Pax6 and Pdx1 are required for production of glucose-dependent insulinotropic polypeptide in proglucagon-expressing L cells

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Fujita Y, Chui JW, King DS, Zhang T, Seufert J, Pownall S, Cheung AT, Kieffer TJ. Pax6 and Pdx1 are required for production of glucose-dependent insulinotropic polypeptide in proglucagon-expressing L cells. Am J Physiol Endocrinol Metab 295: E648–E657, 2008. First published July 1, 2008; doi:10.1152/ajpendo.90440.2008.—Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are incretin hormones that play important roles in maintaining glucose homeostasis and are being actively pursued as novel therapeutic agents for diabetes. GIP is produced by dispersed enteroendocrine cells and interestingly at times is coexpressed with GLP-1. We sought to determine the factors that selectively define GIP- vs. GLP-1-expressing cells. We performed comparative immunostaining of Pax6 and Pdx1 in GIP- and GLP-1-secreting cells. We investigated whether Pax6 and Pdx1 activate the human GIP promoter in control IEC-6 cells and GIP-expressing STC-1 cells. EMSA was performed to assess the binding of these transcription factors to the GIP promoter. Pax6 and Pdx1 consistently colocalized in GIP-immunoreactive cells. Cells that coexpress GIP and GLP-1 were Pax6 and Pdx1 positive, whereas cells expressing only GLP-1 were Pax6 positive but did not express Pdx1. GIP promoter activity was enhanced in IEC-6 cells by exogenous Pax6 or Pdx1 and diminished in STC-1 cells by inhibition of endogenous Pax6 or Pdx1 by dominant-negative forms. Promoter truncation analysis revealed a major loss of promoter activity when the sequence between –184 to –145 bp was deleted. EMSA studies indicated that Pax6 and Pdx1 bind to this proximal sequence of the human GIP promoter. Our findings indicate that concomitant expression of Pax6 and Pdx1 is important for GIP expression. Our results also suggest that the presence of Pdx1 defines whether GLP-1-expressing gastrointestinal L cells also coexpress GIP.

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distribution. The specific factors that regulate this pattern have yet to be fully elucidated. Moreover, the cell-specific expression of GIP and GLP-1 to distinct cell types is overly simplified as there have been reports (30, 31, 37) that some cells produce both hormones. In this series of experiments, we sought to determine why some enteroendocrine cells express GIP, others GLP-1, and yet others evidently produce both. We focused on the transcription factors Pdx1 and Pax6 given that disruption of either gene in mice seems to have a profound effect on incretin cell formation.

### MATERIALS AND METHODS

**Tissue culture.** We used two gut-derived cell lines for this study. IEC-6 cells (ATCC, Bethesda, MD) are undifferentiated cells and STC-1 cells (kindly provided by Dr. D. Drucker, University of Toronto, ON, Canada) are a multiple hormone-positive enteroendocrine cell line. Cells were cultured in high-glucose DMEM (Invitrogen, Burlington, ON, Canada) containing 10% FCS (Invitrogen).

**Animals and tissue preparations.** Gut samples were removed from Wistar rats (~250 g), while the animals were under inhalation anesthesia with 2% Isoflurane (AErrane, Baxter, Mississauga, ON).

### Table 1. Mutation positions, native sequences, and primers

<table>
<thead>
<tr>
<th>Position</th>
<th>Native Sequence</th>
<th>Mutated Sequence</th>
<th>Primers</th>
</tr>
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<tr>
<td>M1 -201  -194 CAGCCAGG</td>
<td>CAAGCAAG</td>
<td>5′-CCC AGA CAG CAG CTG GAA AAA GCC AAA TGT TAA TCA CCA ATT AGC-3′</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>M8 -69   -64 AGATAA</td>
<td>GGGAG</td>
<td>5′-GGG CTT AAT TTC TTG CGC GGA GAT GCT TTT AGG</td>
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**Fig. 1.** Glucose-dependent insulinotropic polypeptide (GIP) is expressed with Pdx1 and Pax6 in human duodenum. Pdx1 (green)/GIP (red) immunofluorescent stains are shown at top. Arrows indicate cells positive for both Pdx1 and GIP, and arrowheads indicate Pdx1 only positive cells. Pax6 (green)/GIP (red) immunofluorescent stains are shown at bottom. Arrows indicate cells positive for both Pax6 and GIP, and arrowheads indicate Pax6 only positive cells. Bars = 20 μm.
Duodenal samples were taken within 2 cm from gastroduodenal junction, jejunal samples were excised 5-cm distal to the ligament of Treitz, and portions of ileum were just proximal to the colon. Samples were fixed in 4% paraformaldehyde in PBS at 4°C overnight and rinsed in 70% ethanol and embedded in paraffin. Human distal duodenum samples were fixed in formalin at 4°C overnight and processed for paraffin embedding and sectioning. Studies were approved by the University of British Columbia.

Immunohistochemistry and immunocytochemistry. Antigen retrieval was performed by wash in Tris-EDTA buffer (pH 9, containing 0.05% Triton X) followed by microwave heating. Sections were treated with a protein-blocking reagent (Dako Cytomation, Mississauga, ON, Canada) for 30 min and incubated with primary antibodies (Supplemental Table S1; supplemental data for this article are available online at the Am J Physiol Endocrinol Metab website) at 4°C overnight. After washes, sections were incubated with conjugated secondary antibodies (Alexafluor 488 or Alexafluor 594, 1:500, Molecular Probes Eugene, OR; AMCA, 1:250, Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. Slides were mounted in aqueous media with/without DAPI (Vector Laboratories, Burlingame, CA). To determine the extent to which GIP and GLP-1 immunoreactivity colocalized, double-stained sections from the intestine of three different animals were examined and scored for either GIP only (K cells), GLP-1 only (L cells), or GIP/GLP-1 co-positive (K/L cells).

Cloning of the human GIP promoter. A 2.9-kb fragment of human GIP promoter (H11002 to H11001 57 bp) was amplified from the human BAC clone RP11–110H20 (BACPAC Resource Center, Oakland, CA) by PCR. The primers used were 5′-ATGCTGGATCTGCTCCTAGG-3′ (sense) and 5′-CAGGCGCGATGAATCACGTC-3′ (antisense). The PCR product was cloned to pNEB plasmid (New England BioLabs, Beverly, MA) and finally transferred to a pGL4.10 luciferase construct (Promega, Madison, WI) between EcoRV and BglII sites. Truncated promoter constructs were produced by restriction digest (see Fig. 5). The two shortest constructs were made using PCR and recloned to pGL4.10. A 2.4-kb fragment of rat proglucagon promoter was kindly provided by Dr. D. Drucker and cloned into pGL4.10.

Plasmids. Human Pdx1 cDNA was kindly provided by Dr. J. Habener (Harvard Medical School, Boston, MA) and cloned to pCDNA3.1 (Invitrogen). The cytomegalovirus (CMV) promoter driven mouse Pax6 expression vector was generously provided by Dr. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria). As a control to adjust the amount of CMV promoter driven vectors, pCDNA3.1 without multiple cloning sites was used. The CMV driven dominant-negative (DN)-Pdx1 plasmid was kindly provided by Dr. D. Stoffers (University of Pennsylvania, Philadelphia, PA; Ref. 36).

Site-directed mutagenesis. The −210-bp human GIP promoter luciferase construct was used as a template and mutated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used are listed in Table 1 (complementary primers were also prepared for mutagenesis). All mutations were confirmed by direct DNA sequencing (PRISM 377 DNA sequencer, Applied Biosystems, Foster City, CA). The DN-Pax6 was also made by site-directed mutagenesis as described above. A stop codon was introduced in the transactivation domain of Pax6 (Thr304; ACA to TAA). The primers used for mutagenesis were 5′-CCCACAGCCCACCTAGCGAGTTTCTCC-3′ (forward) and 5′-GGAGGAGACAGGTCTTTCGCTTGTGGGCTTGT-3′ (reverse).

Dual luciferase assay. One day before transfection, cells were plated into six-well plates at a density of 2 × 10^5 cells per well for
STC-1 cells or 4 × 10^5 cells per well for IEC-6 cells. A mixture of 0.5 µg of pGL4 reporter plasmid, CMV driven vectors, and the control phRL-TK (50 ng for IEC-6 and 16 ng for STC-1 cells; Promega, Madison, WI) was transfected using Lipofectamine (Invitrogen) either for 3 h with Plus Reagent (Invitrogen; IEC-6 cells) or 5 h without (STC-1 cells). After a 48-h incubation with regular media, luciferase and renilla activities were assayed according to the manufacturer’s instruction (Promega) using an LMAXII 384 luminometer (Molecular Devices, Sunnyvale, CA). Firefly luciferase activity was normalized to renilla luciferase expression and is presented as the fold increase in relative light units over samples transfected with pGL4.10. All conditions were analyzed in duplicate in at least three separate experiments.

Adenovirus vector and viral infection of STC-1 cells. An adenoviral vector expressing Pdx1 (Ad-Pdx1) was made using an Adeno-X kit (Clontech Laboratories, Mountain View, CA). The human Pdx1 cDNA was cloned to the shuttle vector and then ligated to pAdeno-X. The production of Ad-GAL was described previously (7). STC-1 cells were plated 2.0 × 10^4 per well on glass slides 1 day before infection. Ad-Pdx1 or Ad-GAL was added to STC-1 cells for 1 h in infection media at 25 multiplicity of infection. Cells were fixed after 48 h of infection and then stained with GIP, GLP-1, and DAPI, and >400 cells were assessed for immunoreactivity in each group, with the investigator blinded to the group assignment.

EMSA. EMSA studies were done with a LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). GIP EMSA probes corresponding to human GIP promoter (−193 to −138) were 5′-CCCCAGACACGAGCTGGAGATGCAATAATTACGACAGTCAGG-3′ (forward) and 5′-CTCTGAACTGTGAATTGGTGATTAACATTTGGCTATCTCCAGCGTCTGGG-3′ (reverse). Probes were biotin-labeled using a 3′-end DNA labeling kit (Promega) and annealed at a concentration of 20 fmol. Nuclear extracts from STC-1 cells were obtained using the NE-PER nuclear and cytoplasmic extraction reagents kit (Promega). Two microliters of nuclear extract (2–4 µg) were incubated in 20 µl of 1× binding buffer containing 0.1 mg/ml BSA (Invitrogen), 5% glycerol, 100 mmol/l KCl, 5 mmol/l MgCl2, 0.1% Nonidet P-40, and the labeled probe with/without competitor (4 pM) for 30 min at room temperature. Super-shift assays were done by preincubating 1 µl of each antibody with nuclear extract for 30 min at room temperature before being mixed with labeled probes. Mixtures were separated by 6% polyacrylamide gel in Tris-borate-EDTA buffer and transferred to nylon membrane (Magna nylon membrane; Osmonics, Westborough, MA). After ultraviolet cross-linking, detection and exposure to X-ray film were performed according to the manufacturer’s instruction (Pierce).

Statistical analysis. Results are means ± SE. Statistical significance was assessed by one-way ANOVA with Bonferroni’s post
hoc test using commercial software (Prism; GraphPad, San Diego, CA).

RESULTS

Double incretin positive cells express both Pdx1 and Pax6, while GLP-1-positive cells express Pax6 but not Pdx1. As previously reported in mice (21), Pdx1 immunoreactivity was identified in GIP-positive cells of both rat and human intestines. However, in proximal duodenum mucosa, nuclear Pdx1 immunoreactivity was also observed in most epithelial cell nuclei, including the absorptive enterocytes (Figs. 1 and 2). In contrast, duodenal Pax6 immunoreactivity was predominantly observed in GIP-positive cells (Fig. 1 and 2). In the jejunum, Pdx1 expression was more limited to GIP-positive cells, which were also immunoreactive for Pax6. In the terminal ileum, we observed GIP immunoreactivity in approximately one-third of GLP-1-positive cells (K/L type; Fig. 3). In this region, we did not find any GIP-positive cells without coexpression of GLP-1. All GLP-1-positive cells expressed Pax6, whether or not they coexpressed GIP. In contrast, Pdx1 expression was restricted to GIP-positive (double incretin positive) cells; GLP-1-immunoreactive cells that did not express GIP were not immunoreactive for Pdx1 (Fig. 3). Thus we observed that GIP-positive cells are scattered throughout the small intestine but always display concurrent expression of both Pdx1 and Pax6.

Forced expression of Pdx1 increases the K/L-type population in STC-1 cells. We used a murine enteroendocrine tumor cell line, STC-1, as a model for GIP and/or GLP-1 expression in vitro. We observed both single GIP or GLP-1 and double GIP/GLP-1 immunoreactive cell clusters in STC-1 cultures.
but none in IEC-6 cells, a rat small intestinal stem cell-like line (data not shown). We identified three distinct populations in STC-1 cells that mimicked those we observed in the rat small intestine in vivo: K-type cells expressing GIP but not GLP-1, L-type cells expressing GLP-1 but not GIP, and a third population of K/L-type cells that were immunoreactive for both GIP and GLP-1 (Fig. 4A). Next, we delivered Pdx1 by adenovirus to STC-1 cells to determine if increased Pdx1 expression could induce a shift of L-type to K/L-type cells. Indeed, Ad-Pdx1-transduced STC-1 cells displayed a greater number and proportion of K/L cells compared with control virus infected cells (Fig. 4, B and C).

Fig. 5. Sequences within the proximal 184 bp of the GIP promoter are critical for GIP transcription. A: map of human GIP promoter luciferase reporter vector (top) and the relative locations of 8 different engineered mutations, indicated by Xs, in –210-bp constructs (bottom). B: truncated human GIP promoter activity in STC-1 cells expressed in relative light units (RLU). C: activity of mutated –210-bp human GIP promoter constructs in STC-1 cells relative to the native [wild type (WT)] sequence. Location and sequences of mutants (M1–M8) are exhibited in A and Table 1, respectively. D: comparison of proximal GIP promoter sequences between human, chimp, macaques, dog, bat, elephant, pig, cow, rat, and mouse. Ex1, exon 1 of GIP gene. Asterisks show identity of DNA sequences for all 10 mammals: *P < 0.05, **P < 0.01, ***P < 0.001, when values were compared with –2.9 kb (B) or WT (C). #P < 0.001 compared with –184-bp construct (B).
Sequences within the proximal 184 bp of the GIP promoter are critical for GIP transcription. When a 2.9-kb human GIP promoter luciferase reporter gene construct (Fig. 5A) was transfected into the cell lines, GIP-expressing STC-1 cells demonstrated an ~40-fold elevated GIP promoter activity over IEC-6 cells (data not shown). To investigate which regions of the GIP promoter are crucial for transcriptional regulation, we generated luciferase reporter gene constructs comprising 5′-deletions and internal mutations of the human GIP promoter and transfected those into STC-1 cells. The greatest promoter activity was detected with the −210-bp construct (Fig. 5B). Notably, GIP promoter activity dropped by ~90% between −184 and −145 bp, clearly indicating that the sequence between −184 and −145 bp of the human GIP promoter is critically important for GIP transcription.

To further resolve which residues within this region are critical for GIP promoter activity, we assessed the function of several internally mutated promoter constructs that were based on the −210-bp construct as a template. The mutations within this region (M3-M6; Table 1) led to significant decreases of GIP promoter activity, the largest drop (~90%) occurring in M5 (Fig. 5C) compared with the nonmutated −210-bp construct. M5 disrupts a TAAT motif like that bound by Pdx1 in the proinsulin promoter (9, 26). M6, disrupting a site similar to a sequence of the rat GIP promoter that has been demonstrated to bind Pdx1 (21), diminished promoter activity by ~80%. M3, which targets a native GATA consensus sequence, resulted in an ~50% reduction of promoter activity. Of the mutations outside the region −184 to −145, M8, which targets a GATA consensus sequence like M3, reduced promoter activity by ~65%. M1 did not alter promoter activity, while M2 and M7 only reduced activity by ~20% relative to the native sequence. A comparison of the proximal GIP promoter (−210 bp) between 10 mammals (Fig. 5D) revealed that this region of the promoter is well conserved; ~90% of the sequences are identical between 8 of the species, while there is ~50% homology between all 10 species.

Pax6 induces both GIP and proglucagon promoter activity, whereas Pdx1 only increases GIP promoter activity in IEC-6 cells. We demonstrated that GIP-secreting cells coexpress Pdx1 and Pax6 in human duodenum and rat small intestines (Figs. 1–3). In contrast, GLP-1-secreting cells express Pax6 but do not always express Pdx1 (Figs. 2–3). To examine whether Pdx1 and Pax6 differentially regulate GIP and proglucagon promoter activity, we transiently transfected CMV-driven Pdx1 and/or Pax6 expression vectors into IEC-6 cells and assessed GIP and proglucagon promoter activity. GIP promoter activity was increased by approximately sixfold by Pdx1 and approximately fourfold by Pax6 in IEC-6 cells (Fig. 6A). When IEC-6 cells were cotransfected with both Pdx1 and Pax6, an approximately sevenfold increase of promoter activity was observed. Similar results were observed in experiments using human HEK 293 cells and human colon cancer Caco2 cells (data not shown). In STC-1 cells, exogenous Pdx1 enhanced GIP promoter activity (~2-fold) as reported previously (21), but exogenous Pax6 induced only a minor increase in GIP promoter activity (~10%; data not shown). The reduced effects of exogenous Pdx1 and Pax6 on GIP promoter activity in IEC-6 cells relative to STC-1 cells likely reflect our observation that Pdx1 and Pax6 are already abundant in untransfected STC-1 cells but not in IEC-6 cells (data not shown). Pax6 transfection increased proglucagon promoter activity by ~24-fold in IEC-6 cells, but Pdx1 transfection had no significant effect (Fig. 6B). In addition, Pdx1 expression did not alter the proglucagon promoter activity induced by Pax6.

DN-Pax6 reduces both GIP and proglucagon promoter activity, whereas DN-Pdx1 only reduces GIP promoter activity in STC-1 cells. We constructed a truncated DN-Pax6 expression vector to determine the effect on GIP promoter activity in GIP-expressing STC-1 cells. This mutant Pax6 has a deleted proline/serine/threonine-rich domain that decreases transactivation potential but increases affinity for DNA binding and can function as a dominant repressor of the transactivation activity of wild-type Pax6 by competing for target DNA-binding sites (34). GIP promoter activity was reduced in a dose-dependent manner by transfection of the DN-Pax6 with a maximal inhibition of ~70% (Fig. 7A). These results are consistent with the concept that endogenous Pax6 activates GIP gene expression. We used a DN-Pdx1 that contains the COOH-terminal DNA-binding domain but lacks the transactivation domain. This COOH-terminal mutant Pdx1 isomorph does not activate transcription and inhibits the transactivation functions of wild-type Pdx1 (36). Like DN-Pax6, DN-Pdx1 also reduced GIP promoter activity but only up to at most ~20% (Fig. 7B). Similar to results obtained with the GIP promoter, DN-Pax6 reduced proglucagon promoter activity in STC-1 cells by up to 70% (Fig. 7C). In contrast, no inhibitory
Effect of DN-Pdx1 on proglucagon promoter activity was detected in STC-1 cells (Fig. 7D).

Endogenous Pax6 and Pdx1 of STC-1 nuclear extracts bind to specific regions within the proximal human GIP promoter. To corroborate the promoter activation studies with transcription factor binding to the proximal region of the GIP promoter (−184 to −145), we used EMSA with/without antibodies against Pdx1, Pax6, GATA4, and GATA6. A biotin-labeled probe was designed to cover the sequence of −184 to −145 bp of the human GIP promoter. We observed retarded bands of this probe (Fig. 8, a, b, and c) with STC-1 nuclear extracts. These complexes proved to be probe specific, because their signals diminished with the addition of ×200 molar excess of the unlabeled probe or a shorter competitor probe (−173 to −145 bp; Fig. 8, lanes 3 and 4). In super-shift assays, super-shifted complexes were observed with antibodies to Pdx6, Pdx1 and GATA4, but not GATA6. Pdx6 antiserum eliminated complex a and weakened bands b and c. Pdx1 antiserum eliminated bands a and b and diminished complex c. GATA4 antibody eliminated only complex a but did not reduce the intensity of the other two bands. These results indicate that Pax6, Pdx1, and GATA4 bind to this specific region of the GIP promoter.

DISCUSSION

Pdx1 is crucial for GIP expression (21), but clearly additional factors are required, as most nuclei of proximal duodenal epithelial cells express Pdx1, yet relatively few of these are positive for GIP. Yamada et al. (40) transfected IEC-6 cells with Pdx1 and subsequently observed multiple endocrine cell related proteins but not GIP. In agreement, we did not observe any GIP immunoreactivity in IEC-6 cells transfected with Pdx1 alone (data not shown). Thus factors in addition to Pdx1 are likely indispensable for GIP expression. We hypothesized that Pax6 may represent one such factor, as GIP expression is diminished in Pax6-null mice (25). Moreover, Pax6 is required for the expression of the related incretin hormone GLP-1 (17, 38). We found that Pax6 immunoreactivity is scattered throughout the proximal duodenum and present in GIP-positive cells. Thus Pdx1 and Pax6 are concomitantly expressed in GIP-positive cells throughout the intestine. We also observed functional activation of a proximal region of the human GIP promoter in IEC-6 cells after transfection with Pdx1 and Pax6 and reduced promoter activity in STC-1 cells after inhibition of endogenous Pdx1 and Pax6 by transfection with the DN forms of these transcription factors. Collectively, these observations suggest that these two transcription factors may be essential for GIP expression.

In agreement with previous observations (30, 31, 37), we noted two populations of GLP-1-secreting cells in the terminal ileum. One population is a GLP-1-positive but GIP-negative cell (L cell), while the other is a double incretin-positive cell (K/L cell). GLP-1-positive cells consistently expressed Pax6 whether or not the cells expressed GIP. Thus, while Pax6 is
promoter luciferase construct reduced activity by ~80%, indicating that it is a very important site for GIP promoter function. The native sequences of mutants M1 and M8 contain “AGATA” and “AGATAA,” respectively, which are GATA consensus motifs (20). These observations suggest that GATA factors may activate multiple sites of the GIP promoter. The distal GATA site resides near the edge of the −184 construct such that GATA factors may be unable to bind to that site of the promoter, perhaps explaining the drop in activity from the −210 to −184 bp of the promoter sequence. GATA4 has previously been identified to bind to a similar sequence in the rat GIP promoter (21). GATA4 is found in STC-1 cells and is widely expressed in murine intestinal mucosa (4, 20). Transient transfection of GATA4 into IEC-6 cells increased human GIP promoter activity (data not shown). We conclude that GATA4 is a GIP transcriptional activator but is not K-cell specific given the wide distribution of GATA4 expression in the gut.

Pax6 has two DNA binding domains, a paired box domain and a homeodomain. A variant of PAX6 (5a), with a 14-residue insertion in the paired box domain, has altered DNA binding activity (13). Epstein et al. (12) reported that the homeodomain recognizes a short DNA motif (TAAT), while the paired box domain recognizes 16- to 20-bp sequences. Pax6 binding sites have been identified within the G1 and G3 elements of rat proglucagon promoter (17). However, we did not observe similar sites within the proximal region (−184 and −145) of the human GIP promoter. In contrast, the human GIP promoter contains the homeodomain motif “TAAT” at −162 and the inverted motif “ATTA” at −153, where Pax6 may be able to bind.

In our EMSA studies, we observed three specific shifted bands that could be super-shifted by preincubation with antibodies to Pdx1, Pax6, or GATA4, confirming that these factors interact with this proximal region of the GIP promoter in STC-1 cells. Our results further suggest that these transcription factors may regulate GIP gene transcription through a protein interaction or complex since multiple specific shifted bands were observed. Pax6 is known to complex with Pdx1 for binding to the somatostatin promoter (2) and with Brn-4 and Cdx2 for binding to the proglucagon promoter (1, 18). Pax6 also interacts with other transcription factors to induce gene expression during eye development (10). Further studies are required to identify specific Pax6 complexes that activate the GIP promoter.

In conclusion, concomitant expression of Pdx1 and Pax6 activates GIP expression in enteroeendocrine cells. Proglucagon-expressing L cells express Pax6; those that express Pdx1 also express GIP. Thus the presence of Pdx1 is a distinguishing feature between L cells and K/L cells. Additional studies are required to determine why proglucagon is not expressed in all GIP-positive cells.

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