Pax-6 Activates Endogenous Proglucagon Gene Expression in the Rodent Gastrointestinal Epithelium

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The proglucagon gene encodes pancreatic glucagon and the glucagon-like peptides, which exert diverse effects on nutrient absorption and assimilation. The therapeutic potential of glucagon-like peptide-1 (GLP-1) has fostered interest in development of cellular engineering approaches to augment endogenous intestinal-derived GLP-1 for the treatment of type 2 diabetes. We have used adenovirus technology to examine the potential roles of the transcription factors Cdx-2/3 and Pax-6 as activators of endogenous proglucagon gene expression in enteroendocrine cell lines and in nontransformed rat intestinal cells. Adenoviral-expressed Cdx-2/3 and Pax-6 activated proglucagon promoter-luciferase activity in baby hamster kidney (BHK) fibroblasts, HEK 293 cells, and enteroendocrine cell lines. Pax-6, but not Cdx-2/3, induced expression of the endogenous proglucagon gene in enteroendocrine cell lines, but not in heterologous fibroblasts. Furthermore, transduction of primary rat intestinal cell cultures in vitro, or the rat colonic epithelium in vivo, with Ad-Pax-6 activated endogenous proglucagon gene expression. These data demonstrate that Pax-6, but not Cdx-2/3, is capable of activating the endogenous proglucagon gene in both immortalized enteroendocrine cells and the nontransformed intestinal epithelium in vivo. *Diabetes* 52: 425–433, 2003

Glucagon and the glucagon-like peptides (GLPs) are encoded by a single proglucagon gene in mammals. Post-translational processing of proglucagon results in the formation of glucagon in the pancreas and GLP-1 and GLP-2 in the intestine (1). Pancreatic glucagon is a key counter-regulatory hormone that opposes the actions of insulin in regulating hepatic glucose production and maintaining physiological levels of plasma glucose. Gut-derived GLP-1 and GLP-2 are secreted into the circulation by enteroendocrine L-cells following nutrient ingestion. GLP-1 regulates glucose homeostasis by stimulating insulin and inhibiting glucagon secretion. GLP-1 also inhibits food intake, delays gastric emptying, and stimulates islet proliferation, differentiation, and neogenesis (2–5). In contrast, GLP-2 maintains the integrity of the intestinal epithelium via effects on intestinal cell proliferation and apoptosis (6).

The pleiotropic effects of the GLPs have fostered interest in their potential use in the treatment of disorders such as obesity, diabetes, and intestinal failure (2,7). GLP-1 and its analogs effectively lower blood glucose in short and long-term studies of diabetic rodents (6). More recent studies have shown that GLP-1 agonists also exert beneficial effects in the treatment of human subjects with diabetes (2,8,9). Hence, understanding the molecular regulation of intestinal proglucagon gene expression may facilitate development of strategies directed at enhancing endogenous production of GLP-1 in vivo.

Our current understanding of the molecular control of proglucagon gene expression is mainly limited to information derived from studies of immortalized pancreatic α-cell lines (10–16). Much less is known about the factors regulating intestinal proglucagon expression. Two of the transcription factors identified in studies of islet proglucagon gene expression—the caudal factor Cdx-2/3 and the paired domain transcription factor Pax-6—have been shown to activate the transfected proglucagon gene promoter in both islet and intestinal cell lines (17–19). As targeted inactivation of the murine Cdx-2/3 gene results in embryonic lethality (20), whether Cdx-2/3 is essential for proglucagon gene transcription remains unclear. In contrast, Pax-$6^{-/-}$ mice exhibit reduced numbers of islet α cells, and mice homozygous for the dominant negative Pax-6 Sey^{neu} mutation exhibited markedly decreased levels of proglucagon mRNA transcripts and immunoreactive proglucagon-derived peptides (PGDPs) in the small and large intestine (19,21). However, these studies do not permit a determination of whether Pax-6 plays a direct role in controlling intestinal proglucagon gene expression or, alternatively, is primarily responsible for specifying enteroendocrine cell development. To investigate the roles of Pax-6 and Cdx-2/3 in the control of intestinal proglucagon gene transcription and GLP-1 biosynthesis, we have now utilized adenoviral vectors for analysis of transcription factor expression in enteroendocrine cell lines and in the normal nontransformed intestinal epithelium. Our data show that Pax-6, but not Cdx-2/3, is capable of activating proglucagon gene expression in the nontransformed intestinal epithelium.

RESEARCH DESIGN AND METHODS

Cell culture. The mouse small bowel– derived intestinal cell line, STC-1 cells (22), were grown in Dulbecco's modified Eagle's medium (DMEM) containing

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DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; GLP, glucagon-like peptide; MOI, multiplicity of infection; PFU, plaque-forming unit; PGDP, proglucagon-derived peptide; RT+, presence of reverse transcriptase; RT−, absence of reverse transcriptase; SI, sucrase isomaltase.

10% horse serum and 2.5% FBS. GLUTag cells, derived from a murine large intestinal glucagonoma, were cultured in DMEM containing 10% FBS as described previously (23). Caco-2 intestinal cells and HEK 293 and baby hamster kidney fibroblast (BHK) cell lines were obtained from American Type Culture Collection (ATCC, Gaithersburg, MD). Cells were trypsinized, distributed into 6-cm plates, and grown to ~90% confluence before transfection or adenoviral transduction. Intestinal epithelial cells were isolated from embryonic day 19–20 fetal rats and cultured in DMEM containing 5% FBS as described (24). Primary intestinal cell cultures were incubated at 37°C under 5% CO_2 and 95% O_2 atmosphere for 24 h before viral transduction studies. Briefly, cell lines or primary cells were incubated with adenoviruses for 1 h and then washed and incubated for an additional 24 h in a culture medium containing 5% FBS before harvesting for RNA or protein analysis.

Preparation of recombinant adenoviruses. cDNAs encoding for wild-type hamster Cdx-2/3, the dominant negative Cdx-2/3 (Cdx-2/3-HD) encoding the homeodomain and COOH-terminal peptide (180–313 amino acids) (30), and mouse Pax-6 and Sey^{neu} genes (kind gifts from Dr. M.S. German, University of California, San Francisco, CA) were subcloned into pACCMV-pLpA vector and cotransformed with pJM17 plasmid into 293 cells (25). Successful homologous recombination between these two plasmids resulted in recombinant viruses encoding the specific transcription factors (Ad-Pax-6, Ad- Sey^{neu}, and Ad-Cdx-2/3, Ad-Cdx-2/3-HD) and the control Ad-βgal. After plaque purification, viruses were amplified in 293 cells and purified by CsCl gradient centrifugation to enrich the viral stocks as previously described (25). Plaque-forming units (PFUs) were assayed using the Adeno-X rapid titer kit (Clontech, Palo Alto, CA). Adenoviral titrations were 2.2×10^{10} 1 PFU/ml and 2.5×10^{10} PFU/ml for Ad-Pax-6 and Ad-Cdx-2/3, respectively. Similar viral titers were obtained for Ad-βgal (2.0×10^{10} PFU/ml) and Ad-Sey^{neu} (2.1×10^{10} PFU/ml). In vitro transductions of viruses into STC, GLUTag, 293, or Caco-2 cell lines were completed after 1-h incubation at 37°C, whereas in BHK cells the transduction time was extended to 2 h.

Northern blotting. Total RNA was isolated by the Trizol method (Life Technologies, Gaithersburg, MD) from STC-1, GLUTag, primary intestinal cell cultures, or rat intestine. RNA was electrophoresed on a 1.2% formaldehyde agarose gel, transferred to Hybond-N plus, and hybridized to ^{32}P-labeled glucagon cDNA probes as described (26,27).

RT-PCR. Semiquantitative RT-PCR was used to determine expression levels of Cdx-2/3, Pax-6, and proglucagon according to the protocol from Clontech (Palo Alto, CA). The following primers were used for PCR amplification: 5′-AAGAGTGGCGACTCCAGAAGTTG-3′ and 5′-ACCACACCTGTATCCTTGCTTCAG-3′ for Pax-6; 5′-AGCTTCAGTCCCACAAGGCAGAAT-3′ and 5′-GGCCGCAGGAGATGTTGTGAAGAT-3′ for proglucagon; 5′-ATGTACGTGAGCTACCTTCTGGAC-3′ and 5′-GGAGCCACCATTGAGGCCGTGGGC-3′ for Cdx-2/3; 5′-CATGACCACAGTCCATGCCATC-3′ and 5′-CACCCTGTTGCTGTAGCCATATTC-3′ for the control transcript GAPDH. A forward primer 5′-GTGTGTACTTGTGGAGCACTGCCC-3′ priming within intron-B sequences of mouse proglucagon gene and a reverse primer 5′-CGGTTCCTCTTGGTGTTCATCAACC-3′ locating in the exon-3 were used in RT-PCR to quantitate newly synthesized proglucagon primary RNA transcripts in GLUTag cells transduced with Ad-βgal or Ad-Cdx-2/3-HD. For RT-PCR experiments shown in Fig. 5, specific primers were designed to amplify cDNA fragments of 297 and 545 bp for Cdx-2/3 and Pax-6, respectively. Semiquantitative measurements of newly synthesized proglucagon primary RNA were obtained by RT-PCR studies. Total RNA (2.5 μg) was digested with 10 units of RNase-free DNase at 37°C for 1 h. After heat inactivation of DNase at 95°C for 10 min, RNA was reverse transcribed with random hexamers in the presence of reverse transcriptase (RT+) or absence of reverse transcriptase (RT−). The resultant cDNA was amplified using Tag polymerase (44).

Electrophoretic mobility shift assays. After adenoviral transduction, STC-1 or GLUTag cells were grown for an additional 24 h and nuclear proteins were isolated as described (17). Synthetic oligonucleotides corresponding to rat proglucagon promoter G1 or G3 sequences (10) were end-labeled with ^{32}P-ATP by T4 kinase, purified by G25 spin column (Pharmacia, Montreal, PQ), and incubated with 2–4 μg nuclear proteins. The reaction mixes were loaded onto a 5% nondenaturing polyacrylamide gel, and, after electrophoresis, the gel was exposed to X-ray film for 16–24 h.

In vivo infusion of Ad-Pax-6 and Ad-βgal into rat colon. A solution of $\sim 1 \times 10^{12}$ virions of either Ad-Pax-6 or Ad-βgal was infused into the large colon of Wistar rats via a modified rectal approach developed in our laboratory. Briefly, animals were fasted for 48 and 16 h before viral infusion, and colonic fecal material was completely cleansed by feeding animals with GoLytely solution (Baxter, Mississauga, ON), followed by three additional bowel irrigations with PBS buffer. To disrupt intestinal mucous, a mucolytic agent, acetylcysteine, was introduced into the rectum and maintained for 30 min before viral infusion. After being mildly sedated with halothane, animals were infused with a viral solution via the rectum by using a 10-cm modified gavage and were kept in the Trendelenburg position for at least 30 min to enhance transduction of adenoviruses into the large intestine. Because the rapid turnover of intestinal epithelium may result in diminished Pax-6 expression in newly formed epithelial cells, animals were killed on day 3 after the viral infusion. The entire colon was excised, cleaned, quickly frozen in liquid nitrogen, ground into fine powder, and used for RNA isolation.

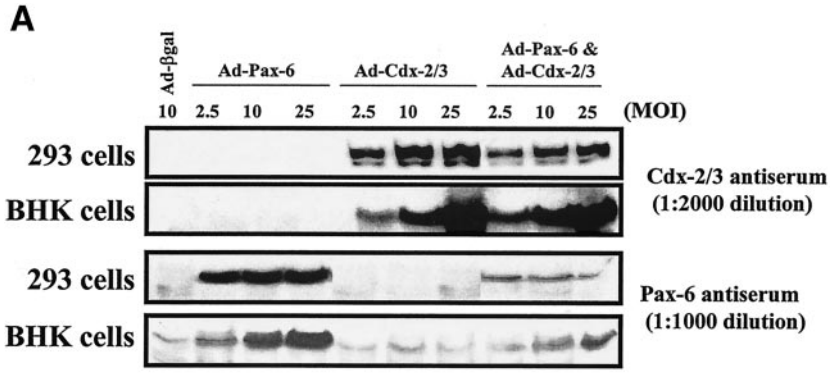
RESULTS

Characterization of recombinant Pax-6 and Cdx-2/3 expression in BHK fibroblasts and 293 cells. To characterize adenoviral-derived Pax-6 and Cdx-2/3, we analyzed expression of these transcription factors in BHK and 293 cells transduced with an increasing multiplicity of infection (MOI of 2.5, 10, and 25). Western blot analyses revealed dose-dependent increases in levels of Cdx-2/3 and Pax-6 in both cell lines (Fig. 1*A*).

To study the transcriptional activation of the rat proglucagon promoter by adenoviral-derived Cdx-2/3 and Pax-6, 293 and BHK fibroblasts were transfected with a reporter plasmid containing 300 bp of the rat proglucagon promoter linked to a luciferase reporter gene [−300]GLU-Luc and transduced with increasing MOI of Ad-Cdx-2/3 and Ad-Pax-6 alone or in combination. Both Cdx-2/3 and Pax-6 increased proglucagon promoter activity (Fig. 1*B*) and, consistent with previous reports (14,28), coexpression of Cdx-2/3 and Pax-6 produced a synergistic activation of proglucagon promoter activity (Fig. 1*B*). Nuclear extracts isolated from 293 or BHK cells transduced with various viruses at different MOI were used in electrophoretic mobility shift assay (EMSA) experiments with proglucagon promoter G1 and G3 probes (Fig. 1*C* and *D*). As shown in Fig. 1*C*, no detectable DNA-protein G3 complex was observed in cells transduced with increasing MOI of Ad-βgal or Ad-Cdx-2/3. By contrast, a dose-dependent increase in the formation of a Pax-6–G3 complex was observed in cells transduced with increasing MOI of Ad-Pax-6. Cells cotransduced with both Ad-Pax-6 and Ad-Cdx-2/3 appeared to contain a somewhat less abundant G3 complex compared with cells expressing Ad-Pax-6 alone. Similar findings were observed using the G1 oligonucleotide probe, which preferentially binds Cdx-2/3 (Fig. 1*D*). Moreover, co-transduction of both Ad-Pax-6 and Ad-Cdx-2/3 into 293 or BHK cells at either low or high MOI did not result in detectable formation of a higher molecular weight complex that might be suggestive of heterodimer formation (Fig. 1*C* and *D*).

Expression of Pax-6, Cdx-2/3, and Sey^{neu} via recombinant adenovirus in enteroendocrine cells. To assess the effect of adenoviral-derived Pax-6 and Cdx-2/3 expression in enteroendocrine cells, we studied mouse GLUTag cells. Basal expression of endogenous Pax-6 and Cdx-2/3 was detectable in GLUTag cells infected with the control Ad-βgal virus (Fig. 2*A*). An approximate three- to fivefold increase in levels of Pax-6 or Cdx-2/3 was observed following viral transduction, with adenovirus-derived Pax-6 and Cdx-2/3 migrating at the same electrophoretic positions as the endogenous GLUTag (Fig. 2*A*) or STC-1 proteins (data not shown).

GLUTag cells transduced with Ad-Cdx-2/3 or Ad-Pax-6 exhibited increased proglucagon promoter activity (Fig. 2*B*), whereas cells infected with a truncated Pax-6 cDNA, Ad-Sey^{neu} did not exhibit significant differences in the transcriptional activity of the identical proglucagon pro-



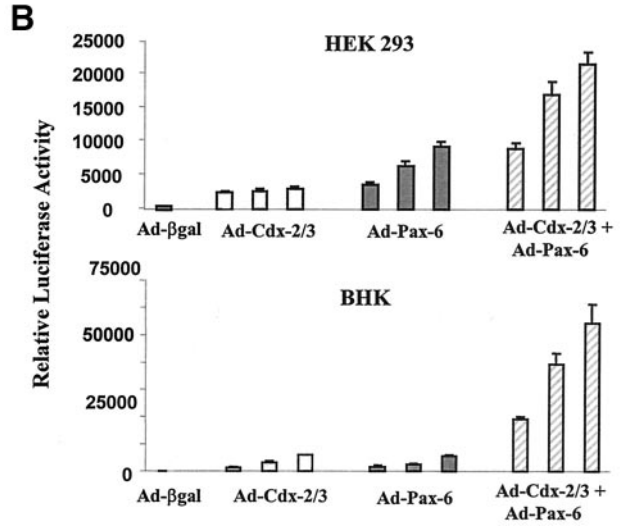
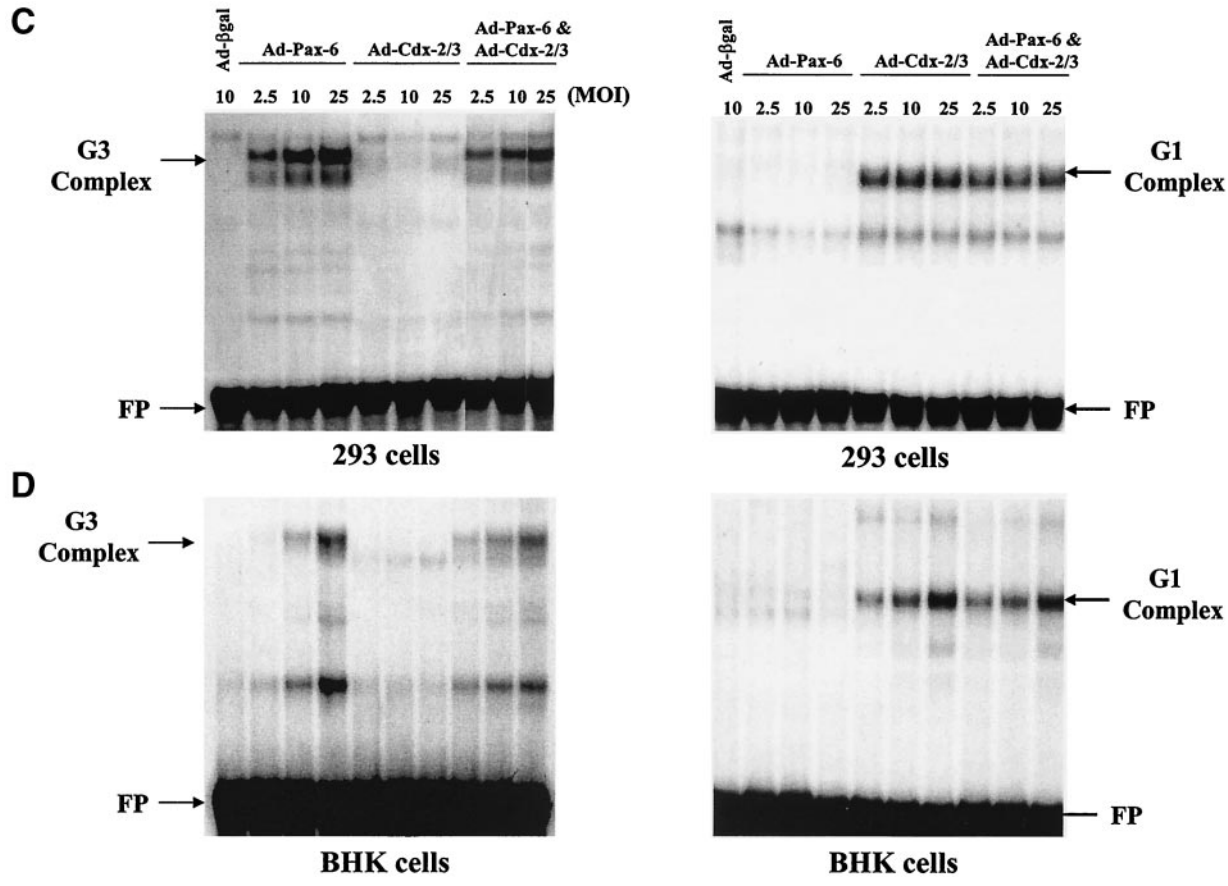


FIG. 1. Characterization of Ad-Pax-6 and Cdx-2/3 in 293 and BHK cells. *A*: Expression of Pax-6 and Cdx-2/3 in 293 and BHK cells by Western blot analysis. Cells were transduced with Ad-Pax-6, Ad-Cdx-2/3, or Ad-Pax-6 + Ad-Cdx-2/3 in three different MOI of 2.5, 10, or 25 for 1 h. Control cells were transduced with Ad-βgal. After a 24-h incubation, cells were suspended in PBS buffer containing 0.1% Triton and sonicated for 5 s at 4°C. Total protein extract (30 μg) was analyzed by 10% SDS-PAGE, and after transfer the membrane was immunoblotted with anti–Cdx-2/3 antiserum (1:2,000 dilution) (upper panel) or anti–Pax-6 antiserum (1:1,000 dilution) (lower panel). *B*: Transcriptional activation of the rat proglucagon gene promoter by recombinant Pax-6 and Cdx-2/3. HEK 293 (upper panel) or BHK cells (lower panel) were transfected with 2.5 μg of a proglucagon promoter-luciferase plasmid [−300]GLU-Luc. After an 8-h incubation, transfected cells were further transduced with various viruses at MOI of 2.5, 10, and 25 for a total of 24 h, after which cells were lysed and assayed for luciferase activity as described (17, 18). Transfection/transduction studies were performed in triplicate and were repeated on three separate occasions. *C*: Transcription factor binding to the proglucagon G3 promoter sequence. Nuclear proteins isolated from virus-transduced cells at different MOI were mixed with [^{32}P]-ATP-labeled proglucagon G3 oligonucleotides and subjected to EMSA. The major complex is designated by an arrow. FP, free probe. *D*: Transcription factor binding to the proglucagon G1 promoter region. Nuclear proteins isolated from virus-infected cells at the MOI shown were mixed with [^{32}P]-ATP-labeled proglucagon G1 oligonucleotides and subjected to EMSA. The major complex is designated by an arrow. FP, free probe.



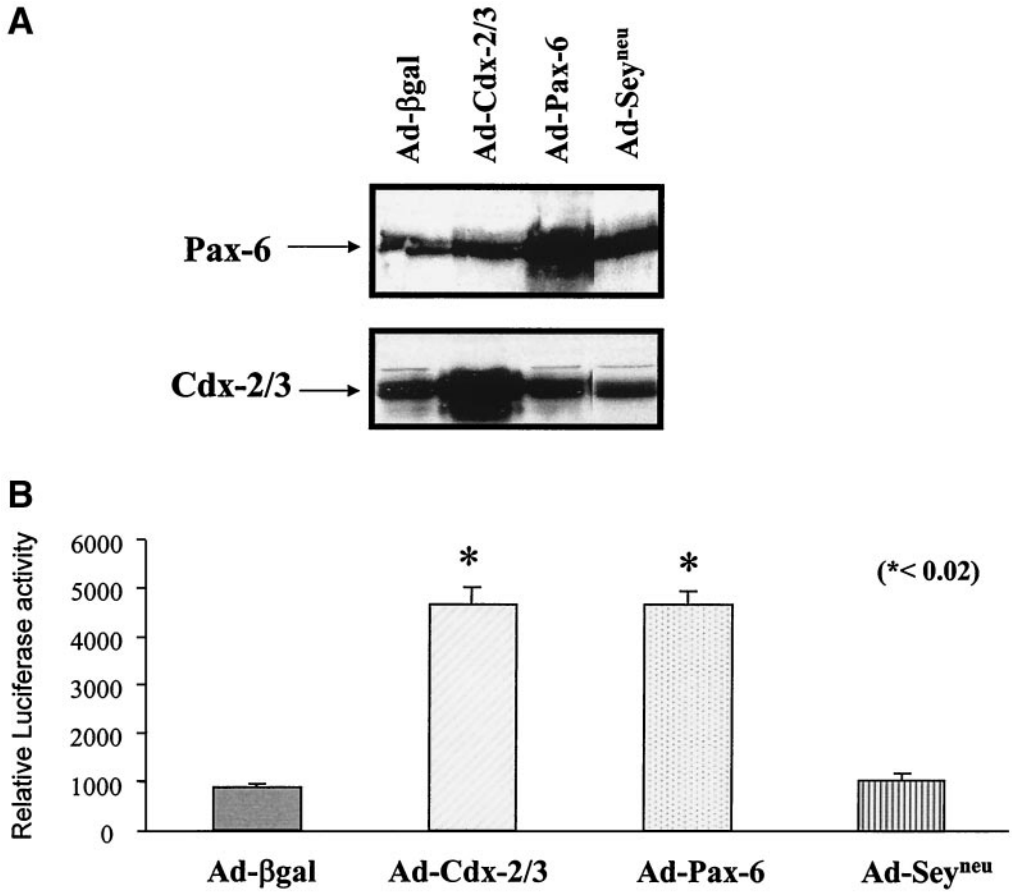


FIG. 2. Expression of Pax-6, Cdx-2/3 and Sey^{neu} in GLUTag cells. *A*: GLUTag cells were transduced with Ad-Cdx-2/3, Ad-Pax-6, or Ad-Sey^{neu} for 24 h, after which cells were lysed for protein isolation. Protein extract (30 μg) was resolved by 10% SDS-PAGE, and after transfer, the membrane was immunoblotted with a 1:1,000 dilution of anti–Pax-6 antiserum (upper panel) (gift from Dr. Saule, Lille, France) or 1:2,000 dilution of anti–Cdx-2/3 antiserum (lower panel). The truncated Sey^{neu} mutant which lacks the COOH-terminal domain of Pax-6 is not detected by this anti–Pax-6 antiserum. *B*: Transcriptional activity of a transfected proglucagon promoter-luciferase plasmid in adenovirus-transduced GLUTag cells. GLUTag cells were first transfected with [−300]-GLU-Luc, cultured for 8 h, and then transduced with Ad-βgal, Ad-Cdx-2/3, Ad-Pax-6, or Ad-Sey^{neu} for an additional 24 h. Cells were collected and assayed for luciferase activity. Transfection/transduction studies were performed in triplicate, and the data shown represent the mean from three different experiments. *P < 0.02.

moter plasmid (Fig. 2*B*). Hence, the adenoviral-derived Cdx-2/3 and Pax-6 function as transcriptional regulators of the proximal rat proglucagon promoter in gut endocrine cells (14,17,28–31).

Activation of endogenous proglucagon gene expression by Ad-Pax-6. To ascertain whether enhanced expression of Pax-6 or Cdx-2/3 leads to increased levels of intestinal proglucagon mRNA transcripts, and not just proglucagon promoter activity, we transduced GLUTag cells with Ad-Cdx-2/3, Ad-Pax-6, or Ad-βgal for 24 h. The levels of proglucagon mRNA transcripts were increased in GLUTag cells transduced with Ad-Pax-6, in a dose-dependent manner (Fig. 3*A*). In contrast, GLUTag cells transduced with Ad-Cdx-2/3 or the control Ad-βgal virus did not exhibit changes in levels of endogenous proglucagon gene expression (Fig. 3*B*). Furthermore, GLUTag cells transduced with Ad-Pax-6 also exhibit increased biosynthesis and secretion of the PGDPs, as assessed by radioimmunoassays for GLP-1 of cell and media contents following viral transduction (data not shown).

To assess whether the Pax-6–dependent activation of enteroendocrine proglucagon gene expression is cell-type specific, we transduced Ad-Pax-6 into HEK 293 cells and BHK fibroblasts, nonendocrine cells that do not normally express the proglucagon gene. In contrast to the activation of endogenous proglucagon gene expression observed in GLUTag endocrine cells, no induction of proglucagon mRNA transcripts was observed in these nonendocrine cell lines following transduction with Ad-Pax-6 (Fig. 3*B*).

Effect of Pax-6 on proglucagon gene expression in cell lines. To determine whether Pax-6 or Cdx-2/3 is capable of activating proglucagon gene expression in enteroendocrine cells derived from both the large and small intestine, we transduced GLUTag cells (derived from endocrine cells of the large bowel) and STC-1 cells (derived from endocrine tumors arising from the small bowel) with Ad-Pax-6, Ad-Cdx-2/3, and Ad-Sey^{neu} viruses. As shown in Fig. 4*A*, both GLUTag and STC-1 cells transduced with Ad-Pax-6 exhibited similar increases in proglucagon mRNA transcripts relative to levels observed in cells infected with Ad-βgal. In contrast, proglucagon mRNA transcripts were not increased in GLUTag or STC-1 cells transduced with Ad-Cdx-2/3 (Fig. 4). Furthermore, we did not observe additive increases in the levels of proglucagon mRNA transcripts in enteroendocrine cells transduced with both Ad-Cdx-2/3 and Ad-Pax-6 (Fig. 4). Indeed, cells transduced with both Ad-Cdx-2/3 and Ad-Pax-6 did not exhibit the increase in proglucagon mRNA transcripts

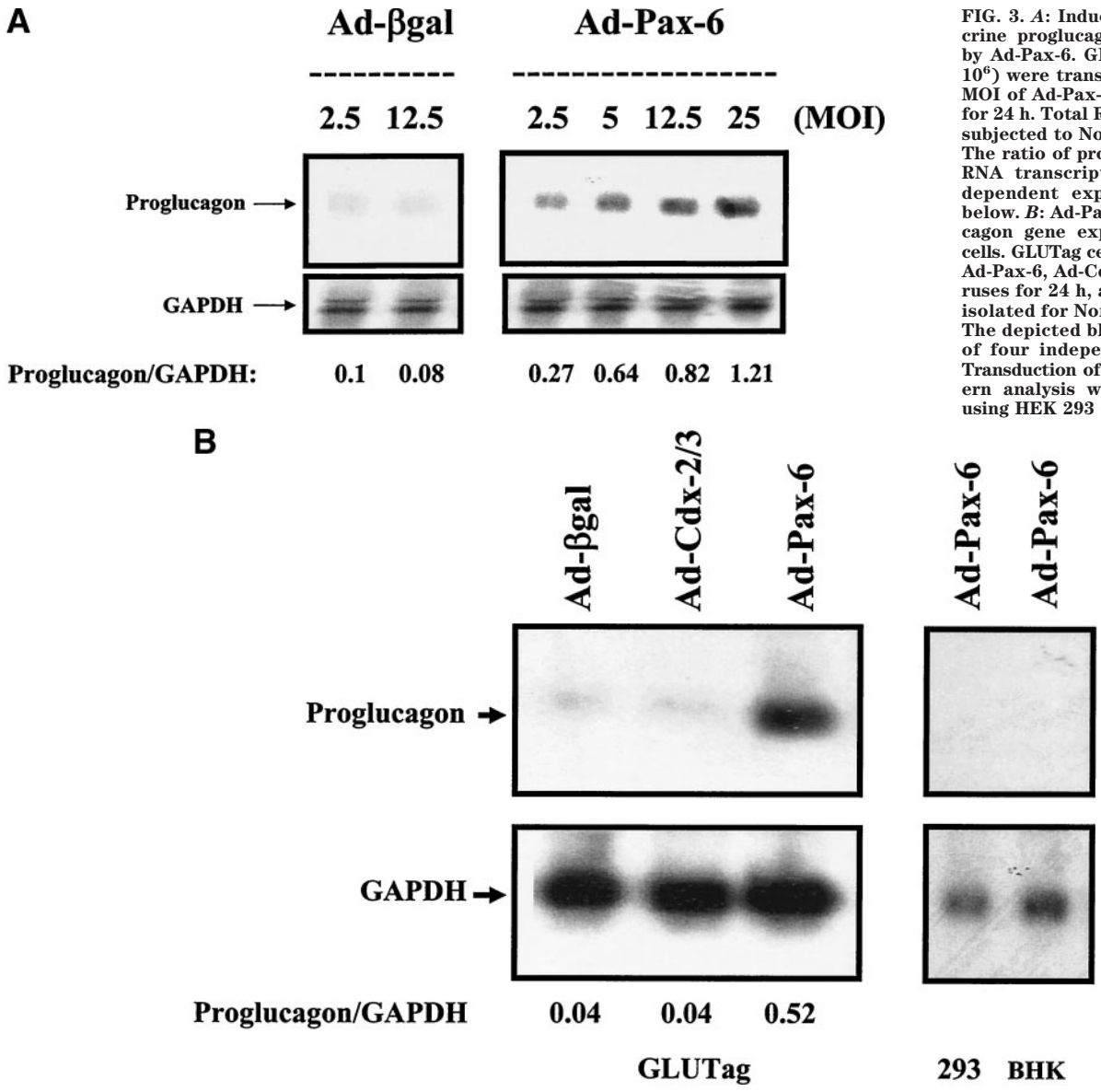


FIG. 3. *A*: **Induction of enteroendocrine proglucagon gene expression by Ad-Pax-6. GLUTag cells (~2.5 × 10^6) were transduced with different MOI of Ad-Pax-6 or Ad-βgal control for 24 h. Total RNA was isolated and subjected to Northern blot analysis. The ratio of proglucagon to GAPDH RNA transcripts from several independent experiments is shown below.** *B*: **Ad-Pax-6 activates proglucagon gene expression in GLUTag cells. GLUTag cells were treated with Ad-Pax-6, Ad-Cdx-2/3, or Ad-βgal viruses for 24 h, after which RNA was isolated for Northern blot analysis. The depicted blot is representative of four independent experiments. Transduction of Ad-Pax-6 and Northern analysis was also carried out using HEK 293 cells and BHK cells.**

detected in cells transduced with Ad-Pax-6 alone (Fig. 4), suggesting that Cdx-2/3 may actually inhibit the stimulatory effects of Pax-6 on the intestinal proglucagon promoter.

To determine whether basal levels of endogenous Cdx-2/3 contributed to control of enteroendocrine proglucagon gene expression, we transduced GLUTag cells with the dominant negative Cdx-2/3-HD virus (Fig. 5). A marked reduction of proglucagon promoter activity was observed following transduction of Ad-Cdx-2/3-HD (Fig. 5*A*). To assess the effect of blocking endogenous Cdx-2/3 activity on newly synthesized proglucagon mRNA transcripts, we performed semiquantitative RT-PCR using specific primers amplifying sequences between intron-B and exon-3 of the mouse proglucagon mRNA transcript. In contrast to the potent inhibition of proglucagon promoter activity (observed in Fig. 5*A*), we did not observe a reduction in levels of endogenous proglucagon RNA transcripts (Fig. 5*B*) in GLUTag cells.

Effect of Pax-6 on proglucagon gene expression in primary intestinal cell cultures and in rat intestine. To ascertain whether Pax-6 activates endogenous proglucagon gene expression in normal intestinal endocrine cells, we isolated small-bowel epithelial cells from fetal rats and cultured cells for 24 h in vitro before adenoviral transduction. Proglucagon mRNA transcripts were clearly increased in a dose-dependent manner following Ad-Pax-6 transduction. In contrast, proglucagon mRNA transcripts were not increased following infection of primary intestinal cells with Ad-βgal, Ad-Cdx-2/3, or Ad-Sey^{neu} (Fig. 6*A*). Similar to findings in GLUTag and STC-1 cells (Fig. 4), coinfection of primary intestinal cell cultures with both Ad-Pax-6 and Ad-Cdx-2/3 abrogated the induction detected with Ad-Pax-6 alone (Fig. 6*A*). These results demonstrate that Pax-6, but not Cdx-2/3, is sufficient for induction of proglucagon gene expression in both transformed and nontransformed intestinal cells.

To determine whether Pax-6 is also capable of increasing endogenous expression of the intestinal proglucagon gene in the intact gastrointestinal tract of a living animal, we performed an in vivo infusion of Ad-Pax-6 virus directly into the rectum and distal portion of the large bowel of

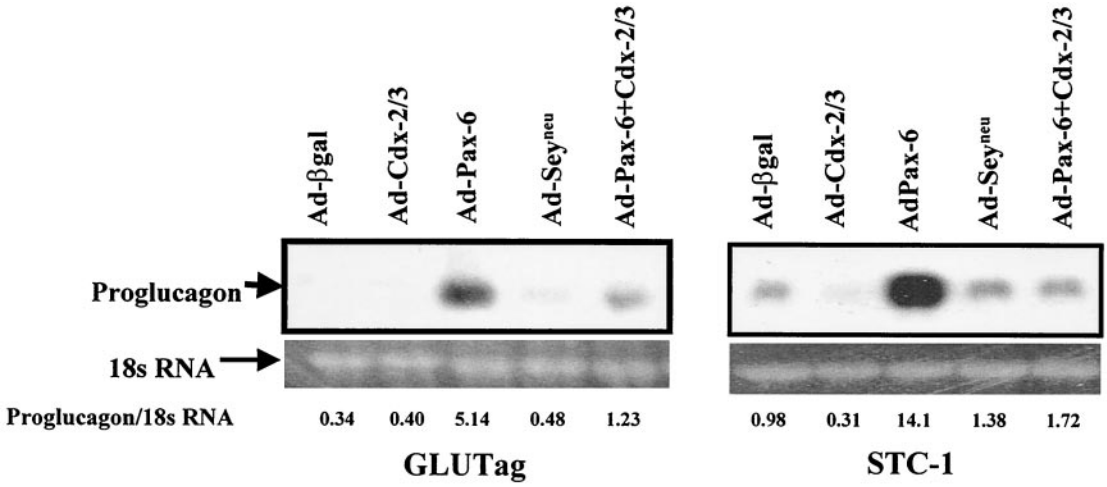
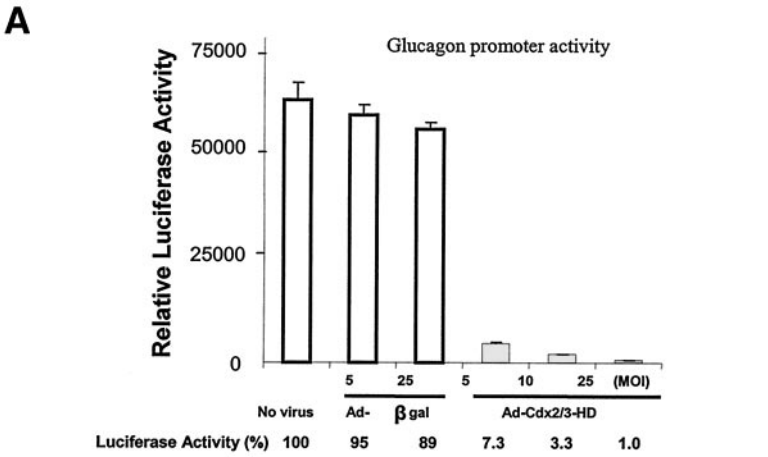


FIG. 4. Ad-Pax-6 activates proglucagon gene expression in small- and large-bowel cell lines. After 24 h of viral transduction, total RNA was prepared from GLUTag and STC-1 cells and was analyzed by Northern blotting. Arrows indicate the position of proglucagon or GAPDH RNA transcripts. The ratio of proglucagon to 18S RNA transcripts from several independent experiments is shown below.

Wistar rats. The large bowel of rats transduced with Ad-Pax-6 exhibited an approximate twofold increase in levels of Pax-6 RNA transcripts, with no difference detected in the levels of endogenous Cdx-2/3 after Ad-Pax6 transduction (data not shown). The results of this in vivo experiment showed that animals given Ad-Pax-6 exhibited an approximate three- to fourfold increase in proglucagon mRNA transcripts in the large bowel relative to control animals infused with Ad-βgal (Fig. 6*B*).

The failure to observe Ad-Cdx-2/3–dependent activation



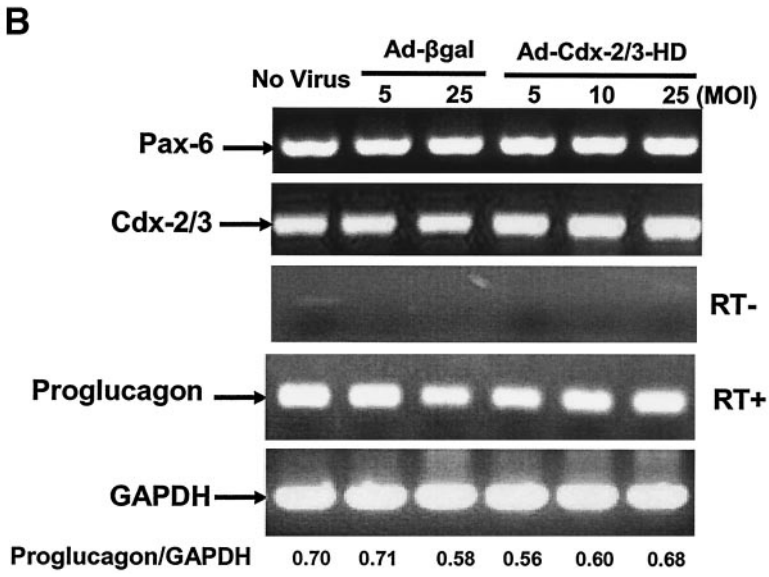


FIG. 5. Expression of a dominant negative Cdx-2/3 cDNA in GLUTag cells. 2.5 μg of proglucagon promoter-luciferase plasmid [−300]Glu-Luc was mixed with lipofectamine reagent (Life Technologies, Gaithersburg, MD) and used to transfect 85% confluent GLUTag cells. Culture medium was changed 6 h later and various MOI of Ad-βgal or Ad-Cdx-2/3-HD viruses were used to transduce cells for 1 h. Transduced cells were cultured for an additional 24 h and used for luciferase assay (*A*) or total RNA isolation and RT-PCR (*B*). Experiments were performed in triplicate and repeated on four separate occasions. The luciferase activity obtained following transduction with different viruses was expressed as a percentage of the activity obtained in transfected GLUTag cells in the absence of adenoviral transduction. The relative densitometric ratios for proglucagon/GAPDH mRNA transcripts from four independent experiments are shown below the RT-PCR data in panel *B*.

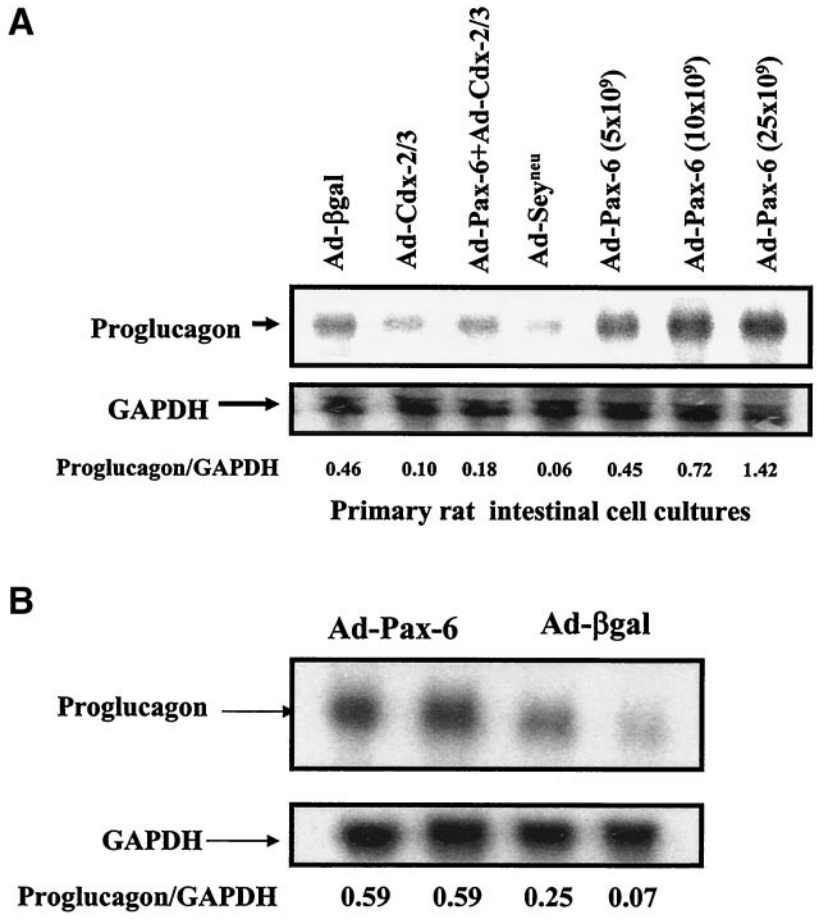


FIG. 6. Proglucagon gene expression in normal intestinal cells and in the colon of Wistar rats transduced with Ad-Pax-6. *A*: Ad-Pax-6 increases proglucagon gene expression in primary rat intestinal cell cultures. The small intestine from fetal rats (E 19–20) was dissociated and used for primary cell culture as described (24). Viral transduction was carried out 48 h before RNA isolation. Identical results were obtained in three separate experiments. *B*: Animals were infused with 1×10^{12} virions of Ad-Pax-6 or Ad-βgal via the rectal approach as described in RESEARCH DESIGN AND METHODS. RNA was isolated from the colon after 3 days and analyzed by Northern blotting. The relative ratios of the proglucagon to GAPDH transcripts are shown below each panel in *A* and *B*.

of proglucagon gene expression in islet and enteroendocrine cells prompted us to assess the transcriptional activity of the Ad-Cdx-2/3 virus in Caco-2 cells, a Cdx-2/3–responsive intestinal epithelial cell line (32). Caco-2 cells transduced with Ad-βgal or Ad-Pax-6 contained comparable levels of endogenous sucrase isomaltase (SI) mRNA transcripts compared with nontreated Caco-2 cells (Fig. 7*A*). In contrast, the levels of SI mRNA transcripts were induced following transduction of Caco-2 cells with Ad-Cdx-2/3 (Fig. 7*A*). These findings demonstrate that the failure of Cdx-2/3 to activate endogenous enteroendocrine proglucagon gene expression was not attributable to the presence of a transcriptionally defective Ad-Cdx-2/3 virus.

To exclude the possibility that the effect of Ad-Pax-6 on intestinal proglucagon gene expression requires coexpression of adenoviral proteins or cofactors that directly or indirectly enhance the levels of proglucagon mRNA transcripts, we performed lipofectamine-mediated DNA transfection of a plasmid vector (pBAT7) containing the Pax-6 cDNA alone or with cotransduced Ad-βgal (expressing adenoviral proteins/cofactors). The results of this experiment (Fig. 7*B*) showed that STC-1 cells transfected with Pax-6 also exhibited increased levels of proglucagon mRNA transcripts compared with cells transfected with empty vector pBAT7 alone (Fig. 7*B*). No additive increases in proglucagon gene expression were observed in cells transfected with Pax-6 in the presence of Ad-βgal. Taken together, these studies demonstrate that Pax-6 alone, whether delivered via a plasmid or adenovirus, is sufficient for activation of proglucagon gene expression in enteroendocrine cells.

DISCUSSION

Transcription factors regulating expression of the proglucagon gene in islet cells include Brn4, Pax-6, Pax-2, Cdx-2/3, Isl-1, CREB, Maf factors, HNF-3β, and HNF-3α (12,16,18,19,31,34–39). The majority of evidence linking these transcription factors to proglucagon gene expression is derived from transfection experiments studying the proglucagon promoter in immortalized islet cell lines. However, there is little genetic evidence from human studies linking mutations in these candidate transcription factor genes to abnormalities in proglucagon gene transcription.

Pax-6 was first identified as a proglucagon gene transcription factor in studies of Pax-6 knockout and Pax-6 Sey^{neu} mutant mice (21,31). Both lines of mice exhibit marked defects in formation of normal islets, with reduced numbers or absent islet α-cells and with concurrent reductions in islet proglucagon mRNA transcripts. Pax-6 binds to rat proglucagon gene promoter sequences and transactivates the promoter in transfection experiments. Intriguingly, our data demonstrate that although abrogation of Pax-6 transcriptional activity markedly reduced proglucagon promoter activity, no effect on proglucagon mRNA transcripts was detected in the same experiment using the mutant Pax6 cDNA. These data illustrate that analysis of relative promoter activity represents only one component

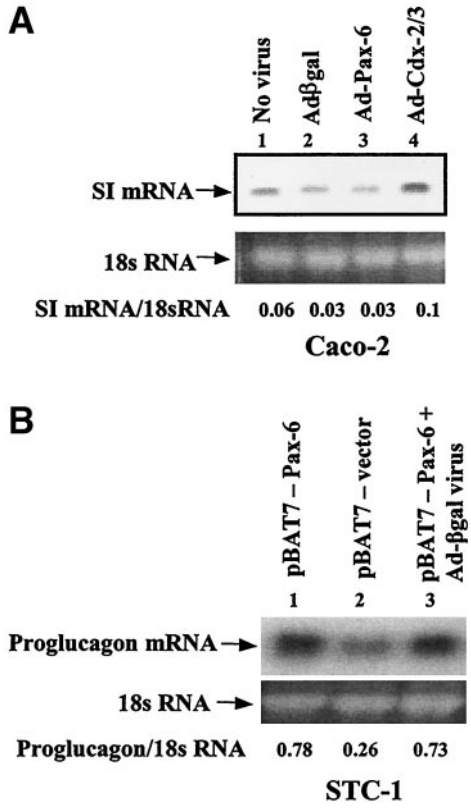


FIG. 7. Differential effects of Pax-6 and Cdx-2/3 on intestinal gene expression. Caco-2 (*A*) cells were transduced with specific viruses, following which RNA was isolated after 24 h and analyzed by Northern blotting for SI gene expression. *B*: Effect of plasmid-derived Pax-6 on intestinal proglucagon gene expression. STC-1 cells were transfected with 10 μg of pBAT7 plasmid vector alone or the pBAT7-Pax-6 plasmid DNA (*lanes 1* and *3*) with (*lane 3*) or without (*lane 1*) coinfection with Ad-βgal (MOI of 5). RNA was isolated from cells after a 24-h incubation. The arrows indicate the migration positions of proglucagon or 18s RNA transcripts.

of the complexity of factors regulating steady-state levels of RNA transcripts. Furthermore, the essential importance of Pax-6 for the developmental formation of islet cells precludes assessment of a potentially independent role for Pax-6 in the control of proglucagon gene expression in the adult pancreas in vivo.

Pax-6 is also essential for the formation of subsets of gut enteroendocrine cells. Pax-6$^{-/-}$ mice exhibit reduced numbers of duodenal gastrointenstinal polypeptide (GIP) cells and decreased numbers of gastric cells immunopositive for gastrin and somatostatin (40). Analysis of endocrine cell populations in the gut of Sey^{neu} mice expressing a dominant negative Pax-6 protein revealed almost complete absence of enteroendocrine cells containing GLP-1 or GLP-2 immunopositivity and a marked decrease in the levels of proglucagon mRNA transcripts in the small and large intestine (19). Nevertheless, it is not possible to infer from these studies whether the reduction in gut proglucagon gene expression is attributable to the importance of Pax-6 as an essential factor for enteroendocrine cell development, or whether the primary role of Pax-6 resides at the level of controlling proglucagon gene expression once enteroendocrine cell development has proceeded normally.

Our results clearly show that Pax-6 alone is sufficient for activation of endogenous proglucagon expression both in vitro and in vivo. This is the first demonstration that enhanced expression of a single transcription factor is capable of activating proglucagon gene expression in isolated cell lines, primary cell cultures, and in the context of the intact intestinal epithelium in vivo. Taken together, these studies provide additional evidence linking the level of Pax-6 expression to the control of intestinal proglucagon gene expression both in vitro and in vivo. In contrast, our data demonstrate that enhanced expression of Cdx-2/3 in primary intestinal cell cultures, or in the intact intestinal epithelium, is not sufficient for activation of enteroendocrine proglucagon gene expression. Hence, although Cdx-2/3 may activate the transfected proglucagon promoter, our data do not support using Cdx-2/3 alone for the enhancement of intestinal proglucagon gene expression.

The finding that GLP-1 agonists ameliorate diabetes in rodents has sparked considerable interest in the potential use of GLP-1 for the treatment of human subjects with type 2 diabetes. Similarly, the intestinotrophic and cytoprotective effects of GLP-2 have prompted studies of the potential use of GLP-2 analogs for the treatment of human patients with intestinal failure (2). An alternative approach to the therapeutic administration of GLPs involves strategies designed to increase the synthesis and secretion of the intestinal GLPs from normal gut endocrine cells in vivo. The gut represents a potentially attractive target for peroral delivery of viral vectors, and several studies have demonstrated the utility of targeting the gut with gene therapy approaches in vivo (41–43). Our studies identify Pax-6 as the first transcription factor capable of enhancing proglucagon gene expression within the context of the normal rodent gut epithelium in vivo. Future studies directed at examining the utility of adenoviral-delivered Pax-6 targeted to the gut epithelium for the enhanced production of GLP-1 and GLP-2 in the setting of experimental diabetes or intestinal disease in vivo appear warranted.

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REFERENCES

1. Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF: Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J Biol Chem* 261:11880–11889, 1986
2. Drucker DJ: The glucagon-like peptides. *Endocrinology* 142:521–527, 2001
3. Holst JJ: Glucagon-like peptide 1 (GLP-1): an intestinal hormone, signalling nutritional abundance, with an unusual therapeutic potential. *Trends Endocrinol Metab* 10:229–235, 1999
4. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CMB, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JPH, Smith DM, Ghatei MA, Herbert J, Bloom SR: A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature* 379:69–72, 1996
5. Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Egan JM, Bonner-Weir S, Habener JF: Insulinotropic glucagon-like peptide-1 agonists stimulate expression of homeodomain protein IDX-1 and increase β-cell mass in mouse pancreas. *Diabetes* 49:741–748, 2000

6. Drucker DJ: Glucagon-like peptide 2. *J Clin Endocrinol Metab* 86:1759–1764, 2001
7. Drucker DJ: The glucagon-like peptides. *Diabetes* 47:159–169, 1998
8. Zander M, Madsbad S, Madsen JL, Holst JJ: Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet* 359:824–830, 2002
9. Agerso H, Jensen LB, Elbrond B, Rolan P, Zdravkovic M: The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. *Diabetologia* 45:195–202, 2002
10. Philippe J, Drucker DJ, Knepel W, Jepeal L, Misulovin Z, Habener JF: Alpha-cell-specific expression of the glucagon gene is conferred to the glucagon promoter element by the interactions of DNA-binding proteins. *Mol Cell Biol* 8:4877–4888, 1988
11. Philippe J: Glucagon gene transcription is negatively regulated by insulin in a hamster islet cell line. *J Clin Invest* 84:672–677, 1989
12. Philippe J, Morel C, Prezioso VR: Glucagon gene expression is negatively regulated by hepatocyte nuclear factor 3β. *Mol Cell Biol* 14:3514–3523, 1994
13. Philippe J, Morel C, Cordier-Bussat M: Islet-specific proteins interact with the insulin-response element of the glucagon gene. *J Biol Chem* 270:3039–3045, 1995
14. Ritz-Laser B, Estreicher A, Klages N, Saule S, Philippe J: Pax-6 and Cdx-2/3 interact to activate glucagon gene expression on the G1 control element. *J Biol Chem* 274:4124–4132, 1999
15. Knepel W, Vallejo M, Chafitz JA, Habener JF: The pancreatic islet-specific glucagon G3 transcription factors recognize control elements in the rat somatostatin and insulin-I genes. *Mol Endocrinol* 5:1457–1466, 1991
16. Hussain MA, Lee J, Miller CP, Habener JF: POU domain transcription factor brain 4 confers pancreatic a-cell-specific expression of the proglucagon gene through interaction with a novel proximal promoter G1 element. *Mol Cell Biol* 17:7186–7194, 1997
17. Jin T, Drucker DJ: Activation of proglucagon gene transcription through a novel promoter element by the caudal-related homeodomain protein cdx-2/3. *Mol Cell Biol* 16:19–28, 1996
18. Jin T, Trinh DKY, Wang F, Drucker DJ: The caudal homeobox protein cdx-2/3 activates endogenous proglucagon gene expression in InR1–G9 islet cells. *Mol Endocrinol* 11:203–209, 1997
19. Hill ME, Asa SL, Drucker DJ: Essential requirement for Pax6 in control of enteroendocrine proglucagon gene transcription. *Mol Endocrinol* 13:1474–1486, 1999
20. Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F: Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* 386:84–87, 1997
21. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P: Pax6 is required for differentiation of glucagon-producing α-cells in mouse pancreas. *Nature* 387:406–409, 1997
22. Rindi G, Grant SGN, Yiangou Y, Ghatei MA, Bloom SR, Bautch VL, Solcia E, Polak JM: Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. *Am J Pathol* 136:1349–1363, 1990
23. Drucker DJ, Jin T, Asa SL, Young TA, Brubaker PL: Activation of proglucagon gene transcription by protein kinase A in a novel mouse enteroendocrine cell line. *Mol Endocrinol* 8:1646–1655, 1994
24. Brubaker PL, Vranic M: Fetal rat intestinal cells in monolayer culture: a new in vitro system to study the glucagon-like immunoreactive peptides. *Endocrinology* 120:1976–1985, 1987
25. Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB: Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol* 43:161–189, 1994
26. Drucker DJ, Asa S: Glucagon gene expression in vertebrate brain. *J Biol Chem* 263:13475–13478, 1988
27. Drucker DJ, Brubaker PL: Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. *Proc Natl Acad Sci* 86:3953–3957, 1989
28. Hussain MA, Habener JF: Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. *J Biol Chem* 274:28950–28957, 1999
29. Laser B, Meda P, Constant I, Philippe J: The caudal-related homeodomain protein Cdx-2/3 regulates glucagon gene expression in islet cells. *J Biol Chem* 271:28984–28994, 1996
30. Trinh KY, Jin T, Drucker DJ: Identification of domains mediating transcriptional activation and cytoplasmic export in the caudal homeobox protein Cdx-3. *J Biol Chem* 274:6011–6019, 1999
31. Sander M, Neubuser A, Kalamaras J, Ee HC, Martin GR, German MS: Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev* 11:1662–1673, 1997
32. Olsen WA, Lloyd M, Korsmo H, He YZ: Regulation of sucrase and lactase in Caco-2 cells: relationship to nuclear factors SIF-1 and NF-LPH-1. *Am J Physiol* 271:G707–G713, 1996
33. Andersen FG, Heller RS, Petersen HV, Jensen J, Madsen OD, Serup P: Pax6 and Cdx2/3 form a functional complex on the rat glucagon gene promoter G1-element. *FEBS Lett* 445:306–310, 1999
34. Wang M, Drucker DJ: The LIM domain homeobox gene isl-1 is a positive regulator of islet cell-specific proglucagon gene transcription. *J Biol Chem* 270:12646–12652, 1995
35. Kaestner KH, Katz J, Liu Y, Drucker DJ, Schutz G: Inactivation of the winged helix transcription factor HNF3α affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes Dev* 13:495–504, 1999
36. Ritz-Laser B, Estreicher A, Gauthier B, Philippe J: The paired homeodomain transcription factor Pax-2 is expressed in the endocrine pancreas and transactivates the glucagon gene promoter. *J Biol Chem* 275:32708–32715, 2000
37. Ritz-Laser B, Estreicher A, Gauthier BR, Mamin A, Edlund H, Philippe J: The pancreatic beta-cell-specific transcription factor Pax-4 inhibits glucagon gene expression through Pax-6. *Diabetologia* 45:97–107, 2002
38. Miller CP, Lin JC, Habener JF: Transcription of the rat glucagon gene by the cyclic AMP response element-binding protein CREB is modulated by adjacent CREB-associated proteins. *Mol Cell Biol* 13:7080–7090, 1993
39. Planque N, Leconte L, Coquelle FM, Benkhelifa S, Martin P, Felder-Schmittbuhl MP, Saule S: Interaction of maf transcription factors with pax-6 results in synergistic activation of the glucagon promoter. *J Biol Chem* 276:35751–35760, 2001
40. Larsson LI, St-Onge L, Hougaard DM, Sosa-Pineda B, Gruss P: Pax 4 and 6 regulate gastrointestinal endocrine cell development. *Mech Dev* 79:153–159, 1998
41. During MJ, Xu R, Young D, Kaplitt MG, Sherwin RS, Leone P: Peroral gene therapy of lactose intolerance using an adeno-associated virus vector. *Nat Med* 4:1131–1135, 1998
42. Lozier JN, Yankaskas JR, Ramsey WJ, Chen L, Berschneider H, Morgan RA: Gut epithelial cells as targets for gene therapy of hemophilia. *Hum Gene Ther* 8:1481–1490, 1997
43. Pandha HS, Lemoine NR: Gene therapy in gastroenterology. *Gut* 38:161–165, 1996
44. Grillo M, Margolis FL: Use of reverse transcriptase polymerase chain reaction to monitor expression of intronless genes. *Biotechniques* 9:262–268, 1990