

# Essential Requirement for *Pax6* in Control of Enteroendocrine Proglucagon Gene Transcription

Mary E. Hill, Sylvia L. Asa, and Daniel J. Drucker

Department of Medicine (M.F.H., D.J.D.)  
The Banting and Best Diabetes Centre  
Toronto General Hospital  
Toronto, Ontario M5G 2C4, Canada

Department of Pathology (S.L.A.)  
The Mount Sinai Hospital  
University of Toronto  
Toronto, Ontario M5G 2C4, Canada

The primary function of islet A cells is the synthesis and secretion of glucagon, an essential hormonal regulator of glucose homeostasis. The proglucagon gene is also expressed in enteroendocrine L cells of the intestinal epithelium, which produce glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2), regulators of insulin secretion and intestinal growth, respectively. We show here that *Pax6*, a critical determinant of islet cell development and proglucagon gene expression in islet A cells, is also essential for glucagon gene transcription in the small and large intestine. *Pax6* is expressed in enteroendocrine cells, binds to the G1 and G3 elements in the proglucagon promoter, and activates proglucagon gene transcription. The dominant negative *Pax6* allele, *SEY<sup>Neu</sup>*, represses proglucagon gene transcription in enteroendocrine cells. Mice homozygous for the *SEY<sup>Neu</sup>* mutation exhibit markedly reduced levels of proglucagon mRNA transcripts in the small and large intestine, and GLP-1 or GLP-2-immunopositive enteroendocrine cells were not detected in the intestinal mucosa. These findings implicate an essential role for *Pax6* in the development and function of glucagon-producing cells in both pancreatic and intestinal endodermal lineages. (*Molecular Endocrinology* 13: 1474–1486, 1999)

## INTRODUCTION

The islets of Langerhans contain distinct populations of specialized endocrine cells that synthesize and secrete insulin, glucagon, and somatostatin, hormones that regulate metabolic processes and, ultimately, glucose homeostasis. Characterization of the molecular

factors important for islet development and islet hormone gene expression is of significant interest, in that inadequate development, dysfunction, or destruction of islet  $\beta$ -cells produces diabetes mellitus in affected individuals. Isolation and analysis of insulin gene transcription factors have provided considerable information about the control of both insulin gene expression and islet cell development and differentiation, as many transcription factors play dual roles in regulating both insulin gene transcription and formation and organization of differentiated islet cell types in the developing pancreas (1–4).

Given the central importance of the  $\beta$ -cell and its product, insulin, for glucoregulation, the majority of studies of islet transcription factors have focused on the insulin gene. One of the first  $\beta$ -cell transcription factors to be isolated, *isl-1*, does not appear to be essential for control of insulin gene transcription (5); however *isl-1* does activate proglucagon gene transcription, and mice homozygous for a null *isl-1* mutation exhibit defective islet development and A cell formation (6, 7). In contrast, the *Pdx-1* transcription factor is required for insulin gene transcription in the adult  $\beta$ -cell (4) and, remarkably, *Pdx-1*<sup>-/-</sup> mice fail to develop a pancreas (8). Furthermore, human subjects with heterozygous loss of function mutations in *Pdx-1* develop maturity-onset diabetes and, similarly, heterozygous *Pdx-1*<sup>+/-</sup> mice develop glucose intolerance, providing important evidence for the central role of homeobox transcription factors for  $\beta$ -cell function *in vivo* (4, 9).

*Pdx-1* is a comparatively weak transactivator of insulin gene transcription and appears to function synergistically with BETA2/NeuroD, a helix-loop-helix protein that together with E47, binds to an adjacent site in the insulin gene promoter (3). Remarkably, BETA2/NeuroD is also essential for both insulin gene transcription and islet development. Mice with a null mutation in the BETA2/NeuroD gene exhibit severe

abnormalities in islet cell formation and a marked reduction in the number of  $\beta$  cells and rapidly develop diabetes and die in the neonatal period (10). Although *Isl-1*, *Pdx-1*, and *BETA2/NeuroD* were originally isolated as insulin gene transcription factors, analysis of genetic mutations in transcription factors not previously implicated in the control of insulin gene expression has revealed an essential and unexpected role for specific genes in islet development.

Targeted inactivation of the gene encoding the Nkx2.2 homeodomain protein results in mice with arrested  $\beta$ -cell development, leading to diabetes in the postnatal period (11). Mice with disruption of both *Pax4* alleles fail to develop mature  $\beta$ - and  $\delta$ -cells and die within 72 h of birth due to growth retardation and dehydration. Intriguingly, *pax4*<sup>-/-</sup> mice have increased numbers of islet A cells, consistent with a possible block in islet cell differentiation (12). In contrast, islets from mice with a targeted inactivation in the *Pax6* gene contain  $\beta$ - and  $\delta$ -cells but no A cells, suggesting that *Pax6* is essential for the formation of the pancreatic A cell lineage (13). Furthermore, mice with the small eye *SEY*<sup>Neu</sup> *Pax6* mutation also exhibit markedly reduced numbers of islet A cells and decreased levels of pancreatic glucagon (2). Taken together, these observations emphasize the essential importance of islet transcription factor genes for development and maturation of mature islet cells *in vivo*.

In contrast to the restricted expression of the insulin gene in the pancreatic  $\beta$ -cell, the proglucagon gene is expressed in both pancreatic islets and enteroendocrine cells of the intestine. Although abnormalities in islet A cell development and proglucagon gene expression are evident in studies of mice with null mutations in islet transcription factors (2, 7, 13), considerably less information is available about the molecular determinants of enteroendocrine cell development and intestinal proglucagon gene expression *in vivo*. The finding that mice with targeted or naturally occurring *Pax6* mutations develop striking abnormalities in the numbers of islet A cells and decreased pancreatic proglucagon gene expression prompted us to determine whether *Pax6* might also be important for proglucagon gene expression in the intestine. We report here that *Pax6* is an essential transcriptional regulator of proglucagon gene expression in enteroendocrine cells of the small and large bowel.

## RESULTS

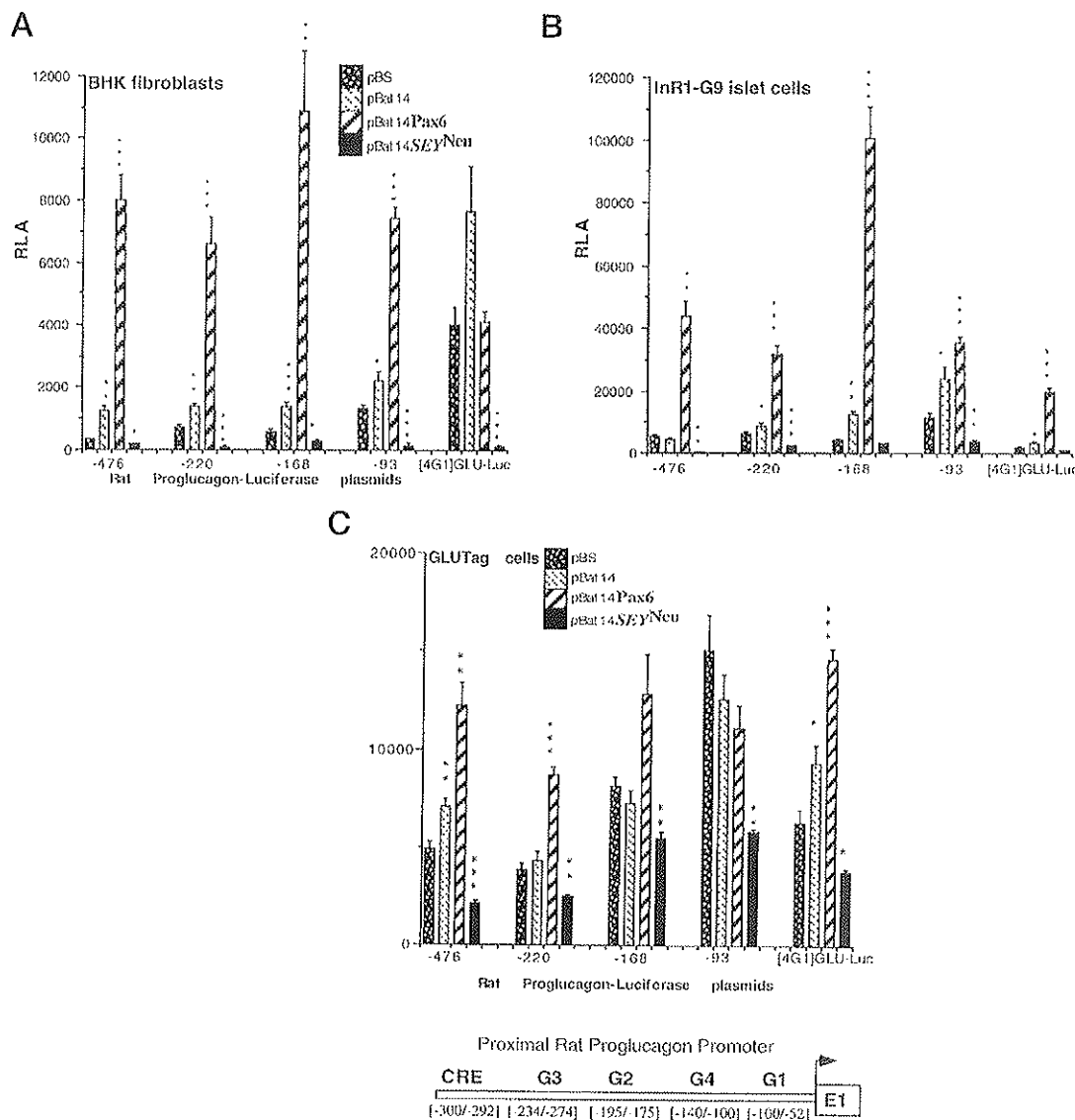
As studies of *Pax6* mutant and wild-type mice localized *Pax6* expression to islet cells, including glucagon-producing A cells (2, 13, 14), we assessed whether *Pax-6* is also expressed in proglucagon-producing intestinal enteroendocrine cells. Northern blot analysis using total and polyA<sup>+</sup> RNA (Fig. 1) detected *Pax6* mRNA transcripts in RNA from the intestinal GLUTag intestinal endocrine cell line and, with a longer expo-



**Fig. 1.** Northern Blot Analysis of Pax-6, Proglucagon, and 18s mRNA Transcripts in Total and polyA<sup>+</sup> RNA from the Enteroendocrine GLUTag and Islet InR1-G9 Cell Lines

The times of exposure were 48 and 12 h for *Pax-6* and glucagon blots, respectively. With a longer exposure (not shown), *Pax-6* mRNA transcripts were detected in the InR1-G9 cell RNA. The relative migration positions of the 28s and 18s RNAs are shown.

sure, InR1-G9 islet cells. After recent studies demonstrating that *Pax6* activates islet proglucagon gene transcription through the proglucagon gene G3 enhancer element (2), we initially assessed the *Pax6*-dependent activation of the proglucagon gene promoter in BHK fibroblasts. A reporter plasmid that contains the G1-G3 proximal rat proglucagon promoter-enhancer elements in their natural genomic orientation (Fig. 2C), [-476]GLU-Luc, was significantly activated by cotransfection with wild-type *Pax6*, but not by the dominant negative *Pax6* mutant allele, *SEY*<sup>Neu</sup>, in BHK fibroblasts (Fig. 2A). Remarkably, deletion of both the G3 and G2 enhancer sequences did not abrogate the *Pax6* activation of proglucagon promoter



**Fig. 2.** Relative Transcriptional Activity of Proglucagon Promoter Luciferase Plasmids in BHK Fibroblasts (A) InR1-G9 Islet Cells (B), and GLUTag Enteroendocrine Cells (C)

The structure of the proximal rat proglucagon promoter and the G1-G4 elements is shown in panel c. GLU-Luc plasmids contained 476, 220, 168, or 93 bp of rat proglucagon gene 5'-flanking sequences (15) fused to the luciferase coding region, or four copies of the G1 proglucagon gene enhancer region ligated upstream of a minimal proglucagon promoter [4G1]GLU-Luc. Relative luciferase activity (RLA) was normalized to the values obtained after transfection of the promoterless expression vector, SK-Luc, in the same experiments. Luciferase reporter plasmids were transfected alone with pBS (Bluescript, Stratagene, La Jolla, CA), with the promoterless expression vector pBAT14 alone, or with either the wild-type *Pax-6* or the dominant negative *Pax-6* *SEY<sup>Neu</sup>* cDNA. All transfections were done in quadruplicate, and the data shown represent the mean  $\pm$  SEM from three different experiments. \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < 0.001$ .

activity, as both [-220]GLU-Luc (lacking G3) and [-168]GLU-Luc (lacking G3 and G2) were activated by *Pax6* (Fig. 2A). Furthermore, deletion of the G3, G2, and G4 enhancer elements did not eliminate *Pax6*-dependent activation of proglucagon promoter activity, as a plasmid containing only G1 sequences, [-93]GLU-Luc, was still activated by *Pax6* (Fig. 2A). These observations, taken together with the presence of a putative *Pax6* binding site at approximately -90 to -60 of the proximal rat proglucagon gene promoter

(15), suggested that the G1 element alone might support *Pax6*-dependent transcriptional activation. These results suggest that the G3 element may not be essential for *pax6*-dependent activation of the proglucagon promoter in fibroblasts.

As studies of islet A cells and pancreatic glucagon biosynthesis in *Pax6* mutant mice infer a major role for *Pax6* in the regulation of pancreatic proglucagon gene expression via the G3 element in islet A cells (2), we assessed the activity of proglucagon promoter-lucif-

erase plasmids, with or without the G3 element, in InR1-G9 islet A cells. In keeping with the data obtained in BHK fibroblasts, *Pax6* activated the proglucagon promoter in InR1-G9 cells, in a G3-independent manner (Fig. 2B). The greatest degree of *Pax6*-dependent proglucagon promoter activation was observed with [-168]GLU-Luc, a reporter that contains the G1 and G4, but not the G2 or G3 enhancer elements. Furthermore, *Pax6* significantly increased the activity of [4G1]GLU-Luc in InR1-G9 cells, and the dominant negative *SEY<sup>Neu</sup>* cDNA markedly repressed the basal activity of [-93]GLU-Luc in islet cells (Fig. 2B). In contrast, the transcriptional activity of a minimal PRL promoter was not inhibited after cotransfection with the *SEY<sup>Neu</sup>* cDNA. These findings clearly demonstrate that *Pax6* activates the proglucagon promoter, not only in transfected BHK cells but also in islet cells, in a G3-independent manner.

To determine whether *Pax6* also regulates proglucagon gene transcription in intestinal endocrine cells, we carried out transfection experiments using the GLUTag enteroendocrine cell line. *Pax6* significantly activated the transcriptional activity of proglucagon promoter plasmids in GLUTag cells (Fig. 2C), demonstrating that *Pax6* is transcriptionally active in intestinal enteroendocrine cells, and not only in islet cell types. Furthermore, the *Pax6*-dependent activation of [-220]GLU-Luc (Fig. 2C) demonstrates that the G3 element is not absolutely required for *Pax6* activation in enteroendocrine cells. Moreover, transfection of the dominant negative *SEY<sup>Neu</sup>* allele significantly inhibited the transcriptional activity of both [-93]GLU-Luc and [4G1]GLU-Luc, consistent with a role for the G1 element in *Pax6*-dependent transcriptional activity in GLUTag enteroendocrine cells.

The finding that *Pax6* activated proglucagon gene transcription via both the G3 (2) and G1 elements prompted us to assess whether *Pax6* was capable of binding both these promoter elements in electrophoretic mobility shift assay (EMSA) studies *in vitro*. After transfection of *Pax6* into BHK cells, a unique DNA-protein complex was detected using the G1 element probe, which was not detected in wild-type BHK cells or cells transfected with the expression vector alone (Fig. 3A, complex G1B). Furthermore, an identically migrating prominent G1B complex was observed in GLUTag nuclear extracts, and a similar, although less abundant complex, was detected using InR1-G9 extract (not shown). Using a G3 element probe containing a previously identified *Pax6* binding site (2), a major DNA-protein complex (G3C) was detected in nuclear extracts prepared from *Pax6*-transfected BHK cells. A similar G3C complex was detected in GLUTag nuclear extracts (Fig. 3A).

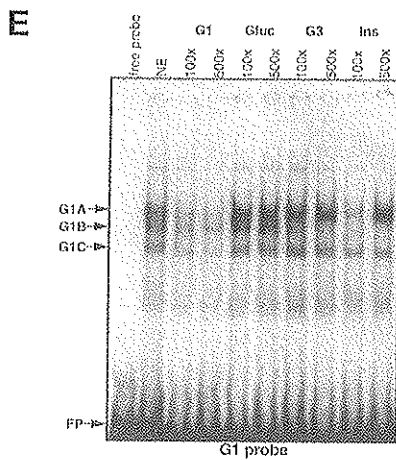
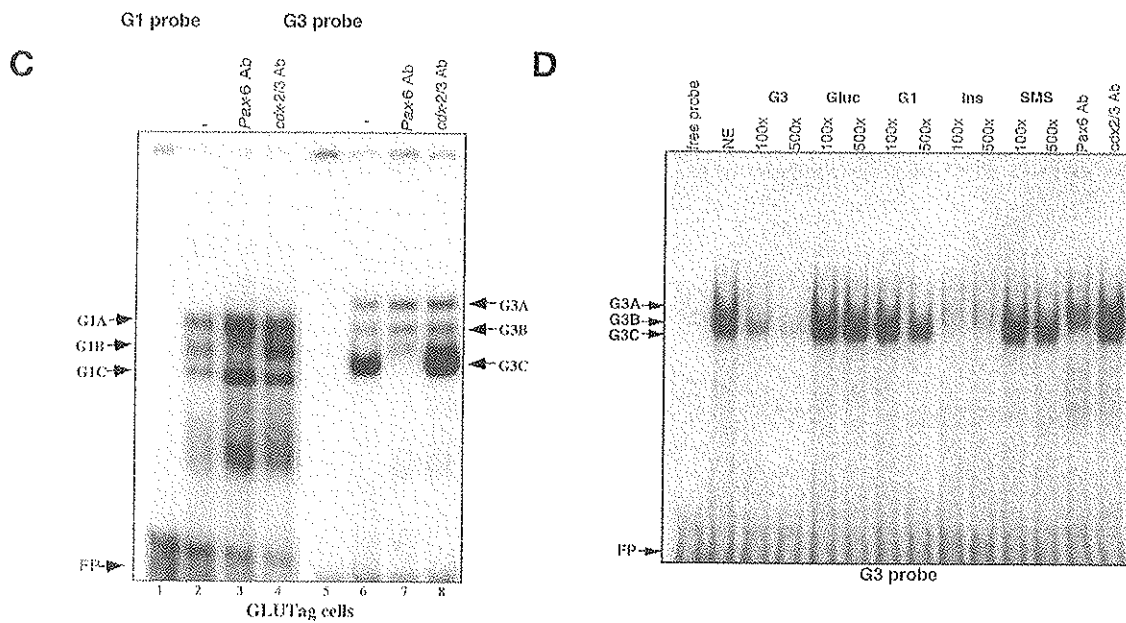
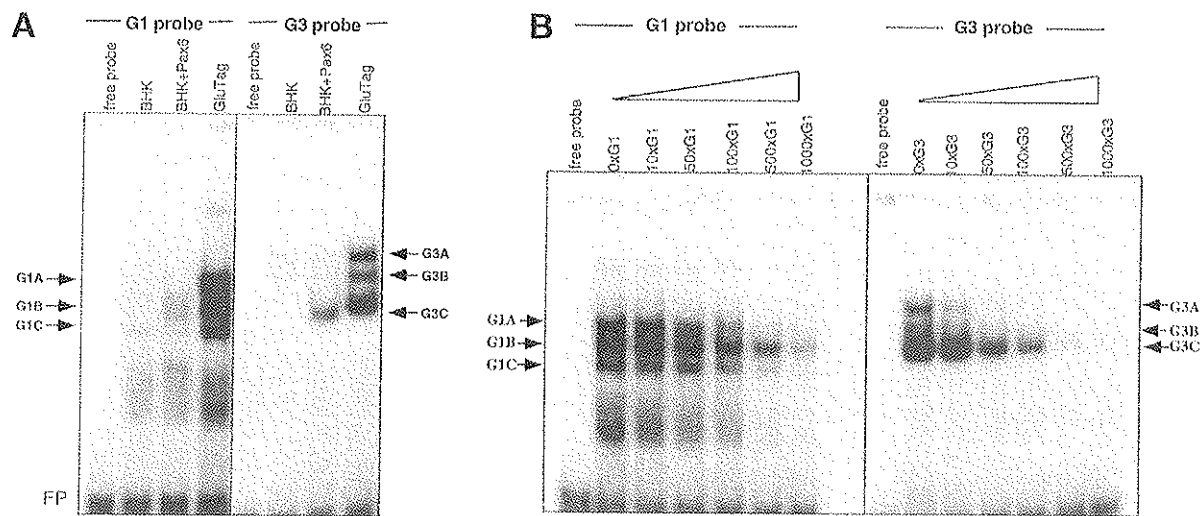
All 3 GLUTag complexes, G1A-C, were diminished after competition for G1 binding with increasing amounts of unlabeled G1 competitor oligonucleotides (Fig. 3B), but no competition was observed with heterologous nonspecific competitor DNA (not shown). Similarly, the three major complexes observed using

GLUTag extracts and the G3 probe, G3A-C, were markedly reduced after competition with increasing amounts of unlabeled G3 sequences (Fig. 3B). Taken together, these experiments strongly suggest that *Pax6* forms a complex with both the G1 and G3 elements in GLUTag enteroendocrine cells.

To provide additional evidence for the presence of *Pax6* in GLUTag DNA-protein complexes detected with either the G1 or G3 probes, EMSA experiments were carried out in the presence or absence of *Pax6* antisera (14). The G1B complex was markedly diminished after preincubation of GLUTag extracts with *Pax6* antisera (Fig. 3C), strongly suggesting that endogenous *Pax6* binds to the proglucagon promoter via the G1 element in enteroendocrine cells. Similarly, the GLUTag G3C complex was almost completely eliminated after preincubation with *Pax6* antisera (Fig. 3C). In contrast, neither the GLUTag G1B nor G3C complexes were diminished after preincubation with antisera against *cdx-2/3* or after incubation with nonimmune sera (not shown).

The finding that *Pax6* binds to both the G3 and G1 elements suggests that *Pax6* recognizes DNA sequences common to both G3 and G1. Furthermore, a core DNA sequence designated the PISCES (pancreatic islet cell-specific enhancer sequence) element has been identified in the 5'-flanking region of the insulin, somatostatin, and glucagon genes (16, 17). To ascertain whether DNA sequences corresponding to putative PISCES elements could compete for the binding to the G3 and G1 elements, a series of EMSA competition experiments were performed (Fig. 3, D and E). An oligonucleotide from the rat glucagon gene (-110/-95) 5'-flanking region just 5' to the boundary of G1 did not compete for complex formation with either the G3 or G1 probes. In contrast, the G1 element modestly diminished the formation of the G3C complex, whereas the insulin gene PISCES element almost completely prevented the formation of the G3C complex (Fig. 3D). Similarly, both the insulin gene PISCES element and the G3 sequence competed for the formation of the G1B complex, whereas the somatostatin element was a comparably ineffective competitor for *Pax6* binding to G3 (Fig. 3D) or G1 (not shown). Taken together, these findings illustrate that the core element common to the G3, G1, and insulin gene PISCES element is capable of competing for *Pax6* binding in GLUTag extracts *in vitro*.

The results of the RNA, transfection, and EMSA experiments demonstrated that *Pax6* is expressed in intestinal GLUTag cells and activates enteroendocrine proglucagon gene transcription via binding to and activation of the G3 and G1 enhancer elements. To explore the relevance of *Pax6* for intestinal proglucagon gene expression *in vivo*, we analyzed enteroendocrine cell populations in mice with a dominant negative *Pax6* *SEY<sup>Neu</sup>* mutation. These mice have previously been shown to exhibit disordered islet formation, markedly reduced numbers of islet A cells, and decreased levels of pancreatic glucagon (2). As *SEY<sup>Neu</sup>* homozygous



mice die shortly after birth, live  $SEY^{Neu} -/-$  mice and both  $+/-$  and  $+/+$  littermates were obtained on neonatal day 1, generally within 1 h after birth, euthanized, and genotyped (Fig. 4A) using PCR and restriction enzyme analysis as described previously (18). We first analyzed proglucagon mRNA transcripts in the small and large bowel of wild-type and mutant mice by RT-PCR. Although proglucagon mRNA transcripts were comparable in RNA from  $+/+$  and  $+/-$  mice, the levels were markedly reduced in both small and large bowel RNA from  $SEY^{Neu} -/-$  mice (Fig. 4B).

We next analyzed the distribution and morphology of endocrine cells in the islets and gut of mice with the homozygous  $SEY^{Neu}$  mutation. Consistent with previously reported findings (2), islets from  $SEY^{Neu}$  mice were small and poorly developed with abnormal distribution of hormone-containing cells (Fig. 5). Cells containing glucagon, although reduced in number, were readily identified (Fig. 5) but did not show the usual pattern of distribution at the periphery of islets as seen in control wild-type animals. Insulin immunoreactivity was reduced and was found most prominently in single cells and small clusters adjacent to ducts rather than in well formed islets (Fig. 5). Somatostatin-containing cells were reduced in number in  $SEY^{Neu}$  mice compared with controls. Staining for pancreatic polypeptide was not markedly different in  $SEY^{Neu}$  and control mice. Cells containing peptide YY (PYY) were numerous in the pancreases of  $SEY^{Neu}$  mice but were not distributed in normal islets as they were in control animals (Fig. 5).

The intestinal morphology of the small and large intestine in neonatal day 1  $SEY^{Neu}$  mice appeared normal, as assessed by conventional light microscopy. However, immunolocalization studies of intestinal endocrine cells showed total absence of glucagon-like peptide 1 (GLP-1) and GLP-2 immunoreactivity in both the small and large bowel (Fig. 6) whereas enteroendocrine cells containing these peptides were readily identified in control  $+/+$  mice (Fig. 6). In contrast to the absence of intestinal endocrine cells expressing GLP-1 or GLP-2, endocrine cells containing immunopositivity for PYY, cholecystokinin, serotonin, and secretin, were found in the usual distribution in the small and large bowel of  $SEY^{Neu}$  mice (Fig. 7). As GLP-2 stimulates growth of the mucosal epithelium in mice (19), we analyzed intestinal morphology of the small bowel in  $-/-$  mice. No significant differences in

small bowel crypt plus villus height were observed after assessment of multiple histological sections of small bowel from  $+/+$  and  $SEY^{Neu}$  mice (data not shown).

## DISCUSSION

Enteroendocrine cells of the small and large bowel exhibit a regional distribution along the small and large bowel that is unique to specific hormonal subtypes (20). As enteroendocrine cells and pancreatic islets are both believed to arise from endodermal origin, it is not surprising that several endocrine genes, including secretin, gastrin, and PYY, are transiently expressed in the fetal endocrine pancreas, after which expression in adult islets is extinguished but is maintained in gastrointestinal endocrine cells throughout adult life (21, 22). Although enteroendocrine cells appear to be derived from a common pluripotential crypt precursor capable of giving rise to multiple differentiated intestinal cell lineages (23), little is known about the molecular determinants of intestinal endocrine cell formation. Furthermore, the hierarchy and complexity of transcription factors that regulate endocrine cell lineage in the gut remain poorly understood.

Analysis of mice with a targeted disruption of the helix-loop-helix transcription factor BETA2 has provided important insights into the genetic control of endocrine cell lineages in the gut (10). The secretin gene, a transcriptional target for BETA2, is transiently expressed in fetal islet  $\beta$ -cells, after which expression in the adult is restricted to enteroendocrine cells (24).  $BETA2 -/-$  mice exhibit striking abnormalities in islet morphogenesis with a severe reduction in mature islet  $\beta$ -cells, and a complete absence of secretin- and cholecystokinin-producing intestinal enteroendocrine cells; in contrast, the numbers of enteroendocrine cells expressing the proglucagon and somatostatin genes were normal despite the lack of BETA2 expression (10). The findings reported here extend our understanding of enteroendocrine cell biology by demonstrating that *Pax6* is essential for expression of the proglucagon gene in the intestinal epithelium. Whether the absence of *Pax6* primarily perturbs endocrine cell formation or, specifically, hormone gene transcription is difficult to determine, as cell lineage markers for the

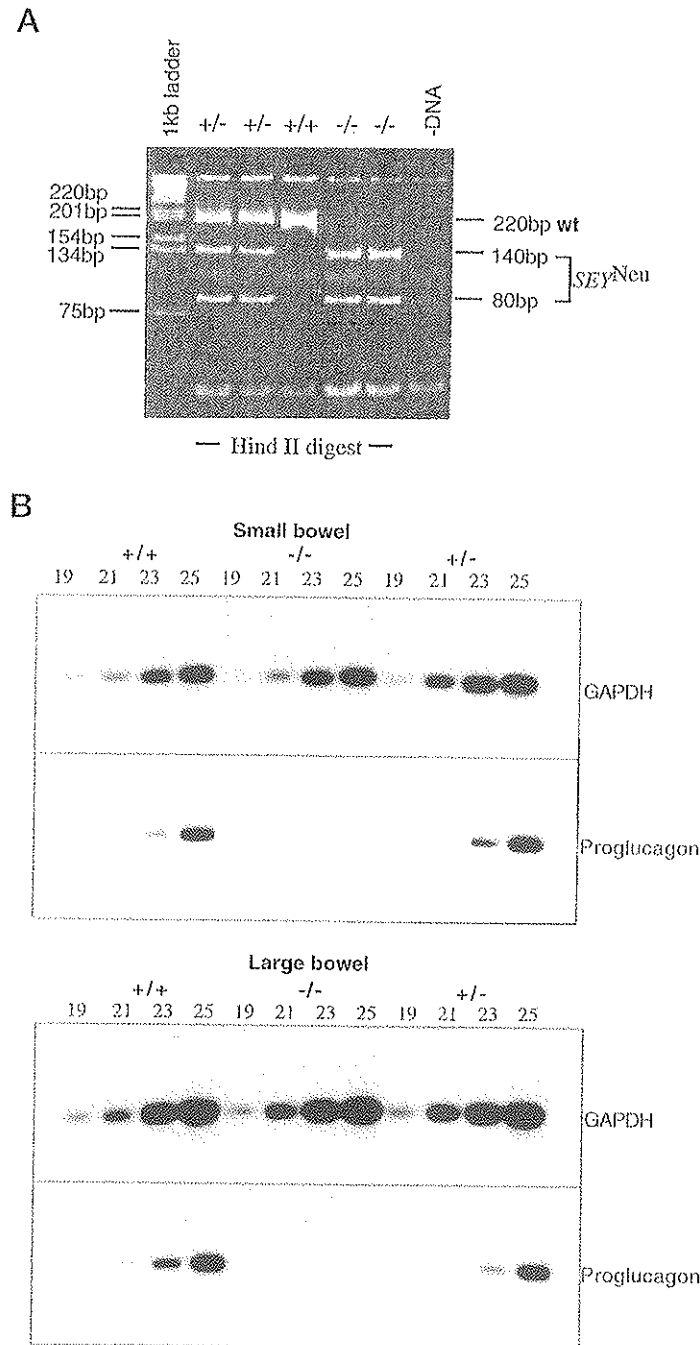
**Fig. 3.** EMSA Experiments with the Proglucagon Gene G1 and G3 Elements

A, Nuclear extracts from wild-type BHK fibroblasts (with or without transfected *Pax-6*) and GLUTag enteroendocrine cells were incubated with either G1 or G3 probes and analyzed as described in *Materials and Methods*. The slightly different migration positions of the G3C complexes are attributable to specific electrophoresis conditions, as evidenced by comparable migration abnormalities of the free probe in the various lanes. Specific complexes are designated by arrows. FP, Free probe. B, Competition for G1 and G3 complex formation in GLUTag cells using increasing (10- to 1000-fold) molar amounts of excess unlabeled homologous competitor oligonucleotide. C, EMSA experiments with *Pax-6* or *cdx-2/3* antisera in GLUTag cell extracts. G1 or G3 probes were incubated with no extract (lanes 1 and 5) nuclear extract only ( $-$ ) or extract plus either *Pax-6* (lanes 3 and 7) or *cdx-2/3* (lanes 4 and 8) antisera. D and E, EMSA experiments using GLUTag extracts and either the G3 (panel D) or G1 (panel E) elements as probes, and the G3, rat proglucagon gene promoter Gluc sequence (-110/-95), G1, insulin (Ins), or somatostatin (SMS) PISCES elements as competitor oligonucleotide sequences. FP, Free probe.

proglucagon enteroendocrine cell population have not been identified. Nevertheless, proglucagon mRNA transcripts were not completely absent from the intestine of  $-/-$  mice, suggesting that  $SEY^{Neu}$  mice contain

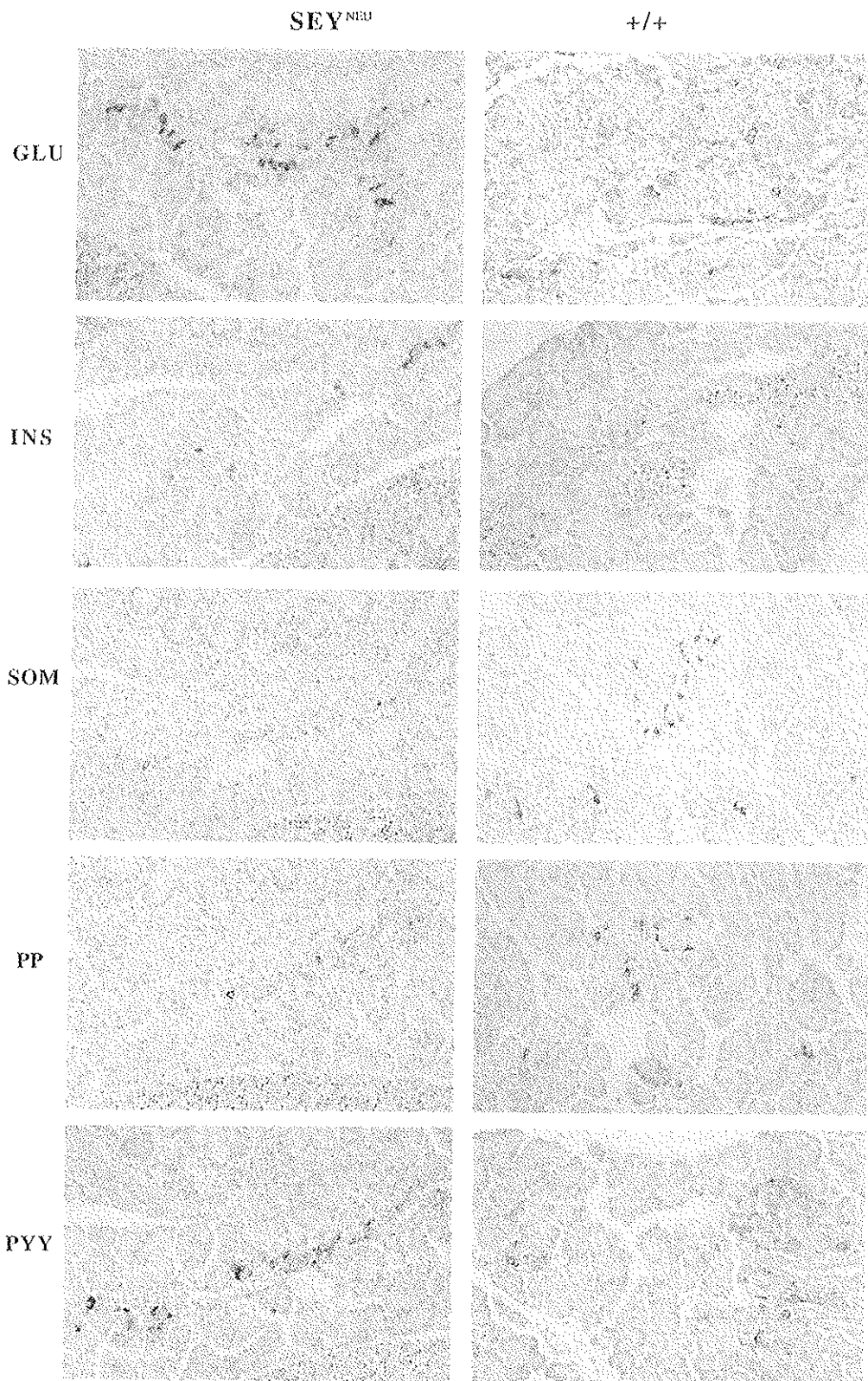
at least a few putative enteroendocrine cells that support proglucagon gene transcription *in vivo*.

The finding that *Pax6* plays an essential role in expression of both pancreatic and intestinal proglucagon



**Fig. 4.** Glucagon Gene Expression in  $SEY^{Neu}$  Mice

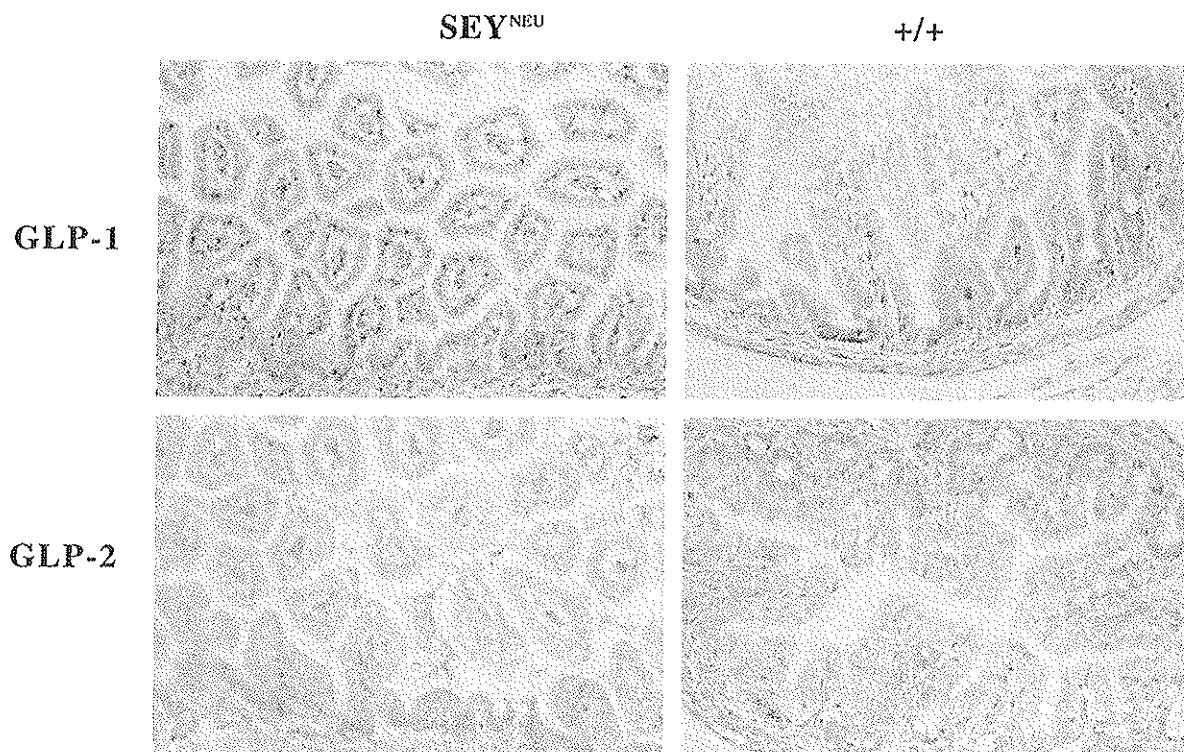
A, PCR analysis of genotyping at the *Pax-6* locus in  $+/+$  wild-type,  $+/-$  heterozygous, and  $-/-$   $SEY^{Neu}$  mice. The  $SEY^{Neu}$  allele contains a unique *Hind*II site that generates 140- and 80-bp fragments after enzymatic digestion. B, RT-PCR analysis of intestinal gene expression in  $+/+$  and  $SEY^{Neu}$  mice. Intestinal RNA from neonatal day 1 age- and sex-matched  $+/+$ ,  $+/-$ , and  $-/-$   $SEY^{Neu}$  littermates. RNA from small and large bowel was reverse transcribed and analyzed by RT-PCR using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and proglucagon, over a range of cycle numbers from 19–25, as described in *Materials and Methods*.



**Fig. 5. Pancreatic Islet Morphology in *+/+* Control and *SEY<sup>Neu</sup>* Mice**

Glucagon immunoreactivity (G) is present in the islets of neonatal day 1 *SEY<sup>Neu</sup>* mice, but the distribution of the immunoreactive cells is distorted compared with the normal peripheral location of glucagon in control (*+/+*) animals. Insulin positivity (I) is reduced in *SEY<sup>Neu</sup>* mice, and it is localized predominantly in scattered individual cells, whereas insulin-containing cells are found in well formed islets in *+/+* controls (d). Somatostatin-containing (S) cells are fewer in the pancreas of *SEY<sup>Neu</sup>* mice than in controls. Staining for pancreatic polypeptide (PP) is not different in *SEY<sup>Neu</sup>* and control mice. Cells containing PYY are numerous in both *SEY<sup>Neu</sup>* and control animals, but the distribution within well formed islets is not seen in *SEY<sup>Neu</sup>* mice.





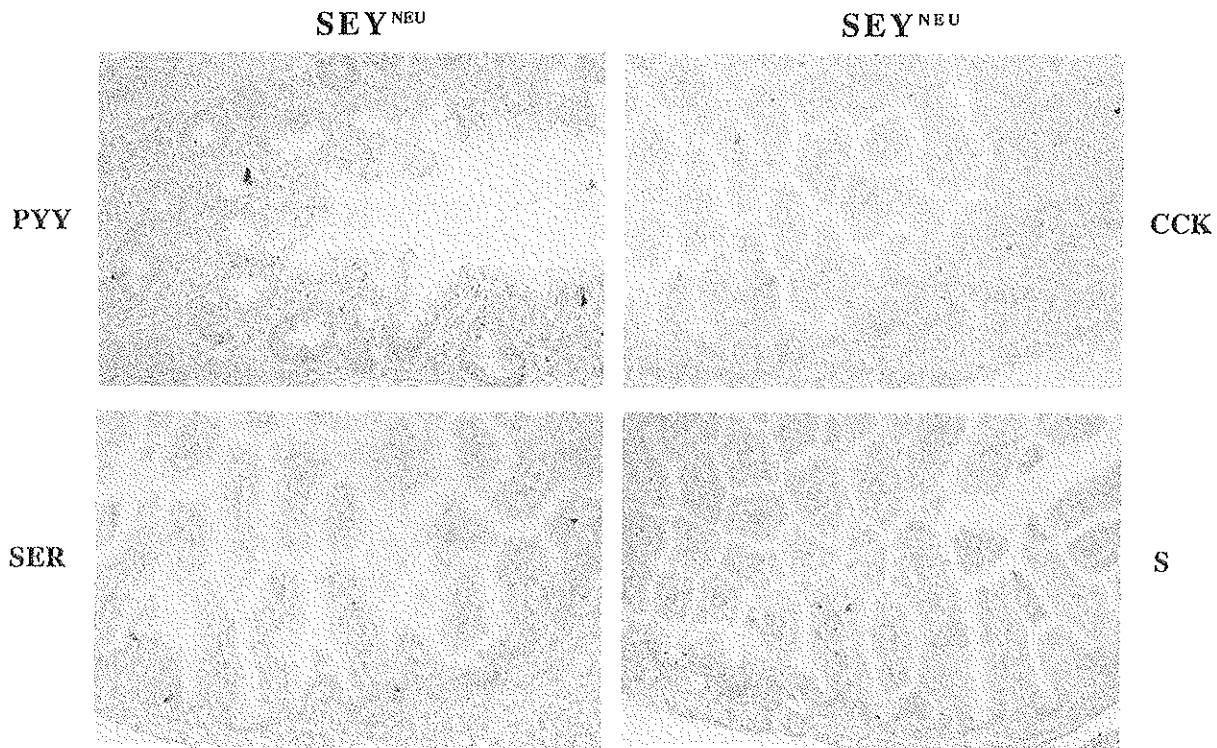
**Fig. 6.** Absence of GLP-1 and GLP-2 immunoreactivity in the Gut of *SEY<sup>Neu</sup>* Mice

GLP-1 immunostaining yields entirely negative results in the bowel of a neonatal day 1 *SEY<sup>Neu</sup>* mouse, whereas a comparable area in the gut of a +/+ control mouse has numerous positive cells. GLP-2 immunostaining is also completely negative in the bowel of a *SEY<sup>Neu</sup>* mouse but several GLP-2-immunoreactive cells are present in the bowel of a +/+ control mouse. No GLP-1- or GLP-2-immunopositive cells were seen in several dozen histological sections from the small and large bowel of three different *SEY<sup>Neu</sup>* mice.

gene expression further supports the existence of shared mechanisms for regulating hormone gene expression in both islet and intestinal endocrine cells. DNA-binding protein(s) from islet cell lines were originally identified that bound to highly similar promoter sequences, designated the PISCES motif, present in the 5'-flanking regions of the insulin, somatostatin, and proglucagon gene promoters (Refs. 16 and 25 and Fig. 8). Multiple DNA-protein complexes have been detected using the glucagon gene G3 element as a probe in EMSA experiments, as shown here and in previous studies (16). Furthermore, the somatostatin and insulin PISCES elements compete for binding with multiple DNA complexes formed by G3 (16). The demonstration that *Pax6* binds to the PISCES element in the insulin, proglucagon, and somatostatin promoters, taken together with the competition for DNA-protein complex formation on G3 using PISCES elements from islet hormone promoters, is consistent with the hypothesis that these elements recognize one or more highly similar, if not identical, transcription factor complexes (16). The major reduction in islet hormone gene expression in *Pax6 SEY<sup>Neu</sup>* mice strongly implicates *Pax6* as a PISCES-binding protein that is functionally essential for transcriptional regulation in the endocrine pancreas (2). Our results extend the functional concept of the PISCES motif to include regulation, via

*Pax6*, of both islet and enteroendocrine gene expression.

*Pax-6* immunoreactive cells have been detected in the mouse fore/midgut endoderm as early as embryonic day 9 (E9.0) (2), and by day E9.5, a few glucagon-positive cells are detected at the time of the appearance of the pancreatic bud that also appear to coexpress *Pax-6*. At E15.5, numerous cells in the endocrine pancreas express *Pax-6* and either glucagon, insulin, somatostatin, or pancreatic polypeptide. The ontogeny of *Pax6* expression in the developing gut has not been reported. The murine intestinal epithelium undergoes a complex pattern of cytodifferentiation beginning at approximately E15, and distinct enteroendocrine cell populations are detectable in the small intestine by E17 (26). Similarly, endocrine cells are first detected in the mouse colon by E15.5, with the earliest cell type detected expressing PYY, followed by the appearance of additional hormonal phenotypes between E17.5 and E18.5 (27). Glucagon-immunopositive cells are infrequent before E19 in the developing small bowel, but detectable by E16.5 in the murine colon (27). Whether intestinal *Pax6* expression is exclusively localized to the glucagon-immunopositive enteroendocrine cell lineage in the developing and adult gut remains to be determined.



**Fig. 7.** Localization of Enteroendocrine Cells in the Gut of  $SEY^{Neu}$  Mice

In the bowel of neonatal day 1  $SEY^{Neu}$  mice, enteroendocrine cells are detected that exhibit immunoreactivity for PYY, cholecystokinin (CCK), serotonin (SER), and secretin (S) in a distribution that is unchanged from control  $+/+$  mice (not shown).

As the levels of proglucagon mRNA transcripts were markedly reduced in  $SEY^{Neu}$  mice, these mutant mice provide an opportunity to assess the importance of the proglucagon-derived peptides for fetal growth and development. Although intestinal-derived GLP-2 has been recently identified as a potent intestinal growth factor in adult mice (19), the role, if any, of GLP-2 (or GLP-1) in the development of the fetal gut is not known. The observation that the small and large bowel appear histologically normal in  $SEY^{Neu}$  mice despite the marked reduction in intestinal proglucagon gene expression (and hence GLP-2 biosynthesis) suggests that normal levels of GLP-2 are not essential for growth and differentiation of the intestinal mucosal epithelium during fetal development *in vivo*. In summary, the results presented here demonstrate that a single transcription factor, *Pax6*, is essential for both islet cell formation and islet and enteroendocrine gene transcription, via interaction with a DNA element common to the promoters of genes expressed in the endocrine cells of the pancreas and gut.

## MATERIALS AND METHODS

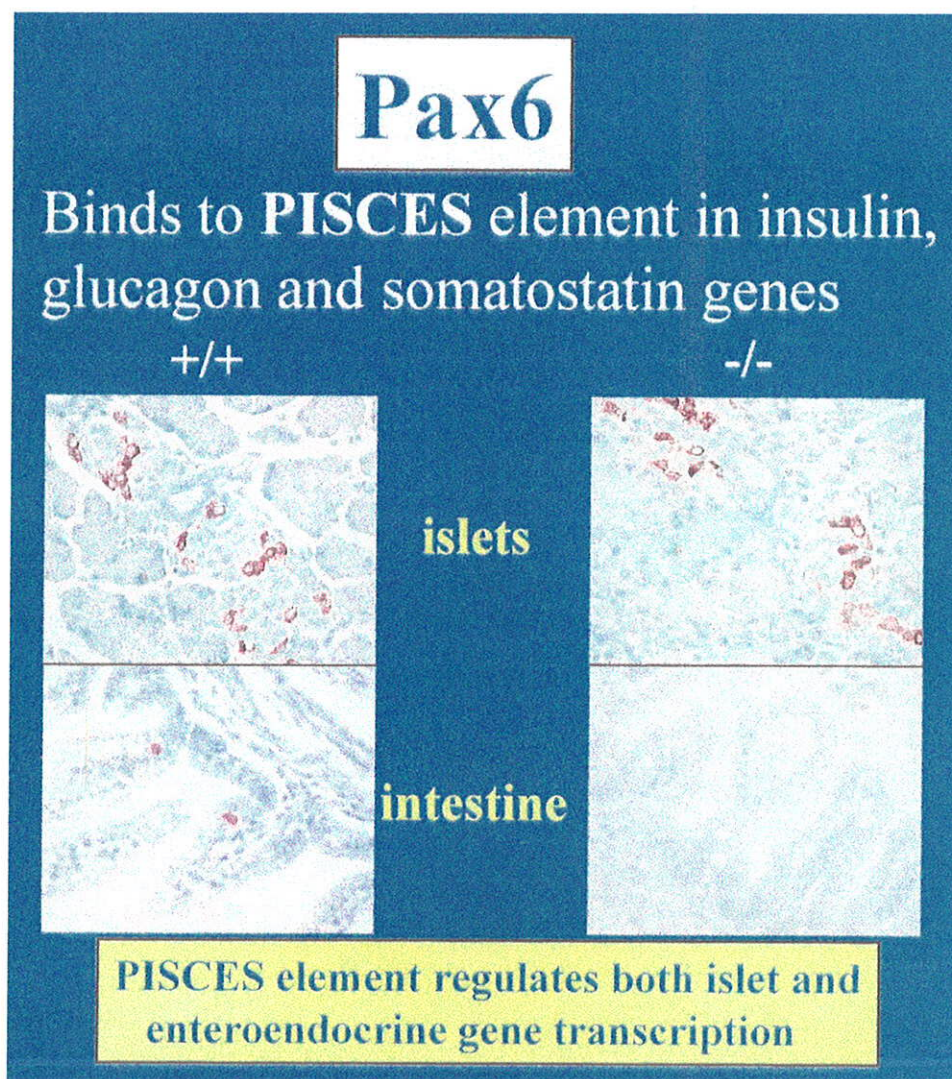
### Plasmids

The plasmids pBat14, pBat14.mPax6, and pBat14.mPax6  $SEY^{Neu}$  were kindly provided by M. S. German (University of

California, San Francisco, CA). These plasmids are eukaryotic expression vectors under the control of the cytomegalovirus (CMV) promoter (2). The 5'-deleted rat proglucagon gene promoter sequences, subcloned in the promoterless plasmid SK-Luc immediately adjacent to the coding sequence of the firefly luciferase reporter gene, have been previously described (28, 29). Synthetic oligonucleotides corresponding to specific rat proglucagon gene G1 sequences (15) were annealed, separated by agarose gel electrophoresis, gel purified, and ligated into the [-82]GLU-Luc plasmid (28). This new plasmid was designated [4G1]GLU-Luc and contained four copies of the G1 sequence in a 5'-3' orientation adjacent to the -82 to +58 fragment of the proximal rat proglucagon promoter. The plasmid [-36]PRL-Luc was a kind gift from H. P. Eisholtz (University of Toronto, Toronto, Ontario, Canada). The CMV promoter plasmid, CMV-Luc, and the promoterless plasmid, SK-Luc, were used as positive and negative controls, respectively, in each transfection experiment.

### Cell Culture and Transfections

All cell culture reagents used were obtained from Life Technologies/Gibco BRL (Toronto, Ontario, Canada). Cell lines were maintained in DMEM (4.5 g glucose/liter). Baby hamster kidney (BHK) fibroblasts and InR1-G9 islet cells (30) were grown in DMEM supplemented with 5% calf serum; mouse enteroendocrine GLUTag cells (31) were grown in DMEM supplemented with 10% FCS. BHK cells and InR1-G9 cells were transfected by the calcium phosphate precipitation method with 5  $\mu$ g reporter plasmid DNA + 5  $\mu$ g expression plasmid DNA per 60-mm dish. GLUTag cells were electroporated with 10  $\mu$ g reporter plasmid + 10  $\mu$ g expression plasmid. All cells were harvested 16-20 h after transfection for analysis of luciferase activity as described previously (28, 32), and values were normalized relative to the background



**Fig. 8.** GLP-1-Immunopositive Cells in the Islets and Intestine of a Newborn Wild-Type (+/+) and *SEY<sup>Neu</sup>* (-/-) Mouse  
The organization and number of GLP-1-immunopositive islet cells is abnormal, and GLP-1- or GLP-2-immunopositive intestinal endocrine cells are not detected in the *SEY<sup>Neu</sup>* mouse.

luciferase activity obtained after transfection of SK-Luc in the same experiment. Statistical analysis was performed using Student's *t* test.

#### RNA Analysis

RNA was prepared from cells and tissue by the acid ethanol precipitation method previously described (33). Poly A+ RNA was isolated using a Qiagen Poly A Kit (Mississauga, Ontario, Canada). Electrophoresis of RNA, transfer to nylon membrane, hybridization, and washing were carried out as previously described (34). For RT-PCR, first-strand cDNA synthesis was generated from total RNA using the SuperScript Pre-amplification System from Life Technologies. Target cDNA was then amplified using specific oligonucleotide primer pairs by the PCR method. Primers for mouse proglucagon were 5'-TGAAGACCATT-TACTTTGTGGCT-3' and 5'-CTGGTGGCAAGATTGTCCA-GAAT-3'; primers for glyceraldehyde-3-phosphate dehydrogenase were 5'TCCACCACCCTGTTGCTGTAG-3' and

5'-GACCACAGTCCATGACATCACT-3'. Reactions were denatured at 94 C for 1 min and then annealed at 66 C for 45 sec, followed by extension at 72 C for 1 min for 19–25 cycles.

#### Mice and Genotyping

Small eye *SEY<sup>Neu</sup>* +/- mice were kindly provided by Brigid Hogan (Vanderbilt University, Nashville, TN) and maintained in the animal facility of the Toronto Hospital. *SEY<sup>Neu</sup>* +/- were mated to generate homozygous, heterozygous, and wild-type offspring, which were distinguished by PCR and restriction enzyme analysis of the amplification product as previously described (18).

#### EMSAs

Nuclear proteins from GLUTag, InR1-G9, and BHK cells were prepared as previously described (28, 32). Synthetic oligonucleotides corresponding to specific proglucagon gene G1 and G3 sequences (15) were annealed, labeled with [<sup>32</sup>P]ATP

using Klenow enzyme, and purified in a G50 spin column. EMSAs were performed as described previously (28, 32). For supershift experiments, nuclear extracts were preincubated with 1:10 diluted anti-Pax6 antiserum (a generous gift from Dr. S. Saule, Lille, France) or nonimmune antisera at 4°C for 30 min before addition of <sup>32</sup>P-labeled DNA probe and subsequent incubation at 30°C for 30 min. All reaction mixes were loaded onto a 5% nondenaturing polyacrylamide gel, and after electrophoresis, the gel was exposed to x-ray film for 24–48 h. Oligonucleotide competitor sequences from the rat insulin and glucagon gene PISCES elements were synthesized as per Fig. 4A in Ref. 16.

### Histology and Immunocytochemistry

Tissues were processed for immunohistochemistry and/or intestinal morphometry as previously described (35, 36). 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 (monoclonal antibody from BioGenex Laboratories, Inc., San Ramon, CA) 1:40 for 30 min; glucagon (polyclonal antiserum from Immunon, Pittsburg, PA) 1:200 for 30 min; GLP-1 (polyclonal antiserum prepared by D. J. Drucker) 1:1500 for 30 min; GLP-2 (polyclonal antiserum prepared by D. J. Drucker) 1:2500 for 30 min; PYY (polyclonal antiserum from Peninsula Laboratories Inc., Belmont CA) 1:2000 for 30 min; somatostatin (polyclonal antiserum from DAKO Corp., Carpinteria, CA) prediluted preparation further diluted 1:40 overnight; pancreatic polypeptide (polyclonal antiserum from DAKO Corp.) 1:6000 for 30 min; cholecystokinin (polyclonal antiserum from Serotec, Oxford, U.K.) 1:1000 for 30 min; serotonin (monoclonal antibody from DAKO Corp.) 1:50 for 60 min; secretin (polyclonal antiserum from BioGenex Laboratories, Inc., San Ramon, CA) prediluted preparation further diluted 1:5 overnight; gastrin (polyclonal antiserum from Immunostain, Los Angeles, CA) prediluted preparation further diluted 1:20 overnight; vasoactive intestinal peptide (polyclonal antiserum from Zymed Laboratories, Inc., San Francisco, CA) prediluted preparation further diluted 1:10 overnight. The reactions were visualized using 3,3'-diaminobenzidine and hydrogen peroxide. Appropriate positive and negative controls were performed in each case. The gastrointestinal tract from newborn +/+ , +/- and *SEY*<sup>Nb</sup> -/- mice was removed and fixed in 10% buffered formalin. Intestinal micrometry was performed with the use of a Leitz (Westar, Germany) microscope with a video camera connected to a computer monitor. The microscope was calibrated at ×10 magnification, and crypt plus villus height in the small bowel was measured by examining at least 10 longitudinally oriented villi from each animal and is expressed in micrometers (mean ± SEM).

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Address requests for reprints to: Dr. D. Drucker, The Toronto Hospital, 200 Elizabeth Street CCRW3-838, Toronto, Ontario M5G 2C4, Canada. E-mail: d.drucker@utoronto.ca.

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