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Pax-2 Activates the Proglucagon Gene Promoter But Is Not Essential for Proglucagon Gene Expression or Development of Proglucagon-Producing Cell Lineages in the Murine Pancreas or Intestine

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Tissue-specific proglucagon gene transcription is achieved through combinations of transcription factors expressed in pancreatic A cells and enteroendocrine L cells of the small and large intestine. Cell transfection and electrophoretic mobility shift assay experiments previously identified Pax-2 as a regulator of islet proglucagon gene expression. We examined whether *Pax-2* regulates gut proglucagon gene expression using enteroendocrine cell lines and *Pax2*^{1NEU} mutant mice. Immunoreactive Pax-2 was detected in STC-1 enteroendocrine cells, and Pax-2 activated proglucagon promoter activity in transfected baby hamster kidney and GLUTag cells. Pax-2 antisera diminished the formation of a Pax-2-G3 complex in electrophoretic mobility shift assay studies using nuclear extracts from islet and enteroendocrine cell lines. Surprisingly, *Pax-2* mRNA transcripts were not detected by RT-PCR in RNA isolated from adult rat pancreas, rat islets, embryonic d 19 or adult murine pancreas and gastrointestinal tract. Furthermore, embryonic d 19 or neonatal d 1 *Pax2*^{1NEU} mice exhibited normal islet A cells and gut endocrine L cells, and no decrement in pancreatic or intestinal glucagon gene expression. These findings demonstrate that *Pax-2* is not essential for the developmental formation of islet A or gut L cells and does not play a role in the physiological control of proglucagon gene expression *in vivo*. (*Molecular Endocrinology* 16: 2349–2359, 2002)

THE PROGLUCAGON GENE encodes structurally similar peptide hormones with unique biological actions important for the control of food intake, gastrointestinal motility, and both nutrient absorption and disposal (1). The liberation of a specific profile of proglucagon-derived peptides (PGDPs) in pancreas and gut is accomplished through a combination of tissue-specific gene transcription and posttranslational processing. Glucagon is produced in the islet A cell, and several PGDPs, including glicentin, oxyntomodulin, and two glucagon-like peptides, GLP-1 and GLP-2, are liberated in endocrine cells of the small and large intestine (2, 3). Cell-specific expression of prohormone convertase enzymes mediates the processing and generation of distinct PGDP profiles in the pancreas, gut, and brain (4–6). In contrast, the identity of the transcription factors that activate proglucagon gene transcription specifically in islet and enteroendocrine cells remains incompletely understood.

The mechanisms regulating tissue-specific transcriptional activation of the proglucagon gene have been approached through structural and functional analyses of the proglucagon gene promoter (7–10), and indirectly via identification of the genes important for developmental formation of glucagon-producing islet and enteroendocrine cells (11–13). In several instances, genes encoding transcription factors that bind and activate the proglucagon promoter also regulate the specification of islet or enteroendocrine cell development. For example, the paired box transcription factor Pax-6 binds to the proximal G3 and G1 rat proglucagon promoter elements alone (14) or in combination with the caudal factor Cdx-2/3 (15, 16) and activates proglucagon promoter activity in transfected cell lines (17). *Pax-6* is expressed early during development of the endocrine pancreas, and targeted inactivation of the murine *Pax-6* gene leads to impairment of islet cell formation with almost complete absence of islet A cells (18). Similarly, mice homozygous for a mutant *Pax-6 Small eye* mutation exhibit abnormal islet morphology, markedly reduced numbers of both islet A cells and gut enteroendocrine cells, and decreased levels of both pancreatic and intestinal proglucagon mRNA transcripts (14, 17).

A role for the related paired domain protein Pax-4 in islet cell formation and proglucagon gene transcription has also been proposed. Pax-4 and Pax-6 exhibit overlapping DNA binding specificities; however, unlike Pax-6, Pax-4 functions as a transcriptional repressor (19, 20). Pax-4 competes for Pax-6 binding to

Abbreviations: BHK, Baby hamster kidney; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP, glucagon-like peptide; H, histone; HA, hemaglutinin; PGDP, proglucagon-derived peptide; STZ, streptozotocin.

the proglucagon gene promoter and inhibits Pax-6-dependent transactivation of proglucagon gene expression *in vitro* (21). Consistent with these findings, *Pax-4* expression extinguishes proglucagon gene expression in islet cells, despite the ongoing expression of *Pax-6* (22). Although *Pax-4* is not expressed in the adult islet, disruption of the murine *Pax-4* gene results in the failure to form δ- and β-cells and increased numbers of glucagon-producing α-cells, consistent with a potential inhibitory action for Pax-4 in control of proglucagon gene expression and A cell development (23).

More recent studies have suggested a role for a third *Pax* gene in the control of islet glucagon gene transcription. *Pax-2* is a member of the group 2 *Pax* family and contains a paired domain, an octapeptide, and a partial paired type homeodomain (24). *Pax-2* expression has been localized to the brain, spinal cord, and subsets of mesenchyme cells and epithelial derivatives in the embryonic kidney (25, 26), after which *Pax-2* expression is down-regulated and subsequently extinguished in the adult kidney. In contrast, *Pax-2* expression persists in the male and female genital tracts of the adult mouse (27, 28) and reappears after the experimental induction of acute tubular necrosis in mice (29), and genetic reduction of *Pax-2* expression attenuates the progression of experimental renal cystic disease (30). *Pax-2* expression may also be reinduced in the human kidney postnatally in young children with dysplasia of the kidney tubules (31) and has been detected in human kidney, breast, and prostate tumors (26, 32–35).

Pax-2 mRNA transcripts were detected by RT-PCR in RNA from adult rat islets and rodent islet cell lines, and two distinct Pax-2 isoforms, Pax-2a and Pax-2b, interact with the proglucagon promoter G1 and G3 elements (36). Both Pax-2a and Pax-2b stimulate proglucagon promoter activity in transfected fibroblast and islet cell lines, implicating Pax-2, together with Pax-4 and Pax-6, as regulators of islet proglucagon gene transcription (36). Despite evidence that Pax-2 may regulate proglucagon promoter activity, the cellular localization and biological action of Pax-2 has not been reported in the normal endocrine pancreas. Furthermore, whether Pax-2, like Pax-6, also regulates intestinal proglucagon gene transcription remains unknown. To determine whether Pax-2 plays a physiological role in the control of islet or enteroendocrine gene expression, we have now studied the actions of Pax-2 in transfected cells *in vitro* and in homozygous mice harboring a mutant *Pax-2* allele *in vivo*.

RESULTS

The identification of *Pax-2* RNA transcripts in rat islets and in two murine islet cell lines, insulin-producing Min6 cells and glucagon-producing αTC1 cells (36), together with the finding that Pax-2 binds to and transactivates the proglucagon promoter (36), prompted us to assess whether Pax-2 regulates proglucagon gene transcription in gut endocrine cells. Western blot analyses detected immunoreactive Pax-2 in nuclear extracts from glucagon-producing mouse (αTC1) and hamster (InR1-G9) islet cell lines (Fig. 1). A Pax-2-immunoreactive protein was also detected in mouse enteroendocrine STC-1 cells (Fig. 1). The Pax-2 proteins from the murine cell lines migrated in an identical position, whereas hamster InR1-G9 Pax-2 migrated more rapidly (Fig. 1A). The Pax-2 antisera recognize both Pax-2a and Pax-2b isoforms in transfected cell extracts using either anti-Pax-2 antisera (Fig. 1B) or anti-hemaglutinin (HA) antisera (Fig. 1C).

Pax-2 binds to the G3 and G1 elements from the rat proglucagon gene proximal promoter and transactivates proglucagon promoter plasmids in baby hamster kidney (BHK) fibroblasts and InR1-G9 islet cells (36). Because Pax-2b was demonstrated to be the more potent transcriptional activator (compared with Pax-2a) of proglucagon promoter activity in transfected cells (36), we cotransfected *Pax-2b* and proglucagon promoter-luciferase reporter plasmids in BHK cells (Fig. 2A) and GLUTag cells (Fig. 2B). Transfection of BHK cells with *Pax-2b* induced a modest but significant activation of [−300] GLU-Luc, a plasmid containing the first 300 bp of the proximal rat proglucagon gene promoter, including the G1–G5 elements in their native genomic orientation (Fig. 2A; Refs. 7, 8). Despite the importance of the proglucagon gene G3 element for Pax-2 binding (36), deletion of the G3 enhancer and additional DNA sequences from −300 to −137 of the rat proglucagon promoter did not abrogate the Pax-2-induction of [−136] GLU-Luc activity in transfected BHK cells (Fig. 2A). Nevertheless, addition of a multimerized G3 element immediately adjacent to the truncated [−136]GLU-Luc promoter did confer greater Pax-2 responsivity beyond that observed with [−136]GLU-Luc alone in BHK cells. The finding that the G3 enhancer element is not essential for Pax-2-mediated transactivation of the proglucagon promoter in fibroblasts may reflect the ability of the more proximal G1 element to functionally interact with Pax-2 in transfected cells (36). To determine whether Pax-2 was also capable of activating proglucagon promoter activity in enteroendocrine cells, the identical reporter plasmids were transfected into mouse GLUTag cells. Consistent with the findings in BHK cells, *Pax-2b* transactivated proglucagon promoter activity in the presence or absence of the G3 element (Fig. 2B). In contrast to the results in BHK cells, however, *Pax-2b* did not activate the transcriptional activity of the [−300]GLU-Luc plasmid alone (Fig. 2B), and no further augmentation of transcriptional activity was observed with 4G3–136Luc compared with the activity obtained with [−136]GLU-Luc alone.

Because Pax-2 was reported to exhibit preferential binding to the rat proglucagon G3 enhancer element in EMSA experiments (36), we examined whether Pax-2 was detected as part of a G3 protein complex using nuclear extracts from fibroblast, islet, and intestinal

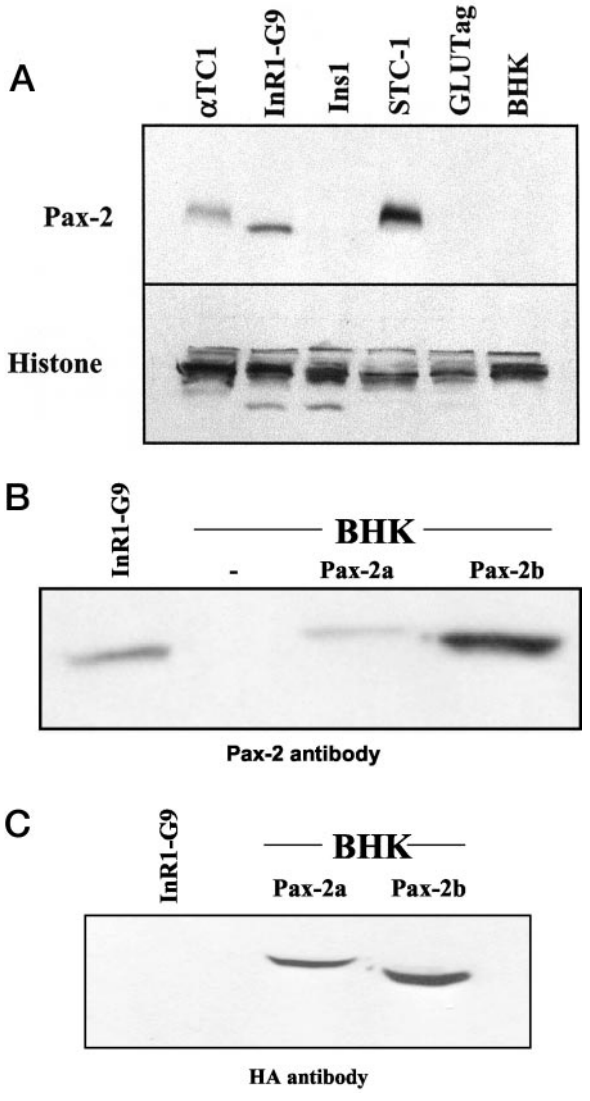

Fig. 1. Western Blot Analysis Detects Pax-2-Immunoreactive Proteins in Rodent Islet and Enteroendocrine Cell Lines

A, Nuclear extracts (40 μg protein) from mouse αTC1, hamster InR1-G9, and rat INS1 islet A cell lines, mouse STC-1 and GLUTag enteroendocrine cells, and BHK fibroblasts were separated by SDS-PAGE electrophoresis, transferred to a nylon membrane, and incubated with antisera directed against Pax-2 (*top*) or against histone proteins (*bottom*) as described in *Materials and Methods*. B, Western blot experiment using Pax-2 antisera for analysis of nuclear extracts from InR1-G9 cells, wild-type BHK fibroblasts, or BHK cells transfected with *Pax-2a* or *Pax-2b* cDNA isoforms. C, The identical nuclear extracts described in *panel* B were analyzed by Western blotting using antisera directed against the carboxy terminal HA sequence present in the murine Pax-2 cDNA open reading frames (65).

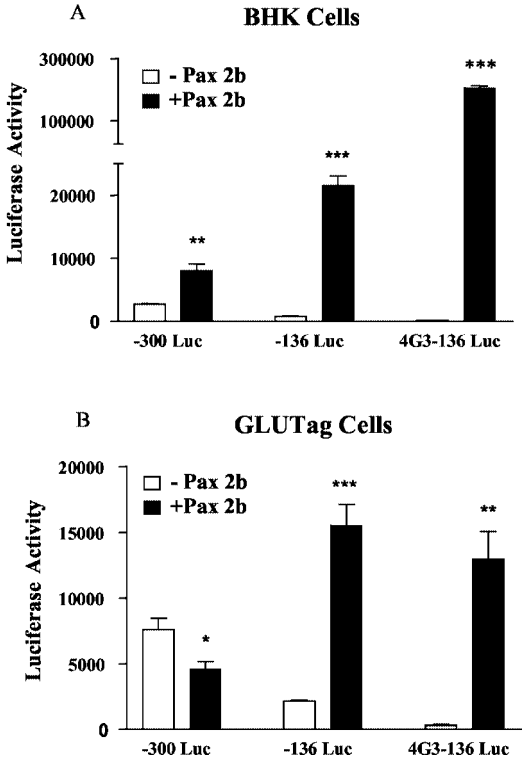

Fig. 2. Transcriptional Activity of Rat Proglucagon Promoter-Luciferase Plasmids after Transfection of BHK Fibroblasts and GLUTag Enteroendocrine Cells

BHK cells (A) and GLUTag cells (B) were transfected with [−300]GLU-Luc, [−136]GLU-Luc, and [4G3–136]GLU-Luc in the presence or absence of cotransfected *Pax-2b*, as described in *Materials and Methods*. Equal amounts of transfected cell extract were analyzed for luciferase activity. Statistical differences were analyzed by *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. Results are the mean of quadruplicates per experiment; n = 4 total experiments.

endocrine cell lines. Transfection of BHK cells with cDNAs encoding either *Pax-2a* or *Pax-2b* generated clearly detectable G3-protein complexes in EMSA experiments that were markedly attenuated in the presence of Pax-2 antisera (Fig. 3A). Nuclear extracts from islet αTC1 and InR1-G9 cells or GLUTag enteroendocrine cells generated G3 complexes that migrated at approximately the same position as the Pax-2-G3 complex detected in transfected BHK cells (Fig. 3, A and B), and the intensity of this complex was also diminished after preincubation with Pax-2-specific antisera (Fig. 3, A and B). In contrast, minimal reduction in the intensity of the GLUTag G3 complex was observed using the identical Pax-2 antisera (Fig. 3A). To provide complementary evidence for the contribution of Pax-2 to the proglucagon gene G3 binding complex in islet or intestinal endocrine cells, G3-protein complexes were transferred to a nylon membrane after EMSA using an unlabeled G3 oligonucleotide probe, and a modified EMSA-Western blot was carried out using Pax-2-specific antisera. Pax-2 was clearly detected as a component of the proglucagon gene G3 complex using αTC1 and STC-1, but not GLUTag nuclear extracts (Fig. 3C).

These findings demonstrated that *Pax-2* is expressed in a subset of proglucagon-producing islet and enteroendocrine cell lines and is capable of interacting with proglucagon promoter elements in trans-

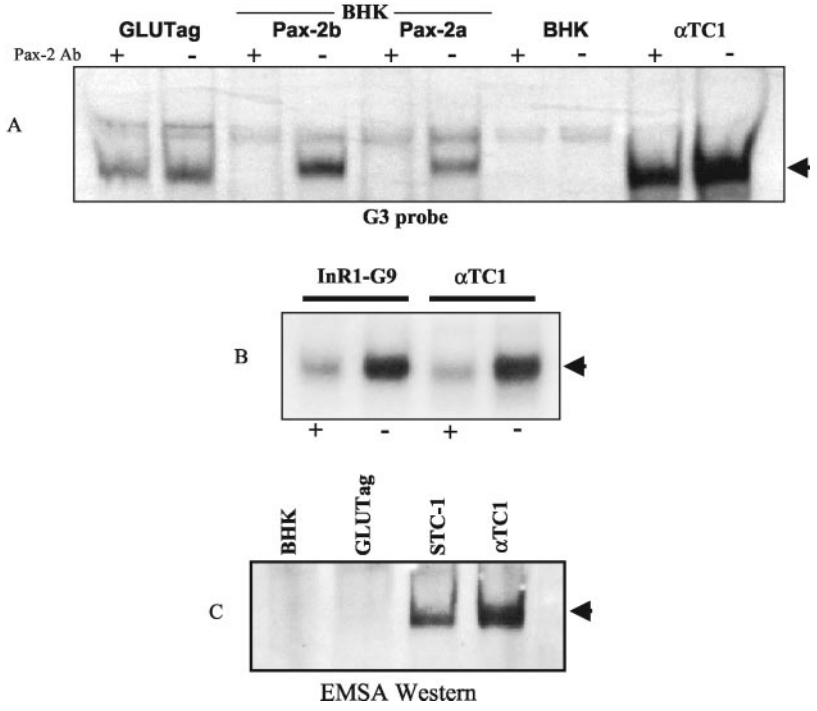

Fig. 3. EMSAs with the G3 Element and Nuclear Extracts from Fibroblast and Endocrine Cell Lines

Nuclear extracts from intestinal GLUTag cells, islet αTC1 cells, and BHK fibroblasts with or without cotransfected *Pax-2a* or *Pax-2b* (A) or islet InR1-G9 and αTC1 cells (B) were incubated with the proglucagon gene G3 oligonucleotide probe as described, in the presence or absence of nonimmune or anti-Pax-2 antisera (Pax-2 Ab). The *arrow* denotes the migration position of the G3 complex that was completely diminished by coincubation with excess unlabeled G3 competitor oligonucleotide (data not shown). C, Nuclear extracts were incubated with unlabeled G3 probe, subjected to gel electrophoresis (EMSA), and transferred to a nylon membrane. Western blot analysis was performed with anti-Pax-2 antisera. The *arrow* designates the major Pax-2-immunoreactive species detected that comigrates in the identical position as the Pax-2-G3 complex detected with labeled G3 probe in *panels* A and B.

fection and EMSA studies. To ascertain the potential relevance of *Pax-2* for control of islet or intestinal proglucagon gene expression *in vivo*, we carried out RT-PCR analysis using RNA from mouse and rat pancreas and different regions of the small and large bowel, the endogenous sites of proglucagon gene expression. Surprisingly, despite the previous assertion that *Pax-2* regulates islet proglucagon gene expression (36), we were unable to detect *Pax-2* mRNA transcripts (using several RNA preparations) in RNA from the adult murine pancreas or gastrointestinal tract (Fig. 4, A and B). *Pax-2* RNA transcripts were not detected in RNA from rat pancreas or isolated rat islets (Fig. 4C). These results clearly imply that *Pax-2* is not a transcriptional regulator of pancreatic or intestinal proglucagon gene expression in the adult rodent.

Because *Pax-2* is transiently expressed during development in tissues that include the central nervous system and kidney (25, 26), we hypothesized that *Pax-2* may be transiently expressed during pancreatic or intestinal development and perhaps play a critical role in the formation of islet A or enteroendocrine cells. Accordingly, we assessed proglucagon gene expression in the pancreas and intestine of *Pax2*^{1NEU} mice that harbor an inactivating mutation of the *Pax-2* gene (37). Mouse embryos were genotyped using a modified PCR strategy that distinguished $+/+$ *vs.* $+/-$ and $-/-$ *Pax-2* alleles (Fig. 5). RT-PCR analysis of RNA isolated from *Pax2*^{1NEU} mice on postnatal d 1 demonstrated comparable levels of proglucagon mRNA transcripts in pancreas (data not shown) and gastrointestinal tract from $+/+$ and *Pax2*^{1NEU} mutant mice (Fig. 6A). Furthermore, *Pax-2* RNA transcripts were not present in pancreatic or intestinal RNA from wild-type mice at embryonic day (E)19; however, *Pax-2* RNA transcripts were detected in RNA isolated from E19 mouse kidney (Fig. 6B). Similarly, although immunoreactive Pax-2 was detected in histological sections of E19 murine kidney (Fig. 6C), we did not detect Pax-2 immunopositive cells in E19 murine pancreas or intestine (data not shown).

To ascertain whether *Pax-2* is required for the formation of differentiated pancreatic endocrine cell types, we examined islet histology in sections from E19 and postnatal d 1 wild-type and mutant mice. Islets from *Pax2*^{1NEU} mice contained a normal distribution of pancreatic endocrine cells exhibiting immunopositivity for insulin, proglucagon, or somatostatin (Fig. 7A). Similarly, GLP-1-immunopositive and somatostatin-immunopositive enteroendocrine cells were

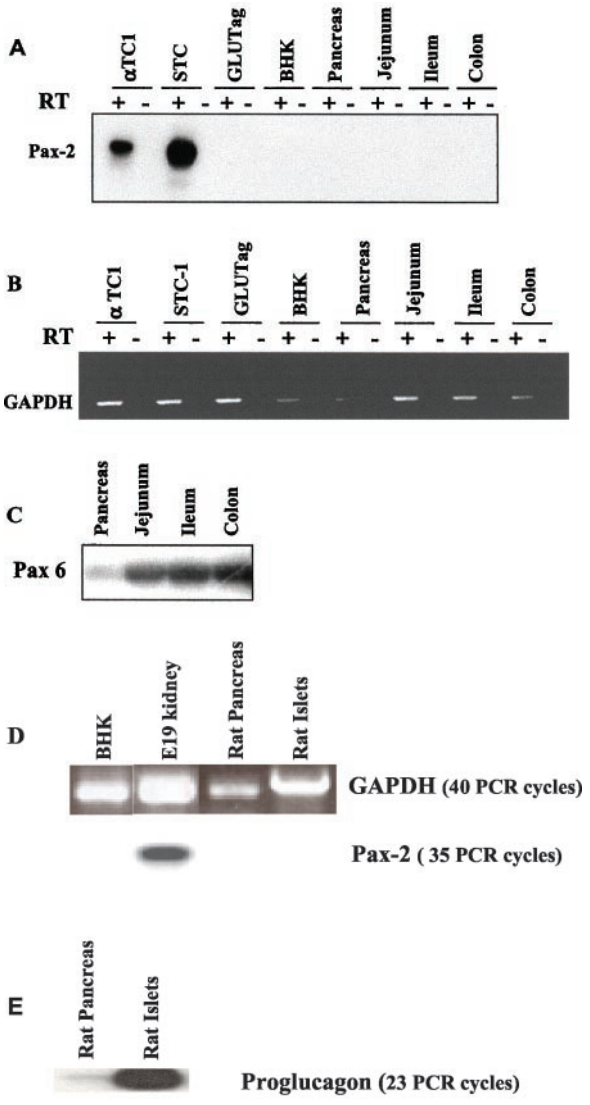

Fig. 4. RT-PCR Analysis of Pax-2 (A), GAPDH (B), and Pax-6 (C) RNA Transcripts in Murine Tissues

RNA from the pancreas and gastrointestinal tract (jejunum, ileum, and colon) of adult male mice, and αTC1, STC-1, GLUTag, and BHK cell lines was analyzed by RT-PCR for *Pax-2* and GAPDH mRNA transcripts as described in *Materials and Methods*. To further verify the integrity of the mouse tissue RNA, RT-PCR for *Pax-6* was performed on the identical samples of first strand cDNA generated from pancreas and intestinal RNA. PCR products were separated on an agarose gel, transferred to a nylon membrane, and hybridized with internal oligonucleotides complementary to *Pax-2* or *Pax-6* sequences. Aliquots of the identical first strand cDNA synthesis from *panel* A were subjected to RT-PCR using primers specific for GAPDH, and the reaction products were visualized by ethidium bromide staining of the agarose gel (B) after electrophoresis of the PCR products. *Plus* and *minus* denote the presence or absence of reverse transcriptase (RT) in the PCR experiments. D, RT-PCR for *Pax-2* and GAPDH was carried out using RNA from BHK fibroblasts, E19 mouse kidney, adult rat pancreas, and RNA from islets isolated from adult male Wistar rats. E, RT-PCR for proglucagon was performed using the same RNA from rat pancreas and islets used for the experiments shown in *panel* C.

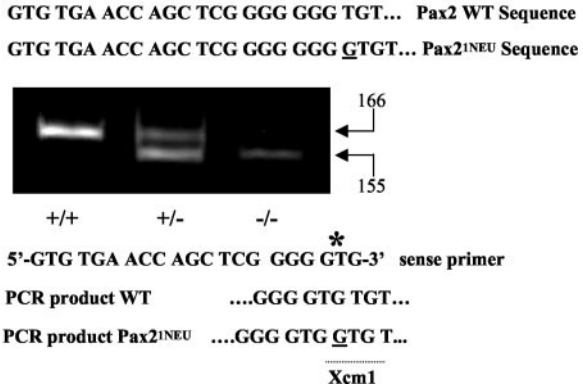

Fig. 5. Genotyping of Wild-Type (+/+) *Pax-2* and Mutant (+/− and −/−) *Pax2*^{1NEU} Alleles

The sequence of the wild-type and mutant *Pax2*^{1NEU} allele in the region of the single base G insertion (*underlined*) is shown. The use of a modified 5′ PCR primer (*) generates an additional *Xcm*I restriction site (*dotted line*) in the PCR product from the mutant allele (see *Materials and Methods*). After PCR using genomic DNA prepared from tail samples, PCR products were digested with *Xcm*I and analyzed by SDS-PAGE electrophoresis. The 166-bp product corresponds to the wild-type allele, whereas the 155-bp product corresponds to the mutant Pax2^{1NEU} allele.

readily detected in the gastrointestinal tract of +/+, +/−, and Pax2^{1NEU} mice (Fig. 7B). Because *Pax-2* expression is induced after induction of experimental injury in the murine kidney (29), we examined the pancreas of mice after treatment with a 5-d course of low dose streptozotocin (STZ), a β-cell toxin known to induce islet injury, but we did not detect evidence for induction of *Pax-2* expression in the pancreas of STZ-treated mice (data not shown). Taken together, these findings suggest that *Pax-2* is not essential for the development of murine islet or gut cell lineages that express the proglucagon gene. Furthermore, the absence of *Pax-2* mRNA transcripts in the rodent pancreas and gut and the comparable levels of pancreatic and intestinal proglucagon mRNA transcripts in +/+ and *Pax2*^{1NEU} mice imply that *Pax-2* is not a transcriptional regulator of pancreatic or intestinal proglucagon gene expression *in vivo*.

DISCUSSION

Members of the *Pax* gene family, characterized by the presence of a 128-amino acid DNA binding paired domain play critical roles in embryonic development and organ formation (38). Although early studies of *Pax* gene biology focused predominantly on the development of mesodermal and neural structures, more recent analyses have identified roles for *Pax* genes in the control of islet cell formation and islet hormone gene expression. Targeted disruption of the *Pax-4* and *Pax-6* genes produces striking defects in formation of

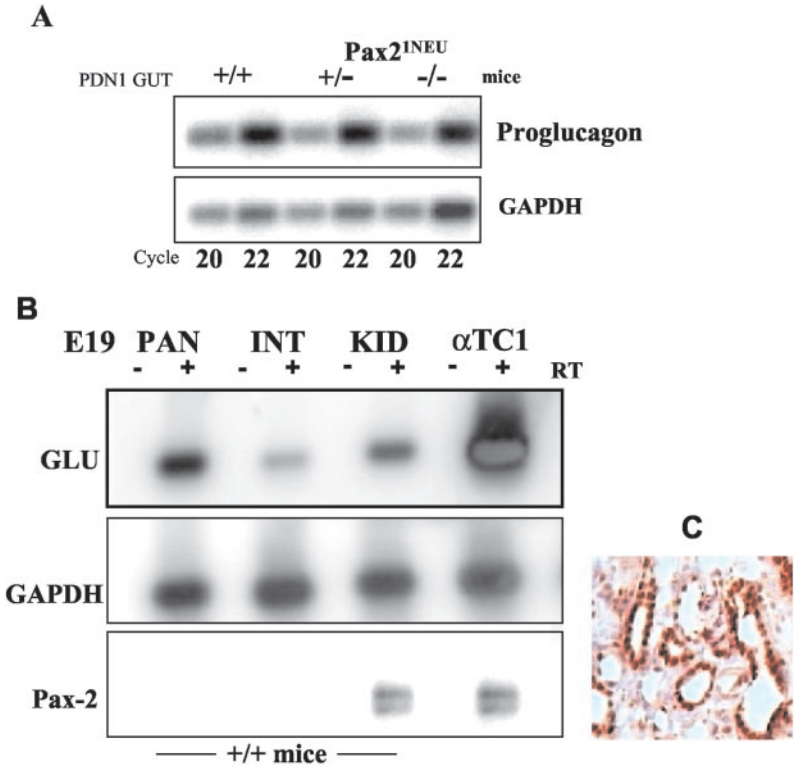

Fig. 6. Proglucagon and *Pax-2* Gene Expression in Wild-Type (+/+) and *Pax2*^{1NEU} (+/− and −/−) Mice

A, RNA isolated from postnatal d 1 intestine (PDN1 GUT) was subjected to PCR using primers specific for proglucagon or GAPDH for either 20 or 22 cycles, respectively, and PCR products were analyzed by Southern blotting using internal oligonucleotides complementary to the proglucagon and GAPDH sequences. B, RNA was isolated from wild-type +/+ E19 murine pancreas (PAN), intestine (INT, small and large bowel), kidney (KID), and αTC1 cells, and analyzed by RT-PCR using primers specific for mouse proglucagon (GLU), GAPDH, and Pax-2. The two PCR products detected for Pax-2 in kidney and αTC1 cells correspond to murine Pax-2a and Pax-2b RNA transcripts as described (26, 36, 65). C, Immunohistochemical detection of immunoreactive Pax-2 in E19 mouse kidney tubules. Magnification, ×400.

pancreatic endocrine cell lineages (18, 23). Whereas *Pax-6* mutant mice exhibit complete loss of glucagon-producing A cells and disorganized islet cell formation, *Pax-4* mutant mice fail to develop islet β- and δ-cells that produce insulin and somatostatin, respectively, and exhibit increased numbers of islet A cells (18, 23, 39). Pax-6 and Pax-4 also regulate islet hormone gene transcription via stimulatory and inhibitory effects on promoter activation, respectively (14–17, 20–22).

In contrast to the demonstrated roles for *Pax* genes in islet development and gene expression, much less is known about the roles of these genes in the formation and differentiated function of intestinal neuroendocrine cells. *Pax-3* promotes the formation of enteric ganglia in part through its regulation of the Hirschprung's disease gene c-RET (40). Mice deficient in Hes-1, the basic helix loop helix transcriptional repressor activated by Notch signaling, exhibit precocious and excessive differentiation of gut endocrine cells in association with enhanced expression of both *Pax-4* and *Pax-6* in the small and large intestine of E18 mutant mice (41). Conversely, the absence of murine *Pax-4* was associated with reduced numbers of endocrine cells in the stomach and duodenum, whereas no significant defects in enteroendocrine cells were observed more distally in the ileum and colon (42). Deletion of the murine *Pax-6* gene resulted in marked reductions in the numbers of antral cells producing gastrin and somatostatin and decreased numbers of glucose-dependent insulinotropic polypeptide-producing cells in the duodenum (42). Furthermore, mutant mice expressing the dominant negative *Pax-6* SEY^{NEU} mutation fail to form glucagon-producing gut endocrine cells in both the small and large intestine (17). Taken together, members of the *Pax* gene family clearly contribute to the control of not only islet, but also enteroendocrine cell development and both pancreatic and intestinal endocrine gene expression (17, 42).

The majority of proglucagon gene transcription factors identified to date have been isolated and characterized using a similar paradigm involving transfection studies of fibroblast and islet cell lines. These experiments have implicated isl-1 (43), the caudal factor cdx-2/3 (16, 44, 45), brain-4 (46), hepatocyte nuclear factor-3 proteins (47), Maf factors (48), helix-loop-helix proteins (49), Pax-6 (14, 15), and Pax-4 (19, 22) as regulators of pancreatic proglucagon gene transcription.

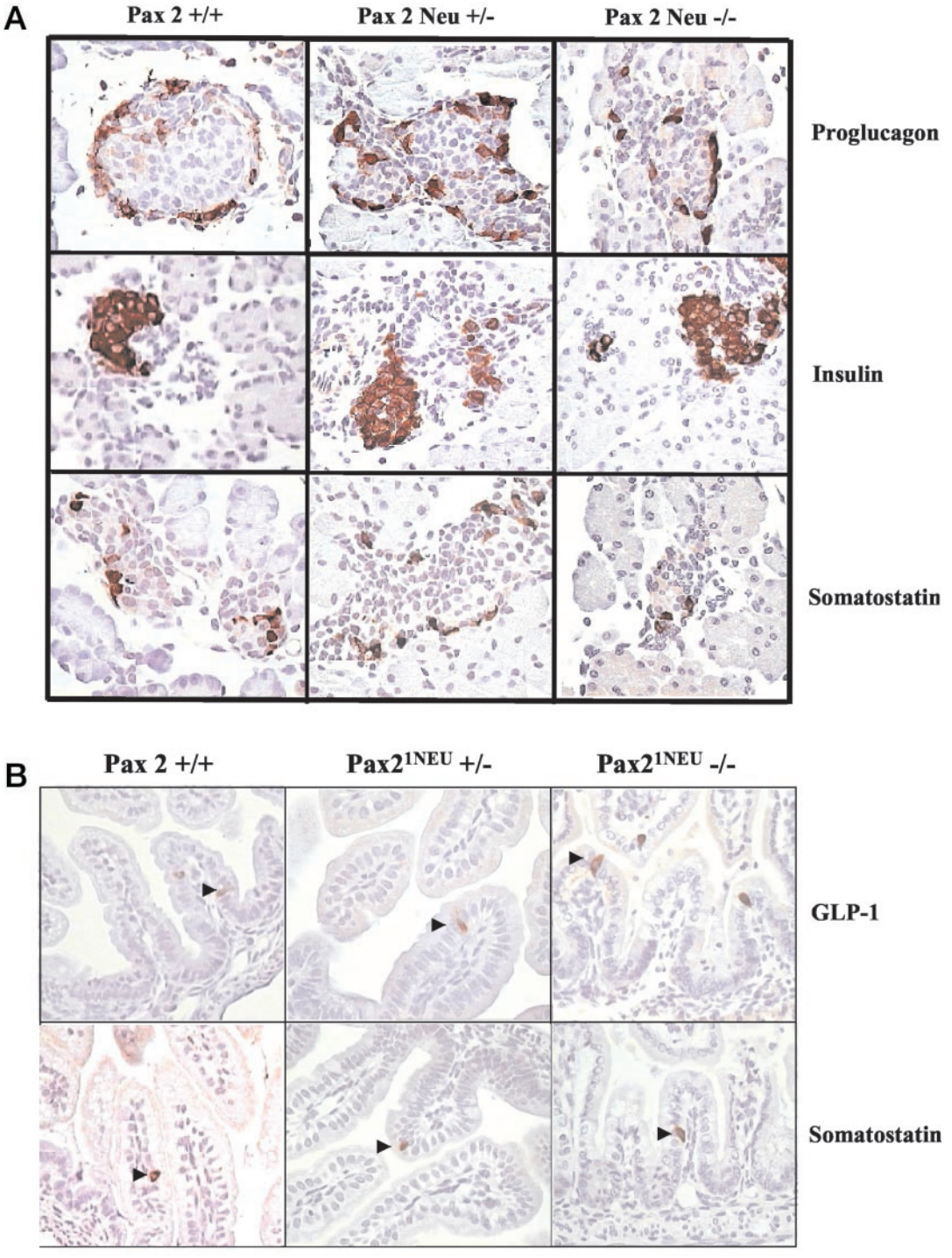

Fig. 7. Histological Analysis of Endocrine Cell Types in Pancreas and Intestine of Wild-Type +/+, +/−, and −/− *Pax2*^{1NEU} Mutant Mice

Histological sections of pancreas (A) were stained with antisera specific for proglucagon, insulin, and somatostatin, whereas sections from murine small bowel (B) were stained with antisera directed against GLP-1 or somatostatin. Tissues were obtained from mice on postnatal d 1 within several hours after birth. *Arrows* denote presence of immunopositive gut endocrine cells. Magnification, ×400 for each panel in A and B.

For some genes such as *Pax-6* (14, 17, 18), complementary evidence, using gain and loss of function strategies in both cells and mice has more firmly established roles for these factors as physiological regulators of proglucagon gene expression. Similarly antisense inhibition of isl-1 expression in an islet α-cell

line reduced levels of endogenous proglucagon mRNA transcripts (43), and islet α-cell formation is defective in mouse embryos with targeted disruption of the isl-1 gene (11). Moreover, hepatocyte nuclear factor-3α (Foxa1) activates the proglucagon promoter, and mice homozygous for a loss of function HNF-3α mutation exhibit reduced levels of pancreatic proglucagon mRNA transcripts (12, 13). In other instances, genes thought to repress proglucagon gene transcription during embryonic development, such as *Pdx-1* (50) and *Pax-4* (19, 21, 22), are not normally expressed in the adult islet α-cell; however, attenuation of Pdx-1 expression *in vivo* leads to increased numbers of islet A cells (51, 52).

Our studies examining the potential importance of *Pax-2* for regulation of intestinal proglucagon gene expression were prompted by the report that *Pax-2* was expressed in rat islets and pancreatic islet cell lines and transactivated the rat proglucagon promoter via the G3 and G1 proximal promoter elements (36). More recent studies have suggested that the Pax-2-dependent activation of proglucagon promoter activity is inhibited by the Pax-2 transactivation domain interacting protein in InR1-G9 cells, but not in fibroblasts, an interaction that may be regulated by the HMG-I/Y-like protein p8 (53). In contrast to data emanating from transfection experiments, no studies have yet identified Pax-2 in a specific differentiated islet endocrine cell lineage, despite the report of *Pax-2* RNA transcripts in RNA from rat islets and islet cell lines by RT-PCR (36). Our inability to detect Pax-2 expression in adult rats or isolated islets, in the fetal E19 or adult mouse pancreas or gastrointestinal tract, or in the injured murine pancreas after STZ administration strongly suggests that Pax-2 is not involved in the control of proglucagon or islet hormone gene expression during late pancreatic development or in the mature animal.

The transient embryonic expression of *Pax-2* in the kidney and nervous system raises the possibility that *Pax-2* might play a transient role in the development of the endocrine pancreas or gut endocrine cell lineages. Because islet A cells and GLP-1-immunopositive gut endocrine cells form normally in the Pax2^{1NEU} mutant mouse, Pax-2 expression is not essential for the developmental formation of these proglucagon-expressing cell lineages in the mouse. Alternatively, the phenotype of reduced or absent *Pax-2* expression in the $+/-$ or $-/-$ *Pax2*^{1NEU} mutant mouse may be hypothetically modified by compensatory up-regulation of related transcription factors such as *Pax-5* or *Pax-6.* Evidence for functional overlap between *Pax-5* and *Pax-2* in other organs derives from their highly similar boundaries of expression and cooperative interactions during development of the mouse central nervous system (54). Expression of a *Pax-5* minigene under the transcriptional control of the Pax-2 locus rescues the majority of defects in midbrain, cerebellum, kidney, genital tract, and eye development that normally occur in Pax-2 mutant embryos (55). Similarly, the expression domains of *Pax-2* and *Pax-6* overlap in ventral zones of the spinal cord, and *Pax-6* is functionally required for correct specification of a subset of *Pax-2*+ interneurons (56). Moreover, studies of eye development in mutant *Pax-6* and *Pax-2* mice reveal reciprocal expansion of expression boundaries and inhibition of promoter activities, indicating interdependent regulation of *Pax-2* and *Pax-6* expression and action during eye development (57). Although the boundaries of *Pax-2* expression during development of the endocrine pancreas remain unknown, the possibility that *Pax-6* may compensate for the absence of Pax-2 expression in the early developing islet merits further consideration.

In summary, although *Pax-2* activates proglucagon promoter activity in transfected cells *in vitro*, its lack of expression in the adult rodent pancreas and intestine rules out a physiological role for *Pax-2* in the control of islet or intestinal proglucagon gene expression. Furthermore, the normal development of islet A cells and gut endocrine L cells in the *Pax2*^{1NEU} mutant mouse indicates that *Pax-2* is not required for the developmental formation and maturation of proglucagon-producing islet or enteroendocrine cell lineages. Whether a functional Pax-2 protein is transiently expressed earlier on in the developing endocrine pancreas and exerts a specific role during islet development or in the control of embryonic proglucagon gene expression remains to be established.

Materials and Methods

Materials

Tissue culture medium, serum, and antibiotics were from Invitrogen Life Technologies, Inc. (Burlington, Ontario, Canada). Chemicals were from Sigma (St. Louis, MO). All electrophoresis reagents were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

Wild-Type and Transgenic Mice

All animal experiments were conducted in accordance with a protocol approved by the Animal Care Committee of the University Health Network. Normal control mice and rats were obtained from Charles River Canada (Toronto, Ontario, Canada). STZ (Sigma; 50 mg/kg body weight, ip injection once daily for 5 d) or vehicle alone was administered as a freshly prepared solution in 0.1 mmol/liter sodium citrate, pH 5.5, to wild-type C57BL/6 mice approximately 8 wk of age, after which mice were euthanized for analysis of pancreatic RNA transcripts within 48 h after the last dose of STZ. For genotyping of Pax2^{1NEU} mice, genomic DNA was extracted from tails using DNeasy tissue kit (QIAGEN, Chatsworth, CA), according to the manufacturer's protocol, and Pax-2 exon 2 sequences were amplified by PCR using DNA from wild-type control or Pax2^{1NEU} mice (37). These mice contain a 1-base insertion in sequences encoding the paired box, resulting in the generation of a prematurely truncated 51-amino acid peptide. The primers used for genotype analysis were: 1Neu sense, 5′-GTGTGA ACCAGCTCGGGGG**T**G-3′; and 1Neu antisense, 5′-GCCCAGGATTTTGCTGACACAGCC-3′. The sense primer has been designed with a point mutation (re-

placing G for T in the original Pax-2 sequence). This change introduces an *Xcm*I restriction site in PCR products generated from the Pax2^{1NEU} mutant allele but not in PCR products generated from wild-type Pax-2 sequence. PCR was performed as follows: 94 C 5 min, 94 C 30 sec, 61 C 45 sec, 72 C 1 min, for 35 cycles, followed by 72 C for 10 min. PCR products were digested with *Xcm*I (New England Biolabs, Inc., Mississauga, Ontario, Canada), and digested DNA fragments were analyzed on a 10% nondenaturing acrylamide gel. The expected products were: a single band of 166 bp for the wild-type allele *vs.* a single band of 152 bp for the Pax2^{1NEU} mutant allele.

Cell Culture and Transfection

Cell lines were maintained in DMEM (4.5 g glucose per liter). BHK fibroblasts and mouse enteroendocrine GLUTag cells were grown in DMEM supplemented with 10% fetal calf serum as described (58). Isolation of rat islets was carried out from male Wistar rats, approximately 250 g, as described (59). Plasmids encoding murine Pax-2a and Pax-2b were a generous gift from G. R. Dressler (University of Michigan, Ann Arbor, MI). The rat proglucagon gene promoter plasmids [−300]GLU-Luc, [−136]GLU-Luc, and [4G3–136]GLU-Luc (17, 44, 60), were transfected alone or with the pCMV *Pax2*-HA plasmid. Transfections were performed in 12-well plates using equal ratios of expression and reporter plasmids to a total of 1 μg of DNA and 2.5 μl of lipofectin (Life Technologies, Inc.) per well, according to the manufacturer's specifications. For control transfections, equal amounts of reporter plasmids and pBlue SK were used to a total of 1 μg DNA. All cells were harvested 48 h after transfection for analysis of luciferase activity as described previously (17, 44, 61). Values for luciferase activity obtained in transfections with proglucagon promoter plasmids and cotransfected transcription factors were normalized relative to the luciferase values obtained after transfection of the identical proglucagon promoter expression plasmid alone. Statistical analysis was performed using *t* test.

EMSAs

Nuclear proteins from GLUTag, InR1-G9, BHK, αTC, and STC-1 cells were prepared as previously described (17, 44, 61). Synthetic oligonucleotides corresponding to specific proglucagon gene promoter G1 and G3 sequences (8, 15) were annealed, labeled with [^{32}-P]ATP using Klenow enzyme, and purified by column chromatography. EMSAs were performed as described (17, 44, 61). For supershift experiments, nuclear extracts were preincubated with anti-Pax-2 antiserum (Zymed Laboratories, Inc., San Francisco, CA) 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.

Western Blot Analyses

Nuclear extracts from GLUTag, BHK, InR1-G9, STC-1, and αTC1 cell lines and from BHK cells transfected with the expression plasmids pCMV*Pax2-a*HA and pCMV*Pax-2b*HA were prepared as described (44), and Western blotting was performed using 40 μg total protein per lane. Blots were probed with anti-Pax-2 antibody that recognizes both Pax-2a and Pax-2b (Zymed Laboratories, Inc.), and with anti-histone (H) antibody that recognizes H1 and core histone proteins H2a, H2b, H3, and H4 (Chemicon, Temecula, CA). Western blotting of nuclear extracts from transfected BHK cells was carried out with anti-HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for analysis of transfected HA-tagged Pax-2 proteins. For analysis of proteins bound to the proglucagon promoter G3 element, nuclear extracts prepared from αTC1, STC-1, GLUTag, and BHK cells were used in EMSA studies with unlabeled annealed G3 oligonucleotide primers. As a control for migration of the G3-protein complexes, protein extracts from αTC1 cells were separately incubated with ^{32}-P-labeled G3 probe and subjected to electrophoresis in adjacent lanes of the same gel. After electrophoresis, the portion of the gel corresponding to the position of the nonradioactive putative G3 complex was transferred onto a Hybond-C membrane (Amersham Pharmacia Biotech, Toronto, Ontario, Canada) for Western analysis using anti-Pax-2 antibody (Zymed Laboratories, Inc.).

RT-PCR

RNA was prepared from cells and tissues using Trizol reagent (Life Technologies, Inc.). First strand cDNA synthesis was generated from total RNA using SuperScript Preamplification System from Life Technologies, Inc. Target cDNA was then amplified using specific oligonucleotide primer pairs by the PCR method. Primers for mouse proglucagon were 5′-TGAAGACCATTTACTTTGTGGCT-3′ and 5′-TGGTGGCAAGATTGTCCAGAAT-3′; primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-TCCACCACCCTGTTGCTGTAG-3′ and 5′-GACCACAGTCCATGACATCACT-3′; primers for Pax-2 were 5′-CAG CCT TTC CAC CCA ACG-3′ and 5′-GTGGCGGTCATAGGCAGC-3′; primers for *Pax-6* were 5′-GCACACGCCCTGGTTGGT-3′ and 5′-CACTGTACGTGTTGGTGAG-3′; primers for *Pax-5* were 5′-CGCAAGAGGGATGAAGGT ATT- 3′ and 5′-AGTATGGGGAGCCAAGCA G-3′. Proglucagon PCR conditions were 94 C 5 min, followed by 94 C 1 min, 66 C 45 sec, 72 C 1 min for 20 or 22 cycles, followed by 72 C for 5 min. *Pax-2*, *Pax-5*, and *Pax-6* PCR conditions were 94 C 5 min, followed by 94 C 1 min, 60 C 1 min, 72 C 1 min, followed by 5 min at 94 C, for a total of 35 cycles for Pax-2, 20 and 22 cycles for Pax-5 and Pax-6. GAPDH PCR conditions were 94 C 2 min, 94 C 1 min, 60 C 2 min, followed by 72 C for 5 min. PCR products were loaded onto a 1% agarose gel, transferred to Nytran Super Charge Nylon Membrane (Mandel Scientific, Guelph, Ontario, Canada), and hybridized using the following internal primers labeled by kinase reaction with γ-^{32}P[ATP] (Amersham Pharmacia Biotech); *Pax-2*, 5′-CAT CGT TCC CAG GGC CTC-3′; *Pax-5*, 5′-GAG AGA CAG CAC TAC TCT GAC-3′; and *Pax-6*, 5′-GGA TGA AGC TCA GAT GCG A-3′. Proglucagon and GAPDH products were hybridized with internal DNA fragments labeled by the Random Primer method with α-^{32}P[dATP].

Histology and Immunocytochemistry

Tissues were processed for immunohistochemistry as previously described (17, 62–64). Formalin-fixed, paraffin-embedded tissue was sectioned at 4 μm for immunohistochemistry using the streptavidin-biotin-peroxidase complex technique. Primary antisera and antibodies were directed against the following antigens and were used at the specified dilutions: insulin 1:40 (monoclonal antibody from BioGenex Laboratories, Inc., San Ramon, CA); GLP-1 1:1500 (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); somatostatin 1:40 further dilution of prediluted aliquot (polyclonal antiserum from DAKO Corp., Carpinteria, CA). The reactions were visualized using 3,3′-diaminobenzidine and hydrogen peroxide. Appropriate positive and negative controls were performed for each antibody, including sections of mouse pancreas for insulin, somatostatin, and GLP-1, and embryonic kidney for Pax-2. The gastrointestinal tract, pancreas, and kidneys were removed from newborn +/+, +/−, and *Pax2*^{1NEU} mice and either fixed in 10% buffered formalin or

frozen (E19) in Optimal Cutting Temperature compound for immunohistochemistry analysis with Pax-2 antibody (Zymed Laboratories, Inc., Markham, Ontario, Canada).

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