathophysiology and treatment of type 2 diabetes. Studies directed at understanding pancreatic endocrine cell development and islet hormone biosynthesis have identified key transcription factors that in many instances play dual roles both as regulators of islet lineage development and as transcriptional regulators (1). Pdx-1, originally identified as an insulin or somatostatin gene promoter binding protein (2–4), has subsequently been shown to play essential roles in pancreatic organogenesis (5, 6) and the regulation of insulin gene expression (7). Ectopic expression of *pdx-1* in the liver induces a pattern of endocrine cell differentiation (8), whereas selective deletion of *pdx-1* in β-cells leads to loss of insulin expression and development of diabetes (7). Furthermore, loss-of-function mutations in the *pdx-1* gene have been identified in a small subset of patients with maturity-onset diabetes of the young (9). Hence, current data strongly support a key role for *pdx-1* in both the development and function of the endocrine pancreas

Several lines of evidence implicate an additional role for *pdx-1* in the endocrine pancreas as a negative regulator of proglucagon gene transcription and/or α-cell development. Reduction of *pdx-1* expression in murine β-cells via expression of an inducible antisense *pdx-1* transgene resulted in progressive loss of β-cells and an increase in the number of α-cells, implying a reciprocal albeit indirect relationship between β-cell Pdx-1 expression and α-cell phenotype (10). Similarly, β-cell-specific deletion of the *pdx-1* gene in mice is associated with a gradual reduction in β-cell mass and an increasing number of islet α-cells in the residual endocrine pancreas (7). Nevertheless, the precise mechanisms linking loss of β-cell Pdx-1 expression to the appearance of increased numbers of islet α-cells remain unclear.

The results of experiments with insulinoma (INS)-1 islet cell-derived subclones have suggested that Pdx-1 may prevent the emergence of the α-cell phenotype by suppression of proglucagon gene expression (11). Induction of *pdx-1* expression in INS-1-derived glucagon-producing subclones potently inhibited proglucagon promoter activity and completely extinguished endogenous proglucagon gene expression (11). Complementary evidence in support of a potential role for *pdx-1* in control of proglucagon gene transcription is derived from experiments identifying Pdx-1 target genes using the chromatin immunoprecipitation assay. Pdx-1 bound strongly to the insulin, islet amyloid polypeptide, *Pax-4*, and proglucagon gene promoters using chromatin isolated from islet β-TC3 cells and *pdx-1*-transfected NIH 3T3 cells (12). Furthermore, Pdx-1 binds directly to the rat proglucagon promoter in EMSA experiments *in vitro*, consistent with a potential role for Pdx-1 as a repressor of proglucagon gene transcription (11). Nevertheless Pdx-1 appears to regulate proglucagon transcription independent of its direct binding to the proglucagon gene promoter (13).

More recent experiments demonstrate that Pdx-1 exhibits a comparatively reduced affinity, relative to Pax-6, for binding to proglucagon promoter elements, suggesting that Pdx-1 exerts inhibitory effects on proglucagon gene tran-

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Abbreviations: BHK, Baby hamster kidney; E, embryonic day; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP, glucagon-like peptide; INS, insulinoma; Luc, luciferase.

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scription predominantly through protein-protein interactions, attenuating the activation of promoter activity by Pax-6 and Cdx-2/3 (13). Furthermore, despite findings that *pdx-1* expression extinguishes the α-cell phenotype in INS cell-derived subclones (11), multiple studies (summarized in Table 1) identified subsets of islet cells coexpressing Pdx-1 and glucagon in the developing or adult endocrine pancreas (14–17). Hence, the available evidence from studies of both islet cell lines and the normal endocrine pancreas is conflicting with respect to the relationship between *pdx-1* expression and the presence or absence of islet proglucagon gene transcription. In contrast, even less is known about the potential importance of *pdx-1* expression for the control of proglucagon gene transcription in enteroendocrine cells.

The proglucagon gene gives rise to multiple biologically active peptides including glucagon released from islet α-cells and both glucagon-like peptide (GLP)-1 and GLP-2 secreted from gut endocrine cells (18). We recently detected the expression of *pdx-1* mRNA transcripts in islet α-cell lines and GLUTag cells, a differentiated murine enteroendocrine cell line that produces both GLP-1 and GLP-2 in a regulated manner (19). Accordingly, to understand the potential relationship between *pdx-1* and specification of the α- or enteroendocrine cell phenotype, we examined the interdependence of *pdx-1* and proglucagon gene expression in islet and enteroendocrine cell lines.

Materials and Methods

Tissue culture medium was from Hyclone (Logan, UT), and fetal calf serum (FCS) was from Invitrogen Life Technologies (Burlington, Canada). Antibiotics and chemicals were from Sigma Chemical Co. (St. Louis, MO). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). The *Pdx-1* adenovirus was a generous gift obtained from Dr. C. Rhodes (Pacific Northwest Research Institute, Seattle, WA) and was generated by subcloning the rat *Pdx-1* cDNA into an adenoviral vector, and functional adenovirus (Ad-*pdx-1*) was generated, amplified, and purified as previously described (20). For viral infections, cells were grown to 70% confluence, virus was added at a concentration of 10^{10} PFU/ml, and cells were exposed to virus for 5 h, after which the medium was replaced and cells cultured for an additional 48 h before harvesting for protein or RNA analyses.

EMSAs

Nuclear proteins from GLUTag, baby hamster kidney (BHK), αTC-1, and βTC-6 cells were prepared as previously described (21–23). Synthetic oligonucleotides corresponding to specific rat proglucagon gene promoter G_1 (5′-AAT TTG AAC AAA ACC CCA TTA TTT ACA GAT GAG A-3′; 5′-AAT TTC TCA TCT GTA AAT AAT GGG GTT TTG TTC A-3′) and insulin (5′-AGC ATT TTC CAC CTC ATT TCC CAG A-3′; 5′-TCT GGG AAA TGA GGT GGA AAA TGC T-3′) promoter sequences (24, 25) were annealed, radiolabeled with [^{32}-P]ATP using the Klenow enzyme, and purified by column chromatography. EMSAs were performed as described (21–23). For supershift experiments, nuclear extracts (10 μg) were preincubated with anti-Pdx-1 antiserum (a generous gift of Dr. C. Wright, Vanderbilt University, Nashville, TN) for 10 min at room temperature before the addition of [^{32}P]-labeled DNA probe and subsequent incubation at room temperature for 10 min. All reaction mixtures were loaded onto a 6% nondenaturing polyacrylamide gel, and after electrophoresis, the gel was exposed to x-ray film for 24 h.

TABLE 1. Experimental models demonstrating coexpression of Pdx-1 and glucagon

Model (Ref.)
Murine embryonic stem cells (14)
Embryonic murine pancreas E9.5 and E13.5 (15)
Murine embryonic pancreas (16)
Murine pancreas E10.5 (17)
Developing and adult pancreas in the PC2 null mouse (45)
Alloxan-treated murine diabetic pancreas (44)
AN697 glucagonoma cells (52)
NHI-6F-GLU islet cells (53)
αTC1.6 glucagonoma cells (54)

Western blot analyses

Nuclear extracts from GLUTag, BHK, and αTC-1 cells were prepared as described (21) and Western blotting was performed using 40 μg total protein per lane. Blots were probed with anti-Pdx-1 antibody (6) at a dilution of 1:5000 and antihistone antibody that recognizes H1 and core histone proteins H2a, H2b, H3, and H4 (Chemicon, Temecula, CA), 1:1000.

RNA isolation, Northern blot analysis, and real-time PCR

RNA was prepared with Trizol reagent, and 10 μg total RNA was loaded onto a 1% agarose gel containing formaldehyde. After electrophoresis, gels were transferred overnight by diffusion (5× saline sodium citrate) to a nylon membrane. The membranes were UV cross-linked and prehybridized for 4 h at 68 C. After prehybridization, the blots were hybridized sequentially with random-primed rat proglucagon (*Sac*I/*Pst*1 fragment from the rat cDNA), *pdx-1* (an internal *Eco*RI fragment from the rat cDNA, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH cDNA probe was generated from a PCR for GAPDH using primers 5′-GACCACAGTCCATGACATCACT-3′ and 5′-TCCACCACCCTGTTGCTGTAG-3′ (sequence described in Ref. 26).

Real-time RT-PCR quantitative detection of glucagon and insulin mRNA transcripts was carried out by real-time PCR using ABI PRISM 7900HT (PE Applied Biosystems, Foster City, CA). PCRs were performed in a total volume of 10 μl. Five microliters SYBR green PCR master mix (PE Applied Biosystems), 0.2 μl (final concentration 1 μM) of primers (forward and reverse), and 0.4 μl 1:10 diluted first-strand cDNA were mixed into 384-well thin-wall PCR plates (PE Applied Biosystems). Primers for insulin were 5′-GGCTTCTTCTACACACCCATGTC-3′ and 5′-AGCTCCAGTTGTGCCACTTGT-3′ and for glucagon were 5′-GCTGATGGCTCCTTCTCTGATG-3′ and 5′-GAAGTCCCTGGTGGCAAGATT-3′. Data were analyzed by ABR PRIM SDS 2.0 (PE Applied Biosystems). All samples were assayed in triplicate, and each real-time PCR experiment was repeated twice on two different occasions.

Cell culture and transfection

Cell lines were maintained in DMEM, 4.5 g glucose per liter (Hyclone). GLUTag cells were originally isolated in our laboratory as described (19), whereas BHK, αTC-1, and βTC-6 cells were obtained from the American Type Culture Collection (Manassas, VA). BHK fibroblasts mouse islet αTC-1, βTC-6, and enteroendocrine GLUTag cells were grown in DMEM supplemented with 10% FCS as described (19). The rat proglucagon gene promoter plasmids [-300]GLU-luciferase (Luc), [-82]GLU-Luc, [4G3]GLU-Luc, and [4G1]GLU-Luc (21, 23, 27) were cotransfected with pBluescript alone (not shown), pcDNA3.1, or cDNAs encoding mouse *Pdx-1* cDNA, *Pax 6*, or *Cdx-2* or were transfected into cells subsequently infected with Ad-Pdx-1. Transfections were performed in 12-well plates using equal ratios of expression and reporter plasmids to a total of 1 μg DNA and 2.5 μl FuGENE (Roche Diagnostics, Laval, Quebec) per well, according to the manufacturer's specifications. All cells were harvested 48 h after transfection for analysis of luciferase activity as described previously (21–23).

Immunocytochemistry

Cells were grown on glass slides and processed for immunohistochemistry as described (23, 28–30). Cells were infected with Ad-*Pdx-1* and 48 h later fixed in methanol/acetone (1:1) mixture at 4 C for 1 h and then rinsed four times in PBS. Before staining, cells were first permeabilized in 0.3% Triton X-100 for 10 min. Endogenous peroxidase and

biotin activities were blocked using aqueous hydrogen peroxide and an avidin-biotin blocking kit, respectively (Vector Laboratories Inc., Burlingame, CA). Cells were then incubated with normal goat serum for 1 h at room temperature with a rabbit polyclonal anti-Pdx-1 antibody at 1:10,000 dilution, followed by sequential 30-min incubations with biotinylated goat antirabbit IgG (Vector Laboratories) and horseradish peroxidase-conjugated streptavidin labeling reagent (Signet Labs. Inc., Dedham, MA). Color development was done with a fresh solution of diaminobenzidine. Slides were washed in running tap water, and specimens were treated again with aqueous hydrogen peroxide solution, blocked with normal goat serum, and then stained overnight with a rabbit polyclonal anti-GLP-1 antibody at 1:5000 dilution. After repeated washings in PBS, cells were incubated with biotinylated goat antirabbit IgG followed by fluorescein isothiocyanate-conjugated streptavidin, counterstained with 4′,6′-diamino-2-phenylindole, and coverslipped with Vectashield (Vector Laboratories).

The GLP-1 antiserum was a polyclonal antiserum (prepared by D.J.D.) that cross-reacts with both proglucagon and processed GLP-1 and hence reacts against both islet A cells and enteroendocrine L cells (23, 31). Pdx-1 polyclonal rabbit antisera were obtained from Dr. C. Wright (Vanderbilt University, Nashville, TN). Appropriate positive and negative controls were performed for each antibody, including sections of mouse pancreas (positive control) for GLP-1 (proglucagon) and Pdx-1.

Murine islet isolation

Islets of Langerhans were isolated from the pancreas of 8-wk-old male C57 BL/6 mice in accordance with guidelines and protocols approved by the University Health Network Animal Care Committee. The pancreas was digested by injection with collagenase type XI solution (0.375 mg/ml) (Sigma) through the bile duct. The inflated pancreas was removed and dispersed homogenously. After washing, islets were isolated in a Ficoll gradient (Sigma) using a modification of the original method of Lacy and Kostianovsky (32). Isolated islets were trypsinized into single cells, plated on slides, and cultured in RPMI 1640 medium supplemented with 11 mM glucose, 10% FCS, 100 IU/m penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies) in the presence or absence of Ad-*pdx-1* for 48 h. For immunohistochemical analysis, slides were fixed and stained with antisera against both GLP-1 and PDX-1 as described above.

Statistical analysis

Statistical significance was assessed by one-way ANOVA followed by the Bonferroni multiple comparison *post hoc* test using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

Results

Studies using inducible Pdx-1 expression in INS-1-derived islet subclones implicated Pdx-1 as a potent negative regulator of the proglucagon gene promoter leading to extinction of proglucagon gene transcription (11). To determine whether the presence of *pdx-1* was invariably associated with elimination of proglucagon gene expression in islet or enteroendocrine cells, we assessed the levels of endogenous proglucagon mRNA transcripts in αTC-1, βTC-6, and GLUTag cells before and after transduction with an adenovirus encoding full-length Pdx-1 (Ad-*pdx-1*). Surprisingly, Pdx-1 immunoreactive protein was detected in nuclear extracts from αTC-1 and GLUTag cells before adenoviral transduction. Furthermore, despite a robust increase in levels of *pdx-1* mRNA transcripts (Fig. 1A) and nuclear Pdx-1 protein (Fig. 1C), the levels of proglucagon mRNA transcripts were not reduced in αTC-1 cells and only modestly reduced in GLUTag cells after infection with Ad-*pdx-1*, as assessed by either Northern blot analysis (Fig. 1A) or real-time PCR (Fig. 1B). In contrast, the levels of insulin mRNA transcripts were increased approximately 3-fold in Ad-*pdx-1*-transduced αTC-1 cells (Fig. 1B). Hence, the presence of nuclear Pdx-1 in

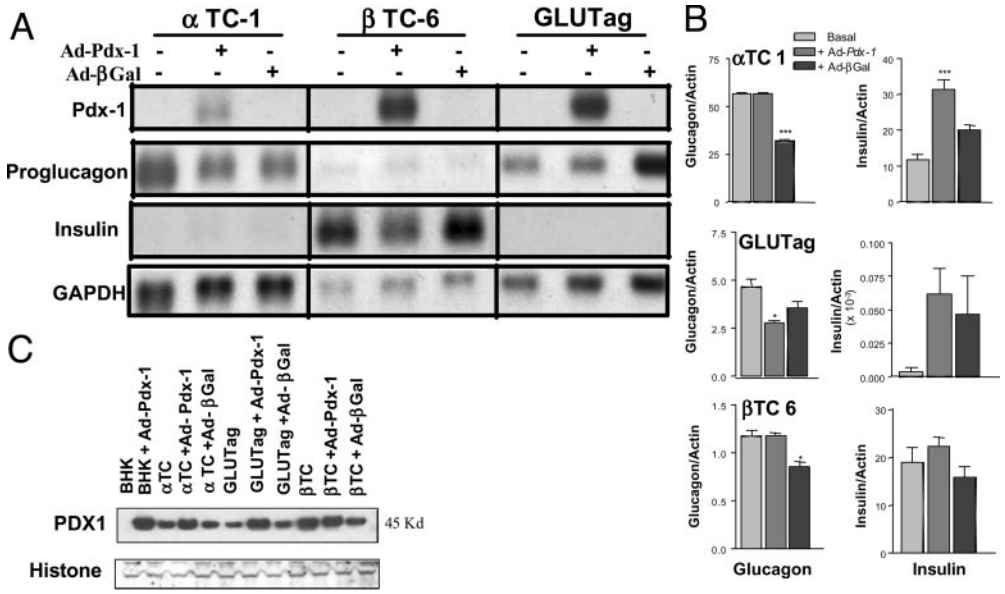

FIG. 1. Northern blot analysis of cell lines after transduction with Ad-*pdx-1*. A, Cells were infected with recombinant adenoviruses (Ad-βGal or Ad-*pdx-1*), and total cellular RNA was collected from cells 48 h after viral infection with adenovirus (+) or after mock infection (−) for analysis as described in *Materials and Methods*. Blots were hybridized with internal [^{32}P]-labeled cDNA probes for *pdx-1*, proglucagon, insulin, or GAPDH. B, Real-time PCR analysis of proglucagon and insulin mRNA transcripts after viral transduction with Ad-βGal or Ad-*pdx-1*. The relative levels of proglucagon and insulin mRNA transcripts, as derived from standard curves generated using genomic DNA, are represented as glucagon/actin or insulin/actin ratios for each experiment. *, $P < 0.05$ *vs.* control. C, Western blot analysis detects nuclear Pdx-1 protein in islet and enteroendocrine cell lines. Nuclear extracts (40 μg protein) from hamster BHK fibroblasts, mouse αTC-1 islet cells, GLUTag enteroendocrine cells, and βTC-6 islet cells isolated before or after transduction with Ad-*Pdx-1* or Ad-βGal were separated by SDS-PAGE, transferred to a nylon membrane, and incubated with antisera specific for Pdx-1 or histone proteins.

both islet and enteroendocrine cells demonstrates that expression of either an endogenous or exogenous Pdx-1 protein is not incompatible with basal expression of the proglucagon gene.

Previous reports demonstrated that although Pdx-1 binds to DNA elements within the proximal rat proglucagon gene promoter and inhibits proglucagon gene transcription, its transcriptional actions appear to be mediated through DNA binding-independent mechanisms (11, 13). To determine whether exogenous Pdx-1 would regulate proglucagon promoter activity in αTC-1 islet cells, we analyzed the transcriptional activity of four different proglucagon promoter-luciferase plasmids (21, 23, 33) in the presence or absence of Ad-*pdx-1* and two additional transcription factors, Pax-6 and Cdx-2/3, known to regulate proglucagon promoter activity. Ad-*pdx-1* alone had no significant effects on the transcriptional activity of [−82]GLU-Luc (Fig. 2A), a truncated proglucagon promoter plasmid containing the proximal 82 bp of the rat proglucagon promoter, including G_1 elements previously shown to mediate the inhibitory actions of *pdx-1* in islet cells (11). Similarly, Ad-*pdx-1* had no significant effects on the transcriptional activity of three additional proglucagon promoter reporter plasmids, [4G1]GLU-Luc, [4G3]GLU-Luc, and [−300]GLU-Luc (Fig. 2, B–D), a plasmid containing the first 300 bp of the rat proglucagon gene promoter including G_1-G_4 promoter elements in their native genomic orientation (Fig. 2) (21, 23, 24, 33, 34). Endogenous Pax-6 was detected in αTC-1, βTC-6, and GLUTag cells by both RT-PCR and Western blotting, whereas Cdx-2/3 RNA and protein were detected in GLUTag but not αTC-1 cells (data not shown). Transfection of Ad-*pdx-1*-transduced αTC-1 cells with cDNAs encoding either Pax-6 or Cdx-2/3 did not significantly change the transcriptional activity of the four GLU-Luc plasmids relative to the activity obtained after transduction with Ad-pdx-1 alone (Fig. 2). Similarly, Ad-Pdx-1 alone or in combination with Pax-6 or Cdx-2/3 failed to repress proglucagon promoter activity in GLUTag cells (data not shown). These findings, taken together with results of previous studies (13), demonstrate variable effects of exogenous *pdx-1* on proglucagon promoter activity, dependent largely on the cell line studied and the specific regions of the proglucagon gene promoter selected for transcriptional analysis.

Our data demonstrated coexpression of *pdx-1* and proglucagon RNA transcripts in murine islet and enteroendocrine cell lines. To determine whether the failure of Pdx-1 to extinguish proglucagon gene expression was due to the inability of the endogenous or exogenous Pdx-1 protein to bind to proglucagon promoter elements, we carried out EMSA analyses using an insulin promoter oligonucleotide and extracts from BHK fibroblasts and islet αTC-1 and enteroendocrine GLUTag cells. No detectable complexes were observed in wild-type BHK cells (Fig. 3A, lane 1). Although some studies detected multiple bands on EMSA using insulin or proglucagon gene promoter fragments, a single prominent DNA-protein complex was observed using extracts from BHK cells transfected with a *pdx-1* cDNA (Fig. 3A, lane 2), which was almost completely eliminated after preincubation of the reaction with Pdx-1 antisera (Fig. 3A, lane 3). A single prominent DNA-protein complex that migrated in an identical position to the complex observed in *pdx-1*-transfected BHK cells was also detected using nuclear extracts (but not cyto-

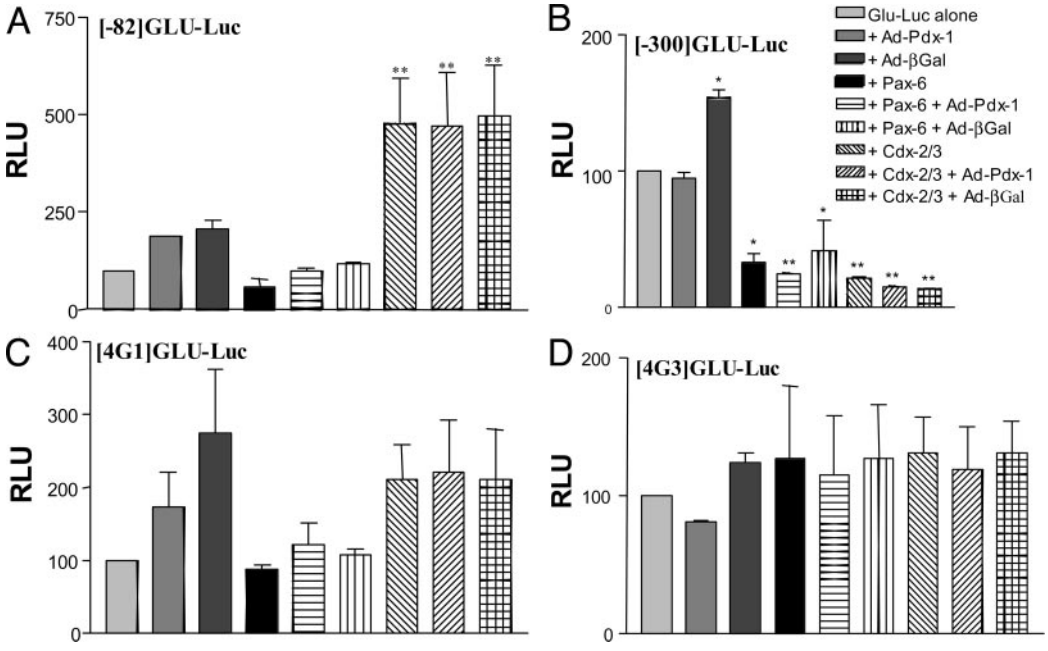

FIG. 2. Transcriptional regulation of rat proglucagon gene promoter-luciferase plasmids. αTC-1 islet cells were transfected with [−82]GLU-Luc, [−300]GLU-Luc, [4G3–136]GLU-Luc, and [4G1–136]GLU-Luc alone (control) or after transduction with either Ad-Pdx-1 or Ad-βGal or in the presence of equal amounts of cotransfected cDNAs for either Pax-6 or Cdx-2/3, with or without coinfection with Ad-Pdx-1 or Ad-βGal, as described in *Materials and Methods*. Equal amounts of transfected cell extract were analyzed for luciferase activity. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ relative to values obtained for GLU-Luc plasmid alone. Results are mean of quadruplicate transfections per experiment (n = minimum 3 experiments for each cell line and set of plasmids).

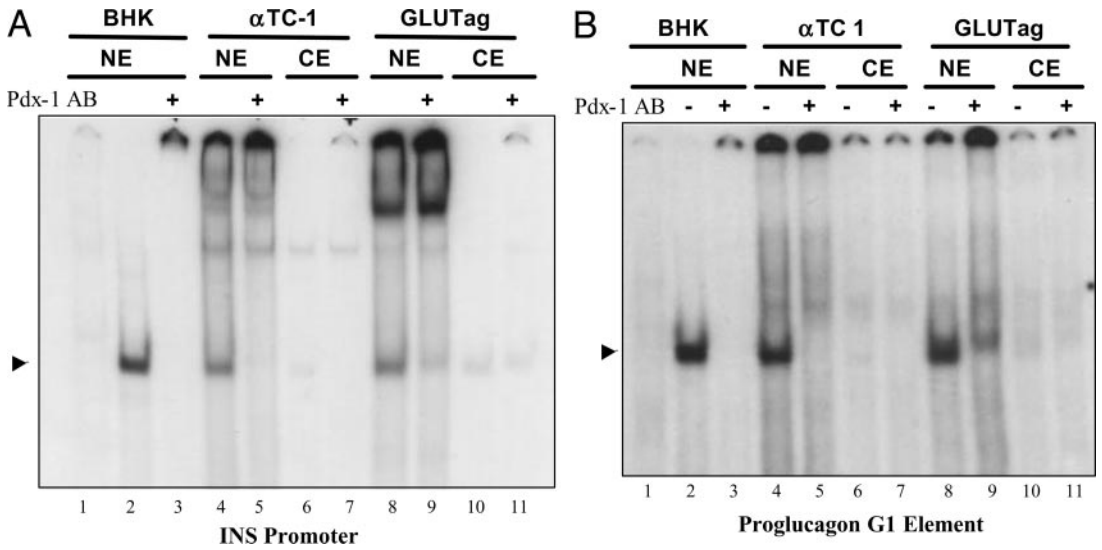

FIG. 3. Endogenous Pdx-1 is capable of binding to DNA promoter elements in islet and enteroendocrine cell lines. A, Nuclear (NE) and cytoplasmic (CE) extracts from nontransfected enteroendocrine GLUTag or islet αTC-1 cells and BHK fibroblasts transfected with the Pdx-1 cDNA (lanes 2 and 3) were incubated with the insulin promoter oligonucleotide probe, as described in *Materials and Methods*, in the presence or absence of anti-PDX1 antisera (Pdx-1 AB). B, NE and CE from cells described in A were incubated with the proglucagon gene G_1 oligonucleotide before gel electrophoresis as described in *Materials and Methods*. Extracts were analyzed in EMSA experiments after incubation radiolabeled DNA probes in the presence or absence of nonimmune sera (−) or anti-Pdx-1 antisera (+). For A and B, lane 1 represents EMSA reactions using nuclear extracts from BHK cells in the absence of *pdx-1* transfection, whereas lanes 2 and 3 reflect EMSA experiments using extracts prepared after transfection of the mouse pdx-1 cDNA. The *arrowhead* denotes the position of the major Pdx-1-DNA complex.

plasmic extracts) from native nontransfected αTC-1 or GLUTag cells (Fig. 3A, lanes 4 and 8). Furthermore, the αTC-1 and GLUTag cell DNA-protein complexes were almost completely attenuated by incubation of the EMSA reaction with Pdx-1 antisera (Fig. 3A, lanes 5 and 9). Similarly, an identically migrating DNA-protein complex was detected in EMSA experiments with nuclear extracts from either *pdx-1*-transfected BHK cells or native αTC-1 or GLUTag cells using the proglucagon gene G_1 element (Fig. 3B). Furthermore, the dominant G_1-protein complex was markedly diminished after preincubation with specific anti-Pdx-1 antisera (Fig. 3B) but was not diminished by incubation with nonimmune control sera (data not shown). Hence, the failure of endogenous *pdx-1* to extinguish proglucagon gene transcription in αTC-1 or GLUTag cells was not attributable to expression of a DNA binding-defective Pdx-1 protein in these cell lines.

We next examined the possibility that failure of viral-derived Pdx-1 to extinguish proglucagon expression (Fig. 1) may be due to expression of a defective Pdx-1 protein. To assess this possibility, we carried out EMSA studies employing cell extracts from cells transduced with Ad-*pdx-1*. A robust increase in the intensity of the Pdx-1-DNA complex was detected in EMSA studies using nuclear extracts from all cell lines (BHK fibroblasts, αTC-1 and βTC-6 islet cells, and GLUTag enteroendocrine cells) transduced with Ad-*pdx-1* (Fig. 4, lanes 3, 7, 11, and 15). Furthermore, the Pdx-1-DNA complex detected in Ad-*pdx-1* transduced cells was markedly diminished and partially supershifted after preincubation of the EMSA reaction with Pdx-1 antisera (Fig. 4, lanes 4, 8, 12, and 16). Hence, adenoviral expression markedly increases the levels of Pdx-1 protein, which retains the ability to interact with DNA promoter elements.

Because endocrine cell lines are known to be heterogeneous with respect to hormone gene expression, promoter analysis using pooled transfected cell lysates or Northern blotting of total cellular RNA may fail to detect suppression of proglucagon promoter activity or proglucagon RNA transcripts in a minority of individually transduced cells. To ascertain whether basal or enhanced expression of Pdx-1 might be associated with reduction or extinction of proglucagon gene expression within individual islet or enteroendocrine cells, we examined the colocalization of Pdx-1 and GLP-1-immunoreactivity in islet αTC-1 and enteroendocrine GLUTag cells before and after Ad-*pdx-1* transduction. Although numerous αTC-1 cells contained either nuclear pdx-1 or cytoplasmic GLP-1 immunopositivity, cells containing both nuclear Pdx-1 and weak yet detectable cytoplasmic GLP-1 immunopositivity were easily observed in the absence of exogenous Ad-*pdx-1* (Fig. 5A, *top panels*). To determine whether increased expression of *pdx-1* would markedly reduce the number of cells exhibiting cytoplasmic GLP-1 immunopositivity, we reexamined αTC-1 cells after Ad-*pdx-1* transduction. A marked increase in the numbers of αTC-1 cells exhibiting strong nuclear Pdx-1 immunoreactivity was detected after Ad-*Pdx-1* infection (Fig. 5A, *lower left panel*); however, in contrast to observations in INS subclones overexpressing *pdx-1* (11), numerous αTC-1 cells containing both nuclear Pdx-1 and cytoplasmic GLP-1 immunopositivity were easily detected (Fig. 5A, *lower panels*). Similar analyses were carried out to ascertain whether Pdx-1 was coexpressed together with GLP-1 in individual GLUTag cells. We detected numerous GLUTag cells that coexpressed both nuclear Pdx-1 and cytoplasmic GLP-1 (Fig. 5B, *top panels*). Furthermore, Ad-*Pdx-1* transduction markedly increased the number of GLUTag cells exhibiting nuclear Pdx-1 immunopositivity, yet many of the Pdx-1+ cells continued to exhibit

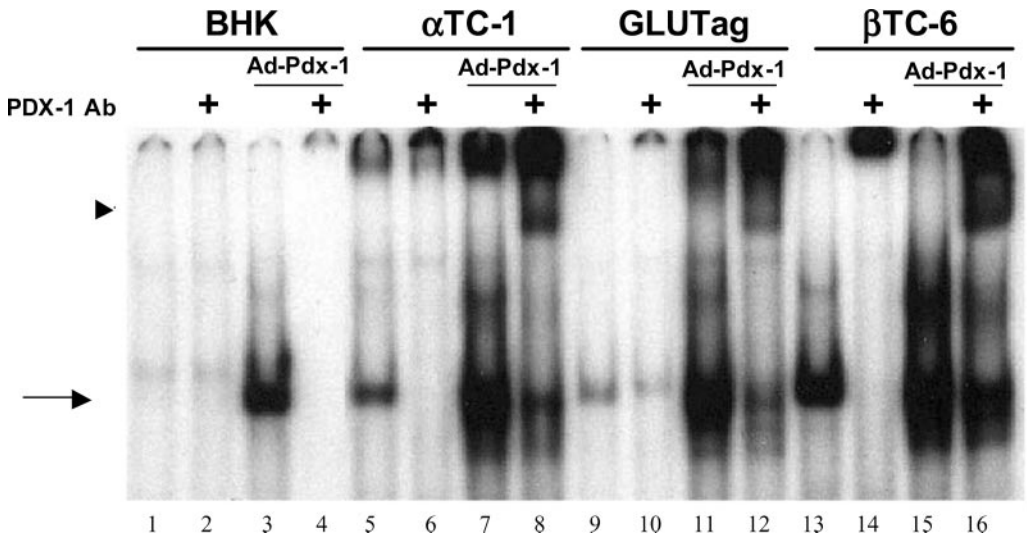

FIG. 4. Transduction of *pdx-1* adenovirus (Ad-*pdx-1*) increases Pdx-1-DNA binding in islet and enteroendocrine cells. EMSA analysis was carried out using nuclear extracts from BHK fibroblasts in the absence of Ad-*pdx-1* transduction (lanes 1 and 2); BHK fibroblasts after Ad-*pdx-1* transduction (lanes 3 and 4), or αTC-1, GLUTag, and βTC-6 cells with or without transduction of Ad-*pdx-1*. Nuclear extracts isolated from nontransduced cells or cells transduced with Ad-*pdx-1* were incubated with an insulin promoter oligonucleotide in the presence or absence of Pdx-1 antibody (+), as described in *Materials and Methods*. Similar results were obtained using the G_1 promoter element (data not shown). EMSA reactions using extracts from cells transduced with the control Ad-LacZ were identical with those obtained using extracts prepared from cells in the absence of viral transduction (not shown). *Arrow*, PDX1:DNA complex; *arrowhead*, supershifted complex.

strong cytoplasmic GLP-1 immunoreactivity (Fig. 5B, *lower panels*). To ascertain whether the detection of cells coexpressing Pdx-1 and proglucagon was restricted to immortalized cell lines, we carried out similar experiments using nontransformed murine islets. A subset of dispersed primary cultured murine islet cells were detected that coexpressed Pdx-1 and proglucagon (GLP-1) immunoreactivity in the absence of Ad-Pdx-1 transduction (Fig. 5C), and enhanced Pdx-1 expression after Ad-Pdx-1 transduction was not associated with extinction of proglucagon expression in individual islet cells (Fig. 5C).

Discussion

The central roles of pancreatic glucagon and gut-derived GLP-1 in the control of glucose homeostasis, together with the more recently delineated actions of GLP-2 on intestinal mucosal growth, have stimulated increasing interest in understanding the control of proglucagon gene expression in both pancreas and intestine. Given the difficulty in procuring sufficient numbers of isolated islet α- or intestinal L cells for studies of proglucagon gene expression in primary cell cultures, the majority of studies directed at identifying signaling pathways and genes important for proglucagon gene expression have used immortalized cell lines; transfected proglucagon promoter plasmids; and, where available, mice exhibiting enhanced expression or targeted elimination of specific transcription factors (13, 23, 25, 33, 35–38).

In contrast to the detailed understanding of the molecular factors important for control of insulin gene expression, comparatively few transcription factors have been identified that play key roles in the regulation of proglucagon gene transcription. Perhaps the strongest evidence implicating a specific transcription factor as an essential regulator of proglucagon gene transcription derives from studies of *pax-6*. *Pax-6* activates the transfected rat proglucagon promoter (25, 39), and enhanced *pax-6* expression increased the levels of endogenous proglucagon mRNA transcripts in not only cell lines but also the gastrointestinal epithelium of rats transduced with Ad-*Pax-6* (40). Conversely, targeted elimination of the *pax-6* gene or expression of a dominant-negative *pax-6* allele in mice is associated with virtual elimination of islet and intestinal proglucagon-producing cell types (23, 41). Hence, multiple lines of complementary evidence support a role for *pax-6* in the control of islet and enteroendocrine cell differentiation and proglucagon gene transcription.

The transcription factor *brn*-4 is also a potent activator of proglucagon gene expression (42), and early developmental expression of *brn*-4 under the control of the *pdx-1* promoter induces ectopic proglucagon gene expression in murine islet β-cells, consistent with a role for *brn-4* in control of proglucagon gene transcription (43). Surprisingly however, targeted elimination of *brn-4* in mice has not been reported to affect development of the α-cell lineage; hence, *brn-4*, unlike *pax-6*, is not essential for the development and function of the differentiated α-cell.

Our studies examining the relationship between *pdx-1* and proglucagon gene expression were prompted in part by reports implicating Pdx-1 as a potent negative regulator of proglucagon gene expression (11, 13). Overexpression of *pdx-1* in a subclone of rat islet INS-1 cells, designated INSrαβ, virtually eliminated proglucagon gene expression, whereas expression of a dominant-negative *pdx-1* cDNA converted a predominantly insulin-producing INS subclone into a glucagon-producing α-cell (11). The *pdx-1*-mediated repression of islet proglucagon gene expression in INSrαβ cells was attributed to suppression of proglucagon promoter activity (11). More recent studies have implicated a DNA binding-independent mechanism whereby Pdx-1 inhibits progluca-

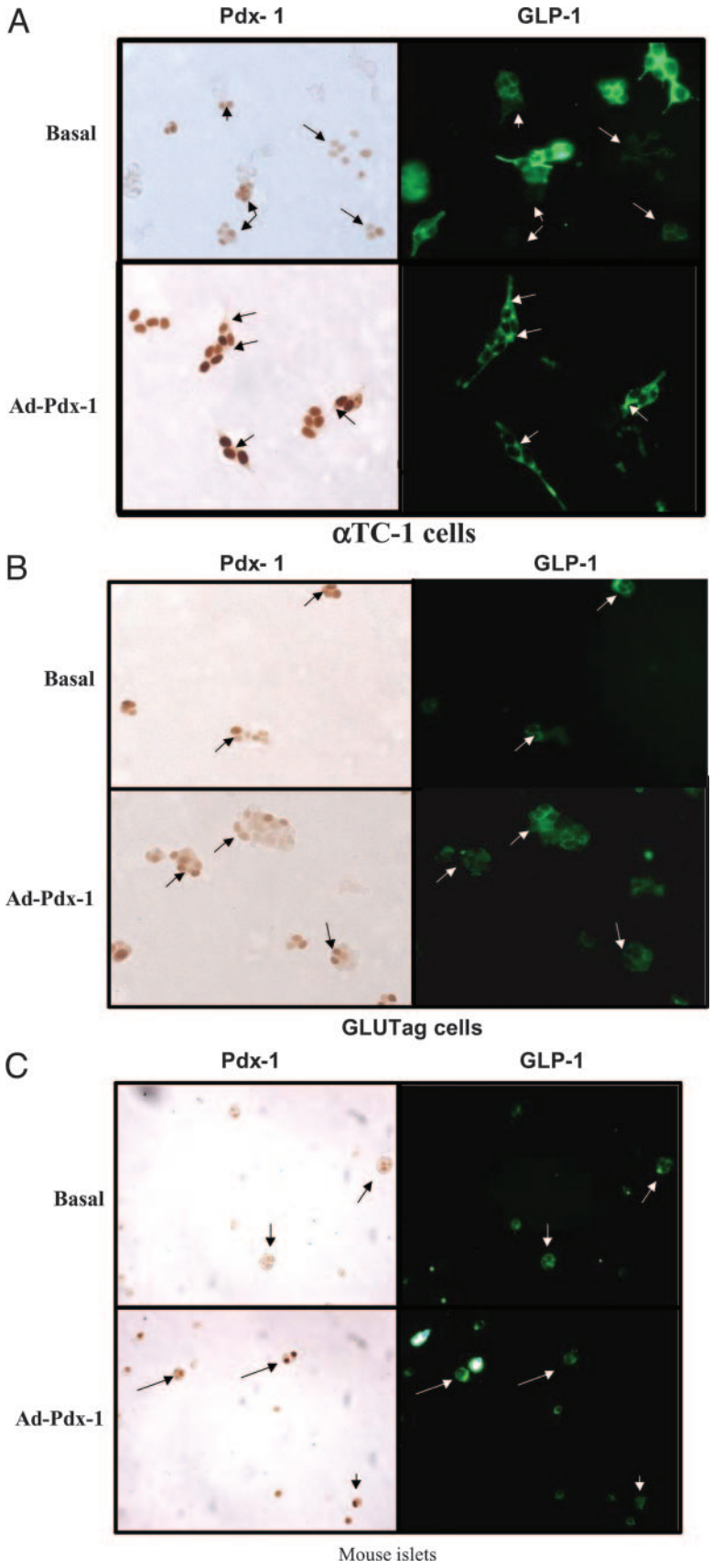

FIG. 5. Coexpression of Pdx-1 and GLP-1 (proglucagon) in individual islet and enteroendocrine cells. αTC-1 islet cells (A), GLUTag enteroendocrine cells (B), and mouse islets (C) were grown on glass slides, and cells were analyzed without (basal) or after Ad-*pdx-1* transduction. Immunohistochemical analysis was carried out in cells isolated 48 h after viral or mock transduction using antisera against Pdx-1 or GLP-1 (proglucagon), as described in *Materials and Methods*. *Arrows* designated individual cells coexpressing nuclear Pdx-1 and cytoplasmic GLP-1 immunoreactivity. No change in the number or intensity of GLP-1-immunoreactive cells was observed in αTC-1 islet cells, GLUTag cells, or islets infected with the control Ad-*Lacz* adenovirus (data not shown).

gon promoter activity via protein-protein interactions (13). Pdx-1 is capable of forming heterodimers with either Cdx-2/3 or Pax-6 in heterologous transfected BHK cells, thereby impairing the Pax-6- and Cdx-2/3-dependent activation of proglucagon promoter activity through the G_1 element (13). Whether this mechanism is also relevant to control of proglucagon gene transcription in islet or gut endocrine cells that express the endogenous proglucagon gene remains to be determined.

Further evidence that expression of Pdx-1 alone does not always eliminate the α-cell phenotype is found in multiple studies documenting coexpression of Pdx-1 and glucagon in developing or normal islet cells and islet cell lines (Table 1). For example, mouse embryonic stem cells cultured *in vitro* differentiate into multiple subsets of phenotypically distinct islet cell types, including cells that express both Pdx-1 and glucagon (14). Similarly, analysis of Pdx-1 expression in the developing murine pancreas revealed islet cells that coexpressed both Pdx-1 and glucagon at both embryonic day (E) 9.5 and E13.5, with a subset (~2.7%) of islet α-cells continuing to express Pdx-1 in the adult mouse pancreas (15).

Analysis of pancreatic islet cells during development arising from neurogenenin-3+ precursor cells demonstrated that although the majority of embryonic glucagon cells did not express Pdx-1, a subset of glucagon+/Pdx-1+ cells was readily detectable in the murine embryonic pancreas (16). Furthermore, coexpression of Pdx-1 and glucagon was also observed in analysis of gene expression in single cells isolated from the developing murine pancreas at E10.5 (17). Similarly, cells coexpressing both Pdx-1 and glucagon were observed in the murine pancreas after treatment with alloxan (44), and islet cells containing nuclear Pdx-1 and cytoplasmic glucagon immunoreactivity were detected in both the developing and adult pancreas of the $PC2^{-/-}$ mouse (45). Hence our data using murine islet and enteroendocrine cells are consistent with multiple studies of nonimmortalized islet cells (Table 1), demonstrating that *pdx-1* expression is not invariably associated with elimination of the α-cell phenotype.

The inability to easily study proglucagon gene transcription in primary cultures of purified islet α- or gut endocrine L cells has fostered a predominant reliance on cell lines for analysis of proglucagon gene transcription. In several instances, the evidence linking a putative transcription factor to the control of proglucagon gene expression is derived strictly from analysis of promoter activity in transfected heterologous cell lines. For example, the transcription factors MafA (46), Pbx-1, and Preb1 (47) have all been implicated in the control of proglucagon gene transcription (47), but none of these factors has yet been shown to modulate the levels of endogenous proglucagon mRNA transcripts in islet or intestinal endocrine cells. Similarly, although *Foxa2* has been reported to repress proglucagon promoter activity in transfected cell lines (48, 49), markedly enhanced expression of *Foxa2* paradoxically increases the levels of proglucagon mRNA transcripts in islet cells (50).

Similar data have emerged from experiments analyzing the putative role of *pax-2* in the control of proglucagon gene transcription. *Pax-2* activates the proglucagon promoter in transfected cell lines, and the HMG-I/Y-related protein p8 has been invoked as a positive modifier of *pax-2* activity on the proglucagon promoter (51). Nevertheless, we have been unable to detect *pax-2* expression in the murine pancreas or intestine, and pancreatic and intestinal proglucagon gene expression is normal despite the complete absence of *pax-2* expression (33). Taken together, these studies emphasize the need for caution when extrapolating data based solely on results from promoter studies in transfected cell lines to the physiological control of endogenous gene expression.

In summary, our data, taken together with the results of previous studies, demonstrate that *pdx-1* expression alone may not be sufficient for extinction of proglucagon gene expression in islet or enteroendocrine cells. One possible explanation that reconciles the existing data are the existence of as-yet-unidentified factor(s), likely expressed during development of the endocrine pancreas, which when coexpressed together with *pdx-1*, represses proglucagon gene expression, leading to elimination of or failure to develop the α-cell phenotype. The loss or reduced expression of the coexpressed factor at specific time points during development or in some immortalized cell lines would result in cells that coexpress *pdx-1* and proglucagon (Table 1). Alternatively, some islet cells may express a factor that inhibits the activity of Pdx-1 on the proglucagon gene promoter, allowing for ongoing coexpression of proglucagon and pdx-1 in the same cell. Future studies directed at identification of the factor(s) modifying the interaction of Pdx-1 with the proglucagon promoter may provide new insights into the control of both islet endocrine cell development and the regulation of proglucagon gene transcription.

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