0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(5):2025–2033

doi: 10.1210/en.2002-0049

Aberrant Regulation of Human Intestinal Proglucagon Gene Expression in the NCI-H716 Cell Line

XIEMIN CAO, GRACE FLOCK, CAROLINE CHOI, DAVID M. IRWIN, AND DANIEL J. DRUCKER

Departments of Medicine (D.J.D.), Laboratory Medicine and Pathobiology (D.M.I., D.J.D.), Banting and Best Diabetes Centre (X.C., G.F., C.C., D.M.I., D.J.D.), Toronto General Hospital, University of Toronto, Toronto, Canada M5G 2C4

Despite interest in understanding glucagon-like peptide-1 (GLP-1) production, the factors important for GLP-1 biosynthesis remain poorly understood. We examined control of human proglucagon gene expression in NCI-H716 cells, a cell line that secretes GLP-1 in a regulated manner. Insulin, phorbol myristate acetate, or forskolin, known regulators of rodent proglucagon gene expression, had no effect, whereas sodium butyrate decreased levels of NCI-H716 proglucagon mRNA transcripts. The inhibitory effect of sodium butyrate was mimicked by trichostatin A but was not detected with sodium acetate or isobutyrate. The actions of butyrate were not diminished by the ERK1/2 inhibitor PD98059, p38 inhibitor SB203580, or soluble guanylate cyclase inhibitor LY83583 or following treatment of cells with KT5823, a selective inhibitor of cGMP-dependent protein kinase. NCI-H716 cells expressed multiple proglucagon gene transcription factors including isl-1, pax-6, pax-2, cdx-2/3, pax-4, hepatocyte nuclear factor (HNF)-3α, HNF-3β, HNF-3γ, and Nkx2.2. Nevertheless, the butyrate-dependent inhibition of proglucagon gene expression was not associated with coordinate changes in transcription factor expression and both the human and rat transfected proglucagon promoters were transcriptionally inactive in NCI-H716 cells. Hence, NCI-H716 cells may not be a physiologically optimal model for studies of human enteroendocrine proglucagon gene transcription. (*Endocrinology* 144: 2025–2033, 2003)

THE PROGLUCAGON GENE is expressed in a highly tissue-restricted manner in the A cells of the endocrine pancreas, L cells of the distal ileum and colon, and brain stem neurons (1–3). Interest in the control of proglucagon gene expression stems from the increasingly diverse set of biological activities attributed to the proglucagon-derived peptides (PGDPs). Posttranslational processing in the pancreas liberates predominantly 29-amino-acid glucagon, which contributes to maintenance of blood glucose through regulation of glycogenolysis and gluconeogenesis (4). In the gut, proglucagon gives rise to glicentin, oxyntomodulin, and two glucagon-like peptides (GLPs), GLP-1 and GLP-2 (1). GLP-1 regulates energy homeostasis via effects on food intake, gastric emptying, and islet hormone secretion resulting in control of glucose disposal predominantly in the postprandial state (5–7). In contrast, GLP-2 acts more proximally to regulate gastric motility, nutrient absorption, and the growth and integrity of the intestinal mucosa (8–10).

Because the biological actions of glucagon, GLP-1, and GLP-2 are potentially relevant for treatment of diseases such as diabetes and intestinal failure secondary to short bowel syndrome, respectively, there is considerable interest in delineating the factors that control PGDP biosynthesis in the pancreas and intestine. The majority of studies of proglucagon gene expression have been carried out using glucagon-secreting rodent islet cell lines. These experiments have identified specific signal transduction pathways, principally activation of adenylate cyclase, leading to cAMP generation and induction of proglucagon gene transcription (11, 12). Similarly, an increasing number of islet transcription factors, including isl-1 (13), cdx-2 (14, 15), brn4 (16), pax-6 (17), and members of the Foxa family (18–20), have been shown to regulate rat proglucagon gene promoter activity in cell transfection studies.

In contrast to the data available on control of rodent proglucagon gene expression, very little information is known about factors regulating the human proglucagon gene. Moreover, recent experiments analyzing the transcriptional activity of the human proglucagon gene promoter illustrate species-specific differences between transcription factors and promoter regions important for rat *vs.* human proglucagon gene transcription (21). Although a stable islet cell line that expresses the human glucagon gene has not yet been described, Reimer *et al.* (22) have recently characterized a human intestinal cell line, NCI-H716 cells, that expresses the proglucagon gene and secretes GLP-1 in a regulated manner. NCI-H716 cells are derived from a poorly differentiated adenocarcinoma of the cecum yet exhibit some degree of endocrine differentiation when propagated *in vitro* (23). To understand the molecular control of human intestinal proglucagon gene expression, we have examined the regulation of proglucagon gene expression in NCI-H716 cells. The results of these experiments demonstrate that although NCI-H716 cells express multiple candidate proglucagon gene transcription factors, they exhibit several differences with respect to factors important for control of human *vs.* rodent proglucagon gene expression.

Materials and Methods

Reagents

Forskolin, 3-isobutyl-1-methylxanthine (IBMX), protoporphyrin IX (PPIX), trichostatin A (TSA), isobutyrate, insulin, phorbol myristate acetate (PMA), serum, and antibiotics were obtained from Sigma (St.

Abbreviations: GLP, Glucagon-like peptide; HNF, hepatocyte nuclear factor; IBMX, 3-isobutyl-1-methylxanthine; PGDP, proglucagon-derived peptide; PKG, cGMP-dependent protein kinase; PMA, phorbol myristate acetate; PPIX, protoporphyrin IX; sGC, soluble guanylate cyclase; TSA, trichostatin A.

Louis, MO); butyrate, LY83583, KT5823, SB203580, PD98059, 8-Bromo-cGMP, and acetate were purchased from Calbiochem (San Diego, CA). [α-^{32}P]-deoxy-ATP and [γ-^{32}P]-ATP (3000 Ci/mmol) were from Amersham Bioscience Corp. (Piscataway, NJ).

Cell culture and transfection

Human NCI-H716 cells, purchased from American Type Culture Collection (Manassas, VA), and hamster islet InR1-G9 cells (24) were grown in RPMI 1640 medium or DMEM, respectively, supplemented with 10% (vol/vol) fetal bovine serum and 4.5 g/liter glucose. For all experiments shown here, cells were grown on plastic dishes. Preliminary studies demonstrated no consistent difference in levels of proglucagon mRNA transcripts, proglucagon promoter activity, or butyrate regulation of proglucagon gene expression, when cells were differentiated and grown on extracellular matrix as described (22). The human and rat proglucagon gene promoter plasmids have been previously described (21, 25, 26). The vector cytomegalovirus-luciferase and the promoterless vector SK-luciferase were used as positive or negative controls, respectively. Cell transfections were carried out in 60 × 15-mm culture dishes using 5.0 μg plasmid DNA and 10 μl Lipofectin (Invitrogen Canada Inc., Burlington, Ontario, Canada) per dish, following the protocol recommended by the manufacturer. All cells were harvested 48 h after transfection for analysis of luciferase activity as described previously (14, 25, 27). Values for luciferase activity obtained in transfection (n = 4–5 dishes/plasmid) with proglucagon promoters were normalized relative to the luciferase values obtained after transfection of the identical control promoterless plasmid alone. Immunocytochemistry for GLP-1 and Pax-6 was performed as previously described (25, 26). The polyclonal rabbit GLP-1 antisera was raised in the Drucker laboratory and used at 1:1500 dilution, whereas the Pax-6 antisera (1:400) was from Zymed Laboratories, Inc. (San Francisco, CA).

RT-PCR experiments

RNA was prepared from human NCI-H716 cells and human LCC-18 cells using Trizol reagent (Invitrogen Canada Inc.) according to the manufacturer's specifications. The human small intestine RNA and human pancreas RNA were purchased from BD Biosciences (Mississauga, Ontario, Canada). First-strand cDNA synthesis was generated from total RNA using SuperScript preamplification system (Invitrogen Canada Inc.). Target cDNAs were then amplified by PCR using specific primer pairs. The sequences for the primers and specific conditions used for RT-PCR experiments are available upon request from the authors.

PCR products were loaded onto a 1% agarose gel, electrophoresed in Tris-acetate-EDTA buffer, transferred onto nylon membranes, and hybridized using internal oligonucleotide probes labeled by T4 kinase reaction with [γ-^{32}P]-ATP (Amersham Bioscience Corp.).

Northern blot analysis

RNA was prepared with Trizol reagent, and 10 μg total RNA were loaded onto a 1% agarose gel containing formaldehyde. Following electrophoresis, gels were transferred overnight by diffusion (5× saline sodium citrate) to a nylon membrane. The membranes were UV cross-linked and prehybridized for 4 h at 68 C. After prehybridization, the blots were hybridized sequentially with random-primed rat proglucagon, 18S cDNA, or glyceraldehyde-3-phosphate dehydrogenase probes in hybridization buffer (BD Biosciences) according to the manufacturer's specifications.

Real time RT-PCR

Quantitative detection of specific mRNA transcripts in RNA isolated from NCI-H716 cells grown in the presence or absence of sodium butyrate for 24 h was carried out by real-time PCR using ABI PRISM 7900HT. Primers were: Brn4, 5′-GCCACAGCTGCCTCGAAT-3′ (forward) and 5′-GCATGGACTAGGGAGGTGGAA-3′ (reverse); ISL-1, 5′-TGCGCCAAGTGCAGCAT-3′ (forward) and 5′-AGCGGGCACGCATCAC-3′ (reverse); Pdx1, 5′-CTGGATTGGCGTTGTTTGTG-3′ (forward) and 5′-CCAAGGTGGAGTGCTGTAGGA-3′ (reverse); Pax2, 5′-GCAAAATAGCGAACATGGTCTGT-3′ (forward) and 5′-AAAAGCGAAAACACGGAATTACA-3′ (reverse); Pax4, 5′-GGGTCTGGTTTTCCAACAGAAG-3′ (forward) and 5′-CAGCTGCATTTCCCACTTGA-3′ (reverse); Pax6, 5′-CAGACACAGCCCTCACAAACAC-3′ (forward) and 5′-TGGTGAAGCTGGGCATAGG-3′ (reverse); CDX-2, 5′-AGTGTCCCAGAGCCCTTGAG-3′ (forward) and 5′-AGGGACAGAGCCAGACACTGA-3′ (reverse); hepatocyte nuclear factor (HNF)-3α, 5′-GTGAAGATGGAAGGGCATGAA-3′ (forward) and 5′-CTCCTGCGTGTCTGCGTAGTAG-3′ (reverse); HNF-3β, 5′-CACCACCAGCCCCACAAA-3′ (forward) and 5′-GGGTAGTGCATCACCTGTTCGT-3′ (reverse); HNF-3γ, 5′-GCGCCGCCAGAAACG-3′ (forward) and 5′-CCGCTGCCCCCTTTTTT-3′ (reverse); Beta2/NeuroD, 5′-CAA GGTGGTGCCTTGCTATTC-3′ (forward) and 5′-GCGCAGAGTCTCGATTTTGG-3′ (reverse); Nkx2.2, 5′-CCTTGGGAGAGGGCTGAAC-3′(forward) and 5′-GCGAAGCTGCGCAAACAT-3′ (reverse); Nkx6.1, 5′-AATAAGCCTCTGGATCCCAACTC-3′ (forward) and 5′-CGCCGCTGCTGGACTT-3′ (reverse). PCRs were performed in a total volume of 10 μl. Five microliters SYBR green PCR master mix (PE Applied Biosystems, Foster City, CA), 0.2 μl (final concentration 1 μM) of primers (forward and reverse), and 0.4 μl 1:10 diluted first-strand cDNA were mixed into 384-well thin-wall PCR plates (PE Applied Biosystems). Data were analyzed by ABR PRIM SDS 2.0 (PE Applied Biosystems). All samples were assayed in triplicate, and each real-time PCR experiment was repeated twice on two different occasions.

Results

NCI-H716 cells secrete GLP-1 in a regulated manner (22, 28). Consistent with these findings, we detected GLP-1-immunopositivity in a large proportion of NCI-H716 cells (Fig. 1A). In keeping with the importance of Pax-6 for rodent proglucagon gene transcription (17, 26), we also observed numerous cells exhibiting Pax-6-immunopositivity (Fig. 1A). Previous studies have demonstrated that activation of the adenylate cyclase pathway increases rodent proglucagon gene expression in both islet and enteroendocrine rat and murine cell models (11, 12, 29). Accordingly, we incubated NCI-H716 cells with forskolin and IBMX and analyzed proglucagon mRNA transcripts by Northern blotting. In contrast to data obtained with rodent cells, forskolin treatment did not increase, and actually modestly decreased, the levels of NCI-H716 proglucagon mRNA transcripts after 12 or 24 h (Fig. 1B). The lack of cAMP-dependent proglucagon gene expression was not due to a generalized defect in NCI-H716 cAMP responsivity because forskolin increased the levels of c-fos mRNA transcripts in comparable experiments (Fig. 1C). Treatment of cells with insulin or PMA, agents previously shown to inhibit (30) or stimulate (31) rat islet proglucagon gene expression, respectively, had no effect on levels of proglucagon mRNA transcripts (Fig. 1D). Surprisingly, incubation of NCIH-716 cells with sodium butyrate, a short-chain fatty acid that increased islet proglucagon gene transcription (32), resulted in a progressive time-dependent reduction in levels of proglucagon mRNA transcripts (Fig. 1D). In contrast, incubation of NCIH716 cells with structurally related molecules such as acetate (Fig. 1E) or isobutyrate (data not shown) did not reduce the levels of proglucagon mRNA transcripts.

To determine the mechanisms underlying the butyrate-mediated reduction in NCI-H716 proglucagon gene expression, we carried out experiments with butyrate alone or butyrate coincubated with specific signal transduction inhibitors. Because butyrate effects on gene expression have been shown, in specific cells, to be regulated in part by the extracellular signal-regulated kinases (33, 34), we coincubated cells with butyrate and the ERK1/2 inhibitor PD98059.

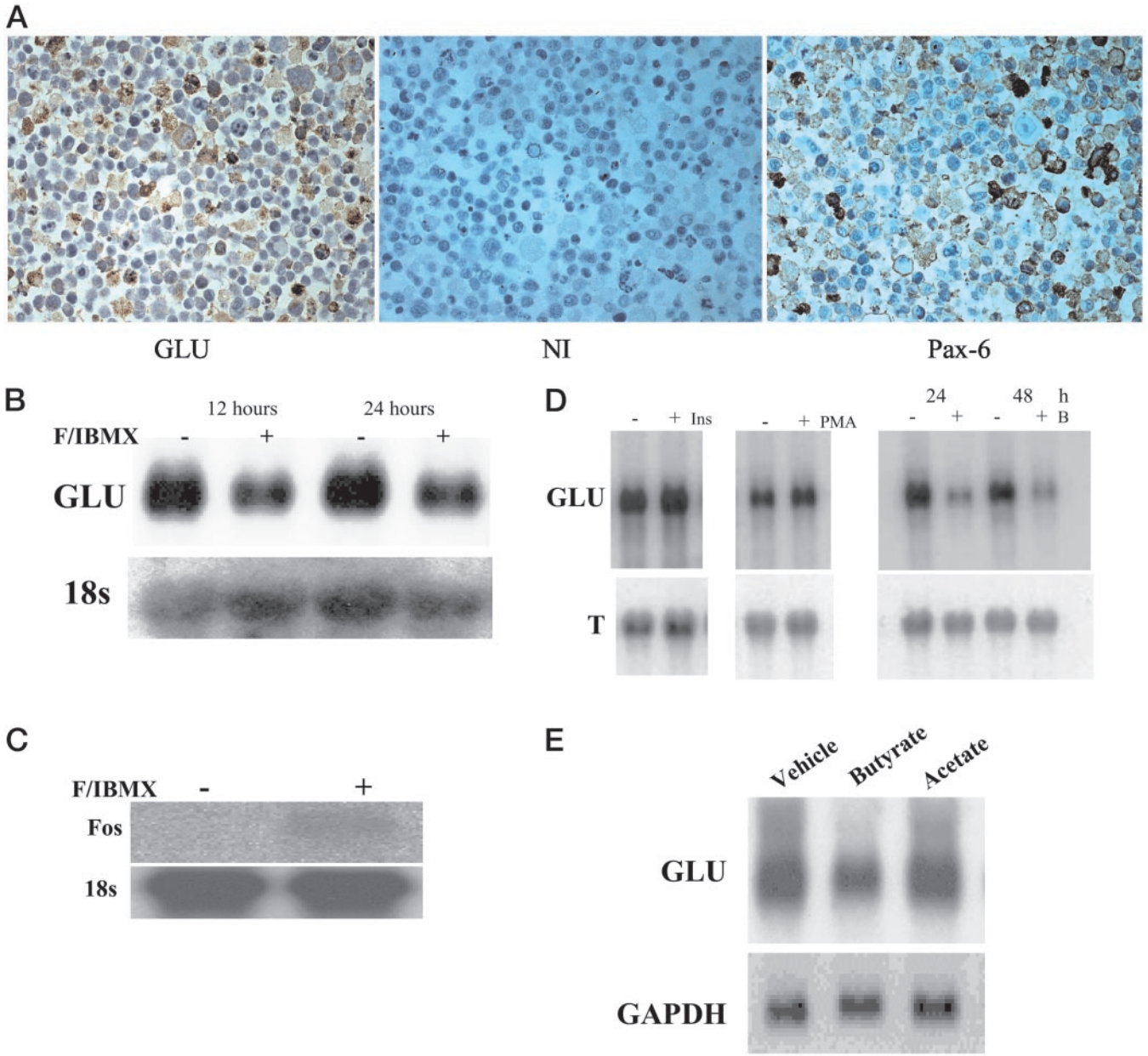

FIG. 1. A, Immunocytochemical analysis of NCI-H716 cells using antisera against GLP-1 (GLU), nonimmune antisera (NI), or antisera against Pax-6 (26). Magnification, ×400. B–E, Northern blot analysis of gene expression in NCI-H716 cells. Cells were incubated with either forskolin (20 μM) and isobutylmethyl xanthine (50 μM; F/IBMX), insulin (100 nM), PMA (100 nM), or sodium butyrate (2 mM) for the designated time periods, and RNA was analyzed by Northern blotting with cDNA probes for proglucagon (GLU), tubulin (T), c-fos (Fos), or 18s RNA (18s). E, NCI-H716 cells treated with either sodium butyrate (2 mM) or sodium acetate (2 mM) for 24 h. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

No diminution of the inhibitory butyrate effect on proglucagon gene expression was observed in cells treated with PD98059 or the p38 inhibitor SB203580 (Fig. 2).

Because the actions of sodium butyrate on globin gene expression appear to be mediated through the soluble guanylate cyclase (sGC) or cGMP-dependent protein kinase (PKG) pathway (35), we examined whether the effects of butyrate on human proglucagon gene expression were mimicked by PPIX, a potent sGC activator. In contrast to results obtained with butyrate, a modest but significant increase in levels of proglucagon mRNA transcripts was detected following incubation of NCI-H716 cells with 8-Br-cGMP, a cell membrane permeable cGMP analog known to activate PKG (Fig. 3). Furthermore, the inhibitory effects of sodium butyrate on proglucagon expression were not abrogated in the presence of the sGC inhibitor LY83583 (36) or following treatment of cells with KT5823, a selective inhibitor of PKG (Fig. 2; Ref. 37).

Several butyrate-regulated genes are sensitive to changes in histone acetylation, in keeping with inhibitory effects of butyrate on histone deacetylase (38). Consistent with these findings, trichostatin A, a histone deacetylase inhibitor, po-

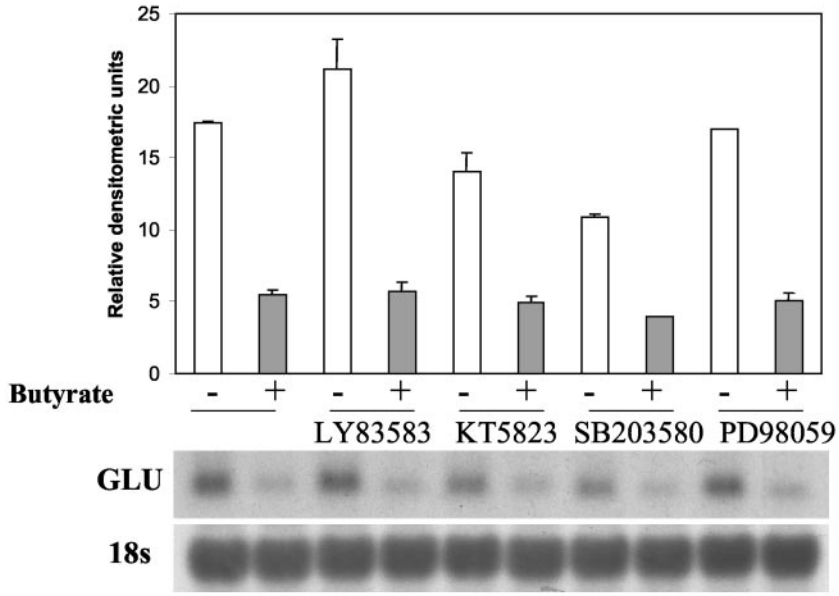

FIG. 2. Northern blot of NCI-H716 cells treated with sodium butyrate alone (2 mM) or in the presence of specific signal transduction inhibitors. Cells were incubated with sodium butyrate with or without LY83583 (1 μM), KT5823 (8 μM), SB203580 (10 μM), or PD98059 (50 μM) for 24 h. The *top panel* depicts the relative densitometric values obtained from three to five representative experiments, and the *bottom panel* shows a representative Northern blot. GLU, Proglucagon; 18s,18s rRNA.

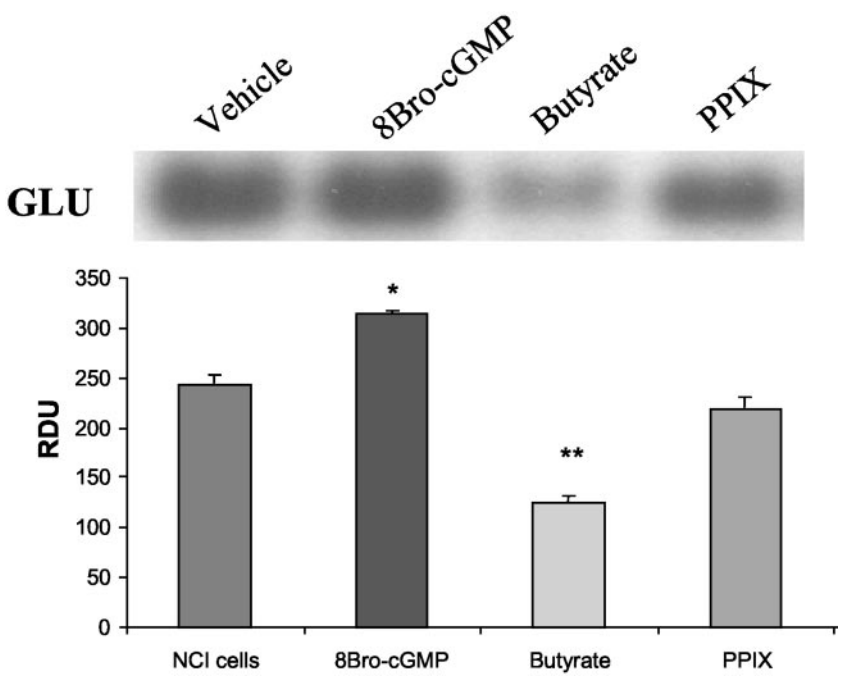

FIG. 3. Northern blot analysis of NCI-H716 cells treated with vehicle, 8-Bromo-cGMP (1 mM), sodium butyrate (2 mM), or PPIX (40 μM) for 24 h. The *bottom panel* depicts the relative densitometric units (RDUs) obtained from three representative experiments. *, $P < 0.05$ *vs.* vehicle-treated cells.

tently reduced the levels of proglucagon mRNA transcripts in NCI-H716 cells (Fig. 4). To determine whether the effects of histone deacetylase inhibitors on human proglucagon gene expression could be mapped to specific proglucagon gene promoter elements, we transfected NCI-H716 cells with a series of human proglucagon gene-luciferase reporter plasmids, containing from 328 to over 5700 bp of human proglucagon gene promoter and 5′-flanking sequences (21) (Fig. 5A). Surprisingly, all human proglucagon promoter plasmids tested, and a control rat proglucagon promoter-luciferase plasmid [−476]GLU-luciferase, known to be transcriptionally active in both islet and gut endocrine cells, were transcriptionally inactive in NCI-H716 cells (Fig. 5A). To determine whether the transcriptional inactivity of the proglucagon promoter plasmids was due to a generalized problem with this specific set of luciferase plasmids, the identical plasmids were cotransfected, on the same day, into NCI-H716, and hamster islet glucagon-secreting InR1-G9 cells (Fig. 5B). The results clearly demonstrated that the rat proglucagon promoter plasmids were active following transfection of the hamster islet cell line (24) but not in human NCI-H716 cells.

Very little is known about the identity of transcription factors necessary for activation of the human proglucagon promoter. Accordingly, we assessed whether specific transcription factors previously implicated in the control of rat proglucagon gene expression were also expressed in NCI-H716 cells. As a control for generalized transcription factor

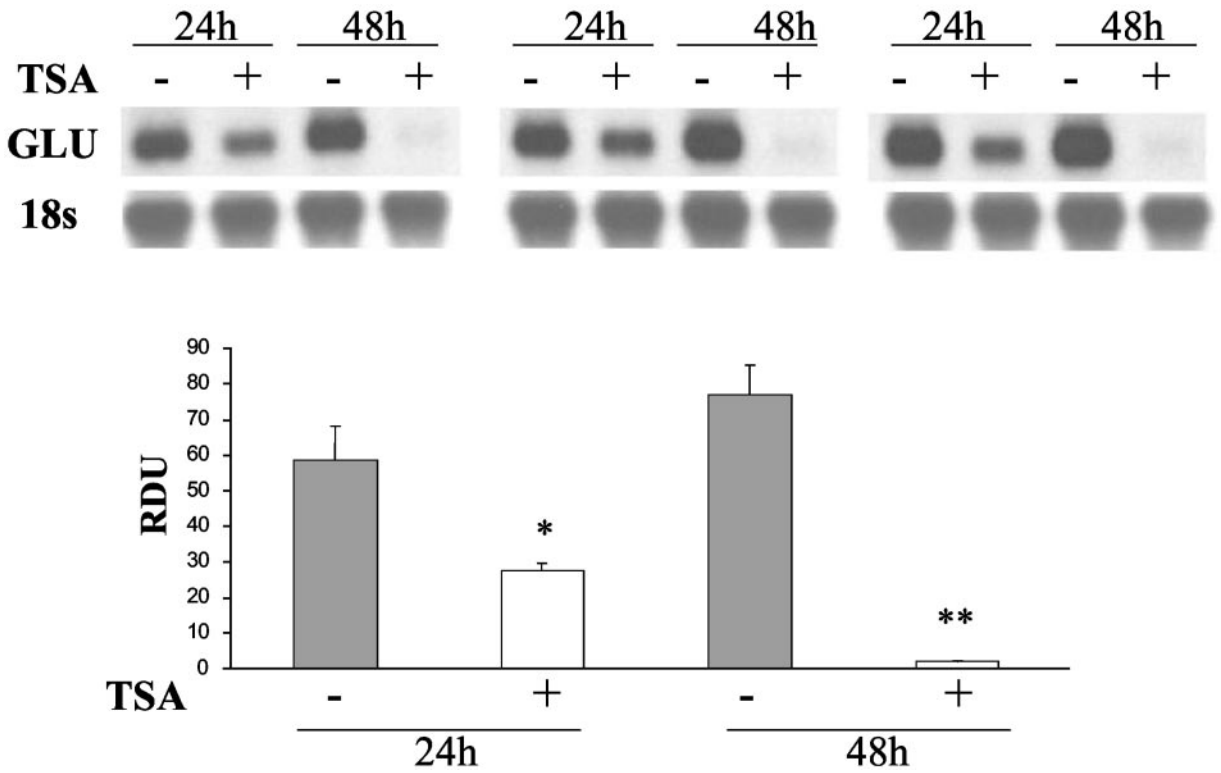

FIG. 4. The histone deacetylase inhibitor TSA inhibits proglucagon gene expression in NCI-H716 cells. Cells were incubated for 24 or 48 h with or without 10 μM TSA, and Northern blot analyses were carried out using cDNA probes for proglucagon (GLU) or 18s rRNA. The *bottom panel* depicts the relative densitometric units (RDUs) obtained from scanning the blots of three separate experiments. *, $P < 0.05$; **, $P < 0.01$.

expression in human colon adenocarcinoma cells, we used RNA from LCC-18 cells, a colon carcinoma with neuroendocrine features including the ability to synthesize and secrete low levels of human glucagon (39). Surprisingly, despite the lack of activity of transfected human or rat proglucagon promoter plasmids in NCI-H716 cells, multiple rodent proglucagon gene transcription factors were detected by RT-PCR analysis of NCI-H716 cells, including Isl-1, Pax-6, Cdx-2, Beta2/NeuroD, Nkx2.2, and members of the HNF-3 (Foxa) forkhead family (Fig. 6). Furthermore, the relative expression of most transcription factors was comparatively greater in NCI-H716 cells relative to levels detected in LCC-18 cells (Fig. 6). Intriguingly, we also detected expression of mRNA transcripts for Pax-4 and Pdx-1, genes previously shown to be involved in the extinguishing of proglucagon gene transcription in islet cells (40, 41). In contrast, we did not detect mRNA transcripts for Brn-4, a positive transcriptional regulator of proglucagon gene expression in the islet A cell lineage (42), in RNA from NCI-H716 cells (Fig. 6).

Despite the lack of activity of the transfected human or rat proglucagon promoter plasmids in NCI-H716 cells, we hypothesized that butyrate may down-regulate expression of the endogenous NCI-H716 proglucagon gene through coordinate repression or activation of positive or negative transcriptional regulators of human proglucagon gene expression. Accordingly, we used real-time PCR to quantitatively examine changes in levels of transcription factor mRNA transcripts following treatment of NCI-H716 cells with sodium butyrate. The results of these experiments (Fig. 7) demonstrated that butyrate treatment was not associated with changes in transcription factor expression that might explain the inhibition of proglucagon gene expression in NCI-H716 cells.

Discussion

Much of our current understanding of mechanisms regulating proglucagon gene expression derives from experiments using rodent islet cell lines. Glucagon-secreting rat (RIN-1056), mouse (αTC), and hamster (InR1-G9) islet cells express the proglucagon gene at high levels and have proven useful for studies of glucagon secretion and proglucagon gene transcription (43–45). In contrast, despite increasing interest in the biology of intestinal PGDPs, principally GLP-1 and GLP-2, comparatively little is currently known about the molecular factors important for intestinal proglucagon gene transcription. Furthermore, the majority of experiments examining proglucagon transcription have used the rat proglucagon gene as the preferred model, and only a single previous report (21) has described the functional characterization of the human proglucagon gene promoter.

NCI-H716 cells, derived from ascitic fluid associated with a human cecal adenocarcinoma, contain secretory granules (46) and express endocrine markers such as chromogranin A (47) and receptors for peptide hormones and serotonin (23). The demonstration that NCI-H716 cells secrete GLP-1 in a regulated manner in response to activation of both the protein kinase A- and C-dependent pathways (22) suggested that these cells may be useful for analysis of factors regu-

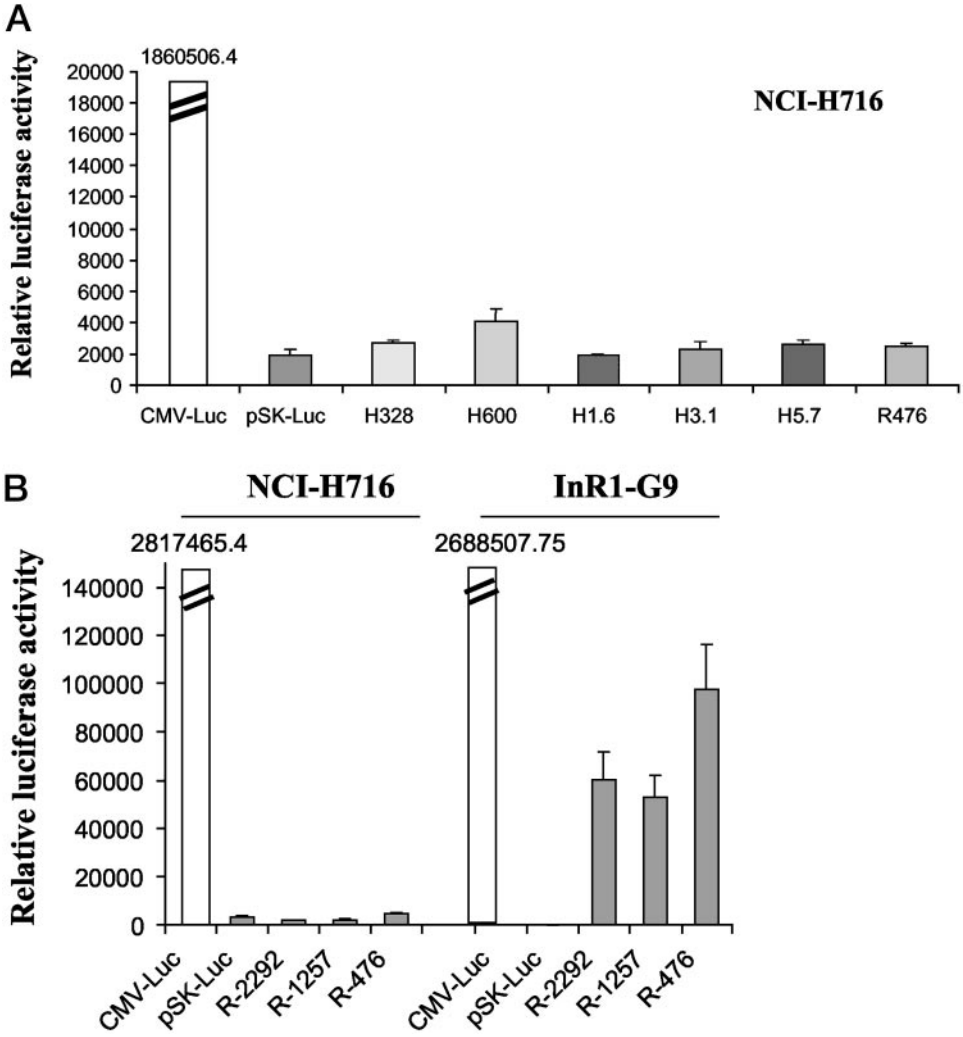

FIG. 5. A, Transcriptional activity of the human proglucagon promoter in NCI-H716 cells. Transient transfection assays were carried out with cytomegalovirus-luciferase, the promoterless empty vector pSK-luciferase, or pSK-luciferase containing from 328 bp to 5.7 kb of the human proglucagon gene promoter (21), or 476 bp of the rat proglucagon gene promoter. The data depicted represent three separate experiments, n = 4 wells per transfected plasmid in each experiment. B, Comparative transcriptional activities of the identical set of rat proglucagon-luciferase plasmids containing 2292, 1257, or 476 bp of rat proglucagon gene 5′-flanking and promoter sequences in NCI-H716 *vs.* InR1-G9 cells. The pSK-luciferase is the promoterless negative control plasmid.

lating human proglucagon gene expression. Nevertheless, we did not observe changes in proglucagon mRNA transcripts in response to forskolin, insulin, or PMA, known regulators of PGDP secretion and rodent proglucagon gene expression. The lack of effect of these agents on human proglucagon gene expression in NCI-H716 cells may reflect a combination of species- and/or cell line-specific differences in regulation of proglucagon gene expression.

The demonstration that two different histone deacetylase inhibitors, sodium butyrate and trichostatin A, down-regulate human intestinal proglucagon gene expression contrasts with previous studies demonstrating up-regulation of rat proglucagon gene transcription following butyrate treatment of islet cell lines (32, 48) or following infusion of short-chain fatty acids, including butyrate, into parenterally fed rats (49). RIN cells exposed to sodium butyrate exhibited reduced cell growth, and increased numbers of glucagon-immunopositive cells, consistent with an effect of butyrate on islet cell differentiation (48). In contrast, NCI-H716 cells exhibited reduced proglucagon gene expression following exposure to either butyrate or trichostatin A. Cell type-specific differences in genomic responses to butyrate have been previously described. For example, sodium butyrate treatment of human HT29 and Caco2 cells increased Cdx-2 expression (50), whereas no effect of butyrate on Cdx-2 expression was observed in AA/C1, HCA7, or RG/C2 colon cancer cells (51). Nevertheless, we did not observe changes in the expression of Cdx-2, a transcription factor previously shown to activate the rat proglucagon gene promoter (14, 15, 27, 52, 53), following butyrate treatment of NCI-H716 cells.

The difficulty in isolating sufficient numbers of GLP-1-secreting human enteroendocrine cells for studies of human intestinal proglucagon gene expression has limited our understanding of the molecular control of human PGDP biosynthesis. We had surmised that characterization of NCI-H716 cells, a human GLP-1-secreting intestinal cell line (22), would provide an opportunity to determine the transcription factors important for human GLP-1 biosynthesis. Surprisingly, despite the expression of several genes encoding transcription factors previously implicated in the control of rodent islet proglucagon expression, NCI-H716 cells did not support the transcriptional activation of either the human or rat transfected proglucagon promoter plasmids. Hence, the mere expression of multiple positive regulators of proglucagon gene transcription, including Pax-6, Pax-2, Cdx-2/3, Isl-1, HNF-3α, and HNF-3γ, is not sufficient to support the

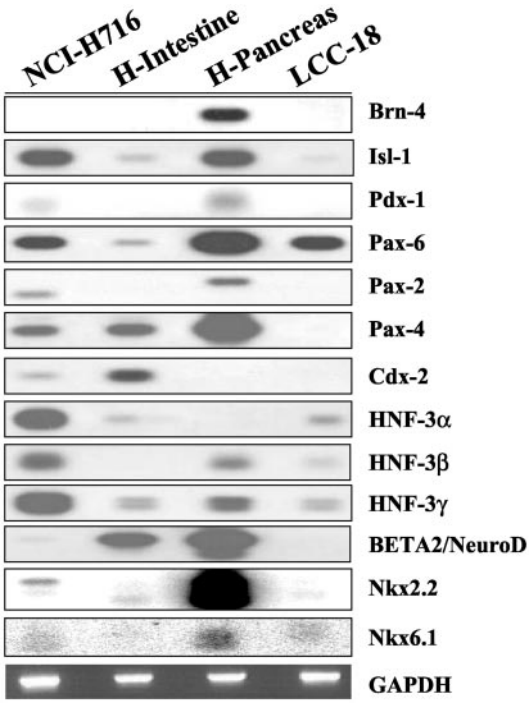

FIG. 6. RT-PCR of transcription factor expression in NCI-H716 cells. RNA was analyzed by RT-PCR as described in *Materials and Methods.* Control RT-PCRs were carried out using RNA from human intestine (H-Intestine), human pancreas (H-Pancreas), and a human LCC-18 adenocarcinoma cell line with features of endocrine differentiation (39).

transcriptional activation of the specific human or rat proglucagon promoter sequences studied in our transient transfection assays. These data differ from results obtained using mouse GLUTag enteroendocrine cells, which do support transcriptional activity following transfection of the identical series of human and rat proglucagon promoter plasmids (14, 21, 27).

Surprisingly, we did not observe coordinate changes in proglucagon gene transcription factor expression in response to treatment with sodium butyrate. Several studies have used a comparatively unbiased microarray approach to identify butyrate-responsive genes in cells derived from colonic epithelium. Sodium butyrate down-regulated 25 and up-regulated 88 genes, from a total of 900 analyzed, in MCE301 murine colonic epithelial cells (54). Similarly, only 21 of 588 mRNA transcripts were down-regulated in butyrate-treated HT-29 colonic adenocarcinoma cells (55). Furthermore, analysis of genes regulated by both butyrate and trichostatin A identified only two genes, lactoferrin and MAPK-activating kinase, which were down-regulated in HT-29 cells (56). None of the butyrate-regulated genes identified in these microarray experiments corresponded to known candidate proglucagon gene transcription factors.

Intriguingly, NCI-H716 cells express two genes, pax-4 and pdx-1, previously associated with extinction of proglucagon gene expression in the islet cell lineage. Pax-4 is not normally expressed in glucagon-producing islet cells and represses proglucagon gene promoter activity both directly and through inhibition of pax-6-mediated transactivation (40,

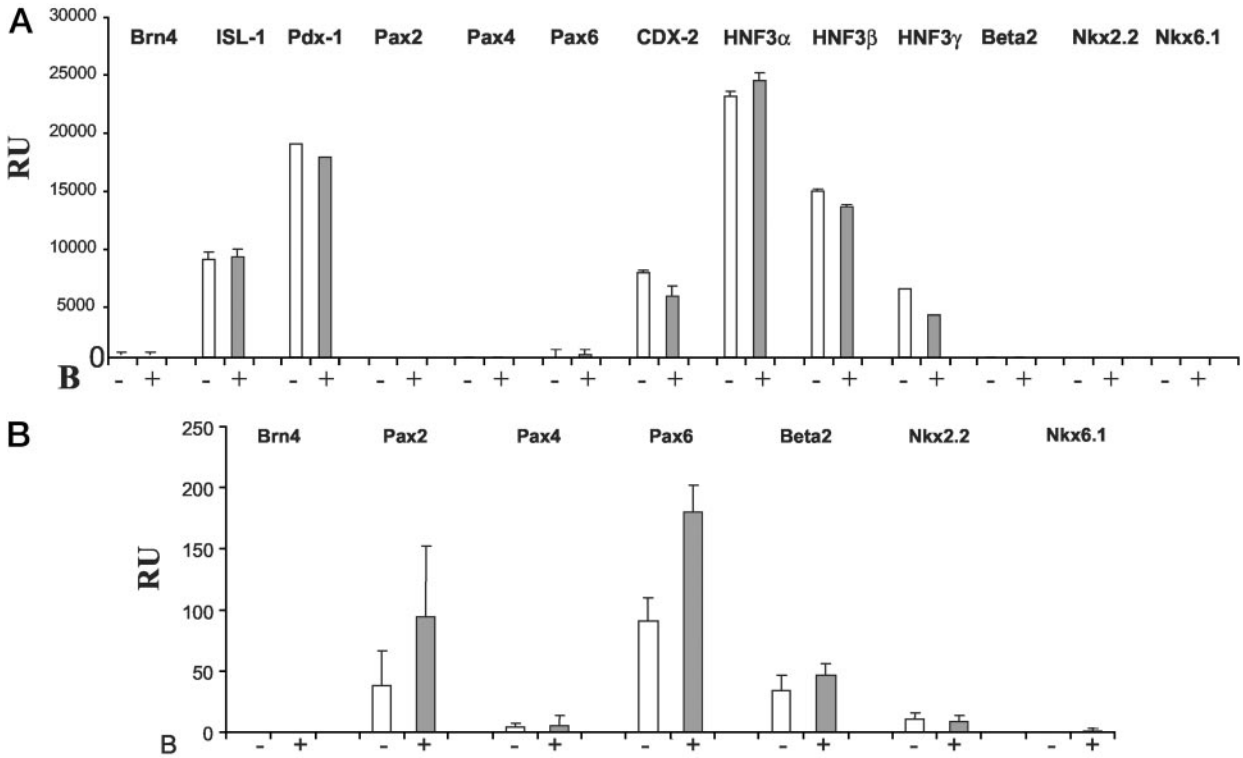

FIG. 7. Real-time PCR analysis of transcription factor gene expression in butyrate-treated NCI-H716 cells. RNA was prepared from three plates of NCI-H716 cells grown in the presence (+) or absence (−) of sodium butyrate (B) for 24 h, and the relative expression of candidate proglucagon gene transcription factors was assessed as described in *Materials and Methods.* RU, Relative expression units derived from real-time PCR experiments. The data for genes expressed at comparatively lower levels as depicted in A were replotted with a different scale in B.

57, 58). Similarly, pdx-1 is not found in nontransformed glucagon-producing islet cells, and induction of pdx-1 expression in α-cells switches the phenotype of islet cells in association with elimination of proglucagon gene expression (41). Although NCI-H716 cells do exhibit some degree of endocrine differentiation and appear useful for studies of GLP-1 secretion (22), the available evidence suggests that these cells exhibit considerable differences in the regulation of proglucagon gene transcription, compared with previous data obtained with islet or enteroendocrine cells (21, 59). Hence, whether NCI-H716 cells represent a suitable physiological model for analysis of human enteroendocrine gene transcription requires further assessment.

Acknowledgments

Received November 18, 2002. Accepted January 23, 2003.

Address all correspondence and requests for reprints to: Dr. D. Drucker, Banting and Best Diabetes Centre, Toronto General Hospital, 200 Elizabeth Street MBRW4R-402, Toronto, Ontario, Canada M5G 2C4. E-mail: d.drucker@utoronto.ca.

This work was supported by operating grants from the Canadian Institutes of Health Research (to D.J.D. and D.M.I.). D.J.D. is a Senior Scientist at the Canadian Institutes of Health Research.

References

1. **Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF** 1986 Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. J Biol Chem 261:11880–11889
2. **Drucker DJ, Asa S** 1988 Glucagon gene expression in vertebrate brain. J Biol Chem 263:13475–13478
3. **Drucker DJ** 1998 The glucagon-like peptides. Diabetes 47:159–169
4. **Unger RH, Aguilar-Parada E, Muller WA, Eisentraut AM** 1970 Studies of pancreatic α cell function in normal and diabetic subjects. J Clin Invest 49: 837–848
5. **Drucker DJ** 2001 Minireview: the glucagon-like peptides. Endocrinology 142: 521–527
6. **Kieffer TJ, Habener JF** 1999 The glucagon-like peptides. Endocr Rev 20:876–913
7. **Holst JJ** 1999 An intestinal hormone, signaling nutritional abundance, with an unusual therapeutic potential. Trends Endocrinol Metab 10:229–235
8. **Drucker DJ** 1999 Glucagon-like peptide 2. Trends Endocrinol Metab 10:153–156
9. **Drucker DJ** 2001 Glucagon-like peptide 2. J Clin Endocrinol Metab 86:1759–1764
10. **Drucker DJ** 2002 Gut adaptation and the glucagon-like peptides. Gut 50:428–435
11. **Drucker DJ, Campos R, Reynolds R, Stobie K, Brubaker PL** 1991 The rat glucagon gene is regulated by a cyclic AMP-dependent pathway in pancreatic islet cells. Endocrinology 128:394–400
12. **Knepel W, Chafitz J, Habener JF** 1990 Transcriptional activation of the rat glucagon gene by the cyclic AMP-responsive element in pancreatic islet cells. Mol Cell Biol 10:6799–6804
13. **Wang M, Drucker DJ** 1995 The LIM domain homeobox gene isl-1 is a positive regulator of islet cell-specific proglucagon gene transcription. J Biol Chem 270:12646–12652
14. **Jin T, Drucker DJ** 1996 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
15. **Ritz-Laser B, Estreicher A, Klages N, Saule S, Philippe J** 1999 Pax-6 and Cdx-2/3 interact to activate glucagon gene expression on the G1 control element. J Biol Chem 274:4124–4132
16. **Hussain MA, Lee J, Miller CP, Habener JF** 1997 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
17. **Sander M, Neubuser A, Kalamaras J, Ee HC, Martin GR, German MS** 1997 Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev 11:1662–1673
18. **Philippe J** 1995 Hepatocyte-nuclear factor 3β gene transcripts generate protein isoforms with different transactivation properties on the glucagon gene. Mol Endocrinol 9:368–374
19. **Kaestner KH, Katz J, Liu Y, Drucker DJ, Schutz G** 1999 Inactivation of the winged helix transcription factor HNF3α affects glucose homeostasis and islet glucagon gene expression *in vivo.* Genes Dev 13:495–504
20. **Liu Y, Shen W, Brubaker PL, Kaestner KH, Drucker DJ** 2002 Foxa3 (HNF-3γ) binds to and activates the rat proglucagon gene promoter but is not essential for proglucagon gene expression. Biochem J 366:633–641
21. **Nian M, Drucker DJ, Irwin D** 1999 Divergent regulation of human and rat proglucagon gene promoters *in vivo.* Am J Physiol 277:G829–G837
22. **Reimer RA, Darimont C, Gremlich S, Nicolas-Metral V, Ruegg UT, Mace K** 2001 A human cellular model for studying the regulation of glucagon-like peptide-1 secretion. Endocrinology 142:4522–4528
23. **De Bruine AP, Dinjens WN, Pijls MM, vd Linden EP, Rousch MJ, Moerkerk PT, de Goeij AF, Bosman FT** 1992 NCI-H716 cells as a model for endocrine differentiation in colorectal cancer. Virchows Arch B Cell Pathol Incl Mol Pathol 62:311–320
24. **Drucker DJ, Philippe J, Mojsov S** 1988 Proglucagon gene expression and posttranslational processing in a hamster islet cell line. Endocrinology 123: 1861–1867
25. **Flock G, Drucker DJ** 2002 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. Mol Endocrinol 16:2349–2359
26. **Hill ME, Asa SL, Drucker DJ** 1999 Essential requirement for Pax6 in control of enteroendocrine proglucagon gene transcription. Mol Endocrinol 13:1474–1486
27. **Jin T, Trinh DKY, Wang F, Drucker DJ** 1997 The caudal homeobox protein cdx-2/3 activates endogenous proglucagon gene expression in InR1–G9 islet cells. Mol Endocrinol 11:203–209
28. **Anini Y, Brubaker PL** 2003 Role of leptin in the regulation of glucagon-like peptide-1 secretion. Diabetes 52:252–259
29. **Drucker DJ, Brubaker PL** 1989 Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. Proc Natl Acad Sci USA 86:3953–3957
30. **Philippe J** 1989 Glucagon gene transcription is negatively regulated by insulin in a hamster islet cell line. J Clin Invest 84:672–677
31. **Philippe J, Drucker DJ, Habener JF** 1987 Glucagon gene transcription in an islet cell line is regulated via a protein kinase C-activated pathway. J Biol Chem 262:1823–1828
32. **Philippe J, Drucker DJ, Chick WL, Habener JF** 1987 Transcriptional regulation of genes encoding insulin, glucagon, and angiotensinogen by sodium butyrate in a rat islet cell line. Mol Cell Biol 7:560–563
33. **Witt O, Sand K, Pekrun A** 2000 Butyrate-induced erythroid differentiation of human K562 leukemia cells involves inhibition of ERK and activation of p38 MAP kinase pathways. Blood 95:2391–2396
34. **Yang J, Kawai Y, Hanson RW, Arinze IJ** 2001 Sodium butyrate induces transcription from the G α(i2) gene promoter through multiple Sp1 sites in the promoter and by activating the MEK-ERK signal transduction pathway. J Biol Chem 276:25742–25752
35. **Ikuta T, Ausenda S, Cappellini MD** 2001 Mechanism for fetal globin gene expression: role of the soluble guanylate cyclase-cGMP-dependent protein kinase pathway. Proc Natl Acad Sci USA 98:1847–1852
36. **Beasley D, Schwartz JH, Brenner BM** 1991 Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. J Clin Invest 87:602–608
37. **Gadbois DM, Crissman HA, Tobey RA, Bradbury EM** 1992 Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells. Proc Natl Acad Sci USA 89:8626–8630
38. **Candido EP, Reeves R, Davie JR** 1978 Sodium butyrate inhibits histone deacetylation in cultured cells. Cell 14:105–113
39. **Lundqvist M, Mark J, Funa K, Heldin NE, Morstyn G, Wedell B, Layton J, Oberg K** 1991 Characterisation of a cell line (LCC-18) from a cultured human neuroendocrine-differentiated colonic carcinoma. Eur J Cancer 27:1663–1668
40. **Ritz-Laser B, Estreicher A, Gauthier BR, Mamin A, Edlund H, Philippe J** 2002 The pancreatic β-cell-specific transcription factor Pax-4 inhibits glucagon gene expression through Pax-6. Diabetologia 45:97–107
41. **Wang H, Maechler P, Ritz-Laser B, Hagenfeldt KA, Ishihara H, Philippe J, Wollheim CB** 2001 Pdx1 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem 276:25279–25286
42. **Hussain MA, Miller CP, Habener JF** 2002 Brn-4 transcription factor expression targeted to the early developing mouse pancreas induces ectopic glucagon gene expression in insulin-producing β cells. J Biol Chem 277:16028–16032
43. **Philippe J, Mojsov S, Drucker DJ, Habener JF** 1986 Proglucagon processing in rat islet cell line resembles phenotype of intestine rather than pancreas. Endocrinology 119:2833–2839
44. **Drucker DJ, Mojsov S, Habener JF** 1986 Cell-specific post-translational processing of preproglucagon expressed from a metallothionein-glucagon fusion gene. J Biol Chem 261:9637–9643
45. **Powers AC, Efrat S, Mojsov S, Spector D, Habener JF, Hanahan D** 1990 Proglucagon processing similar to normal islets in pancreatic α-like cell line derived from transgenic mouse tumor. Diabetes 39:406–414
46. **Park JG, Oie HK, Sugarbaker PH, Henslee JG, Chen TR, Johnson BE, Gazdar A** 1987 Characteristics of cell lines established from human colorectal carcinoma. Cancer Res 47:6710–6718

47. **De Bruine AP, Dinjens WN, van der Linden EP, Pijls MM, Moerkerk PT, Bosman FT** 1993 Extracellular matrix components induce endocrine differentiation *in vitro* in NCI-H716 cells. Am J Pathol 142:773–782
48. **Powers AC, Philippe J, Hermann H, Habener JF** 1988 Sodium butyrate increases glucagon and insulin gene expression by recruiting immunocytochemically negative cells to produce hormone. Diabetes 37:1405–1410
49. **Drozdowski LA, Dixon WT, McBurney MI, Thomson AB** 2002 Short-chain fatty acids and total parenteral nutrition affect intestinal gene expression. J Parenter Enteral Nutr 26:145–150
50. **Domon-Dell C, Wang Q, Kim S, Kedinger M, Evers BM, Freund JN** 2002 Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells. Gut 50:525–529
51. **Qualtrough D, Hinoi T, Fearon E, Paraskeva C** 2002 Expression of CDX2 in normal and neoplastic human colon tissue and during differentiation of an *in vitro* model system. Gut 51:184–190
52. **Hussain MA, Habener JF** 1999 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
53. **Laser B, Meda P, Constant I, Philippe J** 1996 The caudal-related homeodomain protein Cdx-2/3 regulates glucagon gene expression in islet cells. J Biol Chem 271:28984–28994
54. **Tabuchi Y, Arai Y, Kondo T, Takeguchi N, Asano S** 2002 Identification of genes responsive to sodium butyrate in colonic epithelial cells. Biochem Biophys Res Commun 293:1287–1294
55. **Iacomino G, Tecce MF, Grimaldi C, Tosto M, Russo GL** 2001 Transcriptional response of a human colon adenocarcinoma cell line to sodium butyrate. Biochem Biophys Res Commun 285:1280–1289
56. **Della Ragione F, Criniti V, Della Pietra V, Borriello A, Oliva A, Indaco S, Yamamoto T, Zappia V** 2001 Genes modulated by histone acetylation as new effectors of butyrate activity. FEBS Lett 499:199–204
57. **Smith SB, Ee HC, Conners JR, German MS** 1999 Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Mol Cell Biol 19:8272–8280
58. **Petersen HV, Jorgensen MC, Andersen FG, Jensen J, F-Nielsen T, Jorgensen R, Madsen OD, Serup P** 2000 Pax4 represses pancreatic glucagon gene expression. Mol Cell Biol Res Commun 3:249–254
59. **Drucker DJ, Jin T, Asa SL, Young TA, Brubaker PL** 1994 Activation of proglucagon gene transcription by protein kinase A in a novel mouse enteroendocrine cell line. Mol Endocrinol 8:1646–1655