Activation of Proglucagon Gene Transcription by Protein Kinase-A in a Novel Mouse Enteroendocrine Cell Line

Daniel J. Drucker*, Tianru Jin, Sylvia L. Asa, Tara A. Young, and Patricia L. Brubaker

Departments of Medicine, Pathology, and Physiology
University of Toronto
Toronto, Ontario, Canada

The gene encoding proglucagon is expressed predominantly in the pancreas and intestine. The physiological importance of glucagon secreted from the islets of Langerhans has engendered considerable interest in the molecular control of proglucagon gene transcription in the endocrine pancreas. In contrast, little is known about the molecular control of proglucagon gene expression in the intestine. The recent demonstration that glucagon-like peptide-1 (GLP-1) secreted from the intestine is a potent regulator of insulin secretion and glucose homeostasis has stimulated renewed interest in the factors that control GLP-1 synthesis in the intestinal L-cell. To develop a model for the analysis of intestinal proglucagon gene expression, we have targeted expression of a proglucagon gene-simian virus-40 large T-antigen fusion gene to enteroendocrine cells in transgenic mice. These mice develop intestinal tumors that were used to derive a novel cell line, designated GLUTag, that expresses the proglucagon gene and secretes immunoreactive GLP-1 *in vitro*. GLUTag cells demonstrate morphological characteristics of enteroendocrine cells by electron microscopy and are plurihormonal, as shown by immunocytochemistry and RNA analyses. GLUTag cells express the proglucagon and cholecystokinin genes, consistent with the pattern of lineage-specific enteroendocrine differentiation described for mouse intestine. Proglucagon gene expression was induced by activators of the protein kinase-A pathway, and a combination of messenger RNA half-life and nuclear run-on experiments demonstrated that the protein kinase-A-induction is mediated by an increase in proglucagon gene transcription. In contrast, activators of protein kinase-C stimulated secretion, but not biosynthesis of the PGDPs in GLUTag cell cultures. Analysis of proglucagon processing in GLUTag cells demonstrated the liberation of glucagon, oxyntomodulin, glicentin, and multiple forms of GLP-1. These observations provide evidence for the direct induction of proglucagon gene transcription by a cAMP-dependent pathway and suggest that the GLUTag cell line represents a useful model for the analysis of the molecular determinants of enteroendocrine gene expression. (Molecular Endocrinology 8: 1646–1655, 1994)

0888-8810/94$03.00/0
Molecular Endocrinology

INTRODUCTION

Peptide hormones produced by enteroendocrine cells play central roles in the regulation of intestinal motility, nutrient digestion, and metabolism. One of these peptides, designated glucagon-like peptide-1 (GLP-1), has been shown to be an important regulator of glucose-dependent insulin secretion (1–3). The sequence of GLP-1 was elucidated after isolation of the complementary DNAs and genes encoding proglucagon (4–8). Despite the importance of intestinal GLP-1 for the regulation of glucose homeostasis and insulin secretion, the majority of studies of proglucagon (and GLP-1) biosynthesis have focused on analysis of proglucagon gene expression in the endocrine pancreas. These experiments used a variety of islet cell lines for analysis of the signal transduction pathways that mediate activation of proglucagon gene transcription. Islet cell lines have also been employed for gene transfer studies that have led to the identification of several *cis*-acting DNA sequences and transcription factors important for islet cell-specific proglucagon gene transcription (9–11).

Although much has been learned about the molecular control of proglucagon biosynthesis in the islets, less is known about the control of proglucagon gene expression and GLP-1 biosynthesis in the intestine. Studies using fetal rat intestinal cell cultures demonstrated that secretion of peptides derived from the posttranslational processing of proglucagon (PGDPs) was regulated by a protein kinase-A (PKA)-dependent pathway (12). These cell cultures, although valuable for studies of peptide secretion, represent a mixture of endocrine and

nonendocrine cell types (13) and, hence, are not useful for identification of the molecular factors important for proglucagon gene transcription using gene transfer studies *in vitro*. Furthermore, no simple methodologies for the large scale isolation of PGDP-immunoreactive intestinal L-cells have been reported.

To develop a suitable model for analysis of intestinal proglucagon gene transcription, we used an alternative strategy for the generation of an intestinal proglucagon-producing cell line. This approach involves targeting the expression of an oncogene to a specific cell population in the intestine with subsequent tumor formation, ultimately facilitating the isolation of a proglucagon-producing cell line *in vitro*. Although a number of peptide hormone genes (including the gene encoding proglucagon) are expressed in the gastrointestinal tract in a species- and region-specific fashion, the molecular determinants necessary for targeting transgene expression to enteroendocrine cells are not well understood. Initial studies of proglucagon gene expression in transgenic mice used a proglucagon-simian virus-40 (SV40) T-antigen transgene containing ~1.3 kilobases of proglucagon gene 5′-flanking sequences (14). This transgene was expressed in the pancreas and brain, but not the intestine, of transgenic mice (14), suggesting that proglucagon gene sequences important for directing transgene expression to the intestine were different from the sequences sufficient for transgene expression in the endocrine pancreas. We subsequently constructed a larger transgene containing approximately 2.2 kilobases of proglucagon gene 5′-flanking sequences fused to the coding sequence of SV40 large T-antigen, and transgenic mice expressing this GLUTag fusion gene consistently developed proglucagon-producing endocrine tumors of the large bowel (15).

The reproducible development of intestinal endocrine tumors *in vivo* provided an opportunity to isolate an intestinal cell line for studies of enteroendocrine gene expression. Intestinal tumors isolated from glucagon-SV40 T-antigen transgenic mice were passaged sc *in vivo* (16), and used for the derivation of cell lines *in vitro*. We have now propagated one such cell line, designated GLUTag, for more than 18 months *in vitro*. GLUTag cells synthesize and secrete high levels of the PGDPs in a regulated manner, suggesting that this cell line should be a useful model for studies of the molecular determinants of enteroendocrine gene expression.

RESULTS

GLUTag tumors from the large bowel of transgenic mice were propagated sc *in vivo* (16), and one tumor was excised, mechanically and enzymatically dispersed, after which surviving groups of cells that adhered to plastic dishes were clonally expanded, then pooled and continuously propagated *in vitro*. GLUTag cells propagated *in vitro* reproducibly formed glucagon-producing tumors when transplanted back into nude mice *in vivo*. To ascertain the hormonal phenotype of GLUTag cells *in vitro*, we used a combination of Northern blot analysis and immunocytochemistry. For comparative purposes, we also analyzed peptide hormone messenger RNAs (mRNAs) present in a variety of different endocrine cell lines. GLUTag cells expressed the proglucagon and cholecystokinin (CCK) genes, but mRNA transcripts for insulin, peptide-YY (PYY), somatostatin, and amylin were not detected by Northern analysis of total cellular GLUTag RNA (Fig. 1). The sizes of the proglucagon mRNA transcripts in the two mouse cell lines, GLUTag and STC-1, were slightly smaller than the sizes of rat and hamster proglucagon mRNAs (Fig. 1).

By light microscopy, the cultured cells were variable in size and shape. They had moderate amounts of pale eosinophilic cytoplasm and pleomorphic nuclei with prominent nucleoli. Immunohistochemical staining for chromogranin-A demonstrated variable numbers of cytoplasmic granules in the majority of cells; however, some cells did not exhibit chromogranin immunopositivity (not shown). The majority of cells contained immunopositivity for GLP-1 (Fig. 2a), glucagon, and pancreatic polypeptide (not shown), but the staining intensity was variable from cell to cell. Focal or faint staining was also detected for PYY, secretin, and somatostatin (data not shown). No positive immunostaining was detected with the CCK antisera directed against the first 39 amino acids of CCK. The cells were negative for the remainder of the antisera tested (see *Materials and Methods* for complete description of antisera tested).

The ultrastructural features of the cultured GLUTag cells are demonstrated in Fig. 2b. The cells had moderately well developed cytoplasmic organelles, including short profiles of rough endoplasmic reticulum, juxtanuclear Golgi complexes, numerous mitochondria, and abundant glycogen. Secretory granules were variable in number, but were well defined by a closely apposed, double limiting membrane. Although some granules were large, measuring up to 350 nm in diameter, the majority were smaller than 150 nm, with contents of variable electron density. These features confirm endocrine differentiation and are consistent with cells of enteroendocrine lineage.

The identification of proglucagon mRNA transcripts and GLP-1 immunopositivity in GLUTag cells as well as the demonstration by electron microscopy of abundant secretory granules prompted us to assess whether PGDP synthesis and secretion were regulated in GLUTag cells. Experiments with fetal rat intestinal cell (FRIC) cultures have shown that whereas both cAMP and phorbol 12-myristate 13-acetate (PMA) stimulate the secretion of PGDPs, an increase in proglucagon mRNA transcripts was detected only with activators of the PKA-dependent pathway (12). To identify the factors important for the regulation of intestinal PGDP secretion in GLUTag cells, cultures were incubated with different agents for 2 h, after which the medium was assessed by RIA for glucagon-like immunoreactivity (GLI), immunoreactive glucagon (IRG), glucagon-like peptide-1

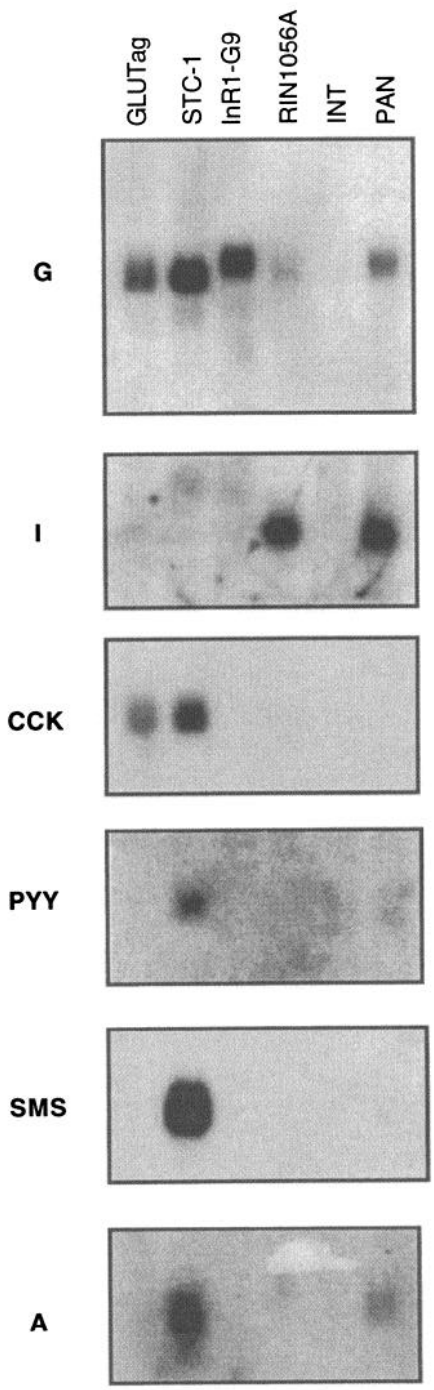


Fig. 1. Northern Blot Analysis of Hormone Gene Expression in the GLUTag Cell Line

Ten micrograms of RNA from GLUTag cells, STC-1 cells, hamster InR1-G9 islet cells, RIN1056A rat islet cells, fetal mouse large intestine (INT), and fetal mouse pancreas (PAN) were size-fractionated on an agarose gel, transferred to a nylon membrane, and hybridized with complementary DNA probes for proglucagon (G), insulin (I), CCK, PYY, somatostatin (SMS), and amylin (A). To avoid overexposure of the autoradiographs, blots were generally exposed to film for 12–36 h. With longer exposures, mRNA transcripts for proglucagon, somatostatin, PYY, and CCK could be detected in RNA from intestine (not shown).

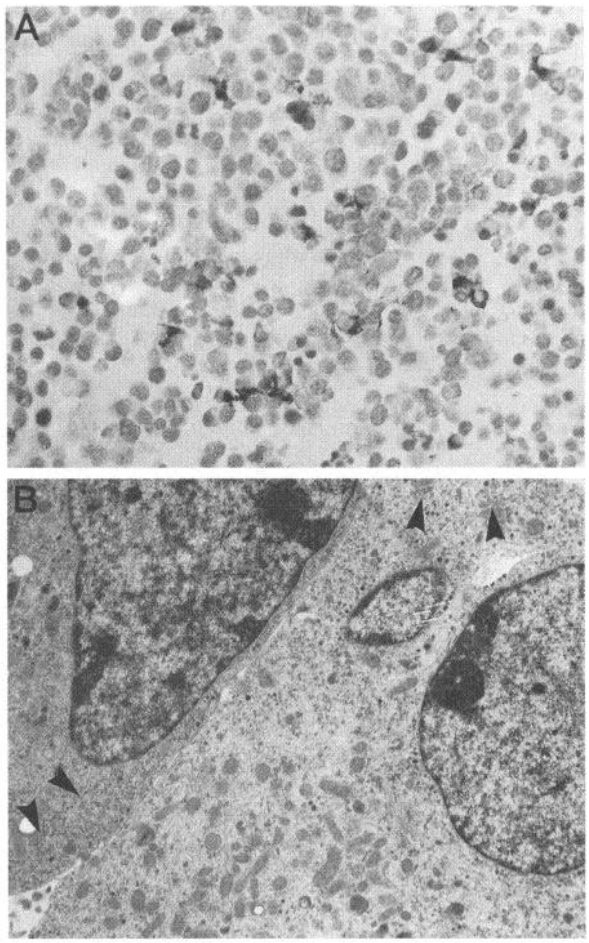


Fig. 2. a, GLP-1 Is Localized with Variable Intensity in Cultured GLUTag Cells

Some are strongly immunopositive with diffuse cytoplasmic reactivity. b, Ultrastructural examination of cultured cells reveals moderately well developed cytoplasmic organelles, including short profiles of rough endoplasmic reticulum (*arrowheads*) and numerous small secretory granules of variable electron density

[GLP-1-(x-37),[1] and GLP-1-(x-36)NH_2. PGDP secretion was stimulated up to 5-fold by activation of the PKA-dependent pathway with forskolin-isobutylmethylxanthine (IBMX), cholera toxin-IBMX, or $(Bu)_2$cAMP (523 ± 28%, 404 ± 20%, and 241 ± 28% of control values, respectively; $P < 0.001$; Fig. 3). In contrast, sodium butyrate, a control for the effects of $(Bu)_2$cAMP and an agent previously shown to stimulate PGDP synthesis and secretion in RIN1056A cells (17), had no effect on secretion of PGDPs in GLUTag cells. The phorbol ester PMA also significantly stimulated PGDP secretion (to 348 ± 12% of the control value; $P < 0.001$). To ascertain whether these agents also stimulated the synthesis of PGDPs, the total PGDP content (the sum of 2 and 22 h media plus cellular peptides) was assessed after

[1] x-37 or x-36 denotes GLP-1 peptides with N-teminal sequences beginning at amino acids 1 or 7.

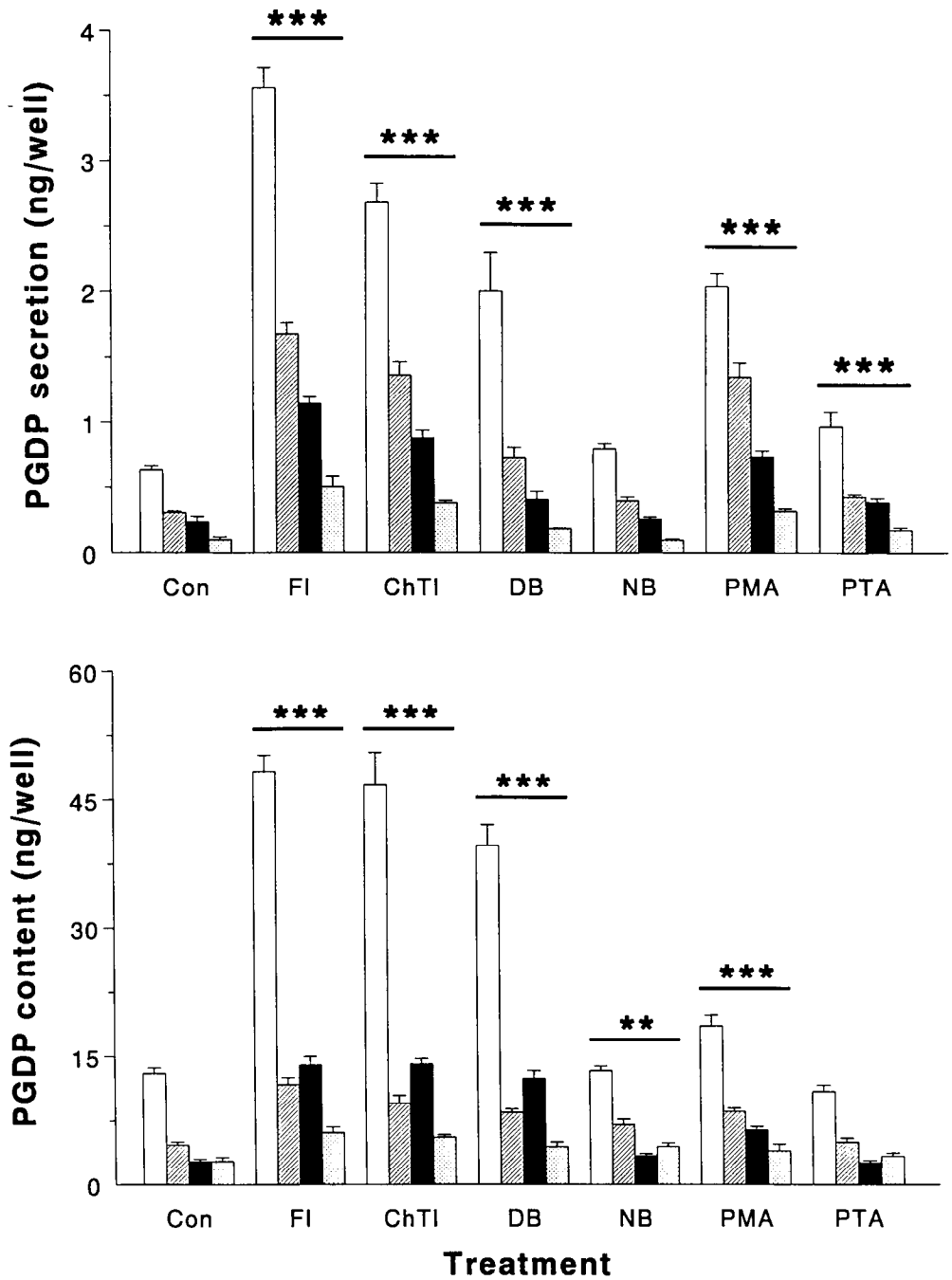


Fig. 3. Effects of Different Agents on the Secretion and Synthesis of PGDPs in GLUTag Cells

GLUTag cells were incubated with control medium (Con) or 10 μM forskolin plus 10 μM IBMX (FI), 5 ng/ml cholera toxin plus 10 μM IBMX (ChTI), 5 mM $(Bu)_2$AMP (DB), 5 mM sodium butyrate (NB), 1 μM PMA, or 1 μM TPA (n = 6 for each treatment). PGDP secretion was determined as a function of the medium content of the PGDPs after a 2-h incubation period, whereas PGDP content was calculated as the 2 and 22 h media plus the cell content of the PGDPs after a 24-h incubation. □, GLI; ▨, IRG; ■, GLP-1-(7–37); ■, GLP-1-(7–36)NH_2. **, $P < 0.01$; ***, $P < 0.001$.

24-h incubation with the test agents (Fig. 3). Activation of PKA clearly stimulated the synthesis of PGDPs in GLUTag cells (to 284 ± 14%, 276 ± 9%, and 269 ± 21% of control values for forskolin-IBMX-, cholera toxin-IBMX-, and $(Bu)_2$cAMP-treated cells, respectively; $P < 0.001$). Small increases in PGDP synthesis were also noted with sodium butyrate and PMA treatment (135 ± 8% and 170 ± 9% of the control value, respectively), but not with phorbol 12,13,20-triacetate.

As RIA does not establish the molecular identity of the specific PGDPs processed from proglucagon in GLUTag cells, HPLC analysis of the cellular peptides

was carried out after 24-h incubations under control conditions or after stimulation with either forskolin-IBMX or PMA. As the profiles obtained for each treatment group were identical, all data were pooled to make n = 3. The normal intestinal L-cell cleaves proglucagon to generate predominantly glicentin, oxyntomodulin, and GLP-1-(7–36)NH_2 (18, 19). The GLUTag cell line differed from the normal L-cell in that significant amounts of glucagon (57 ± 2% of the total GLI) were produced in addition to glicentin, oxyntomodulin, and small amounts of the putative 9-kilodalton (kDa) peptide (17 ± 2%, 20 ± 2%, and 5 ± 1% of the total GLI, respectively; Fig. 4). As in the normal L-cell, however, GLP-1-(7–36)NH_2 was the predominant GLP-1-containing peptide synthesized (78 ± 7% of the total GLP-1 immunoreactivity), whereas only smaller amounts of the biologically active GLP-1-(7–37) and inactive GLP-1-(1–36)NH_2 and GLP-1-(1–37) were produced (10 ± 2%, 10 ± 5%, and 2 ± 1% of the total GLP-1, respectively). Finally, as similar amounts of the N-terminal PGDPs (as assessed by total GLI) and the C-terminal PGDPs (as assessed by total GLP-1) were found after HPLC analysis, these data suggest that processing of proglucagon to different molecular forms of GLP-1 in GLUTag cells is complete, with little or no high mol wt GLP-immunoreactive forms detected.

To ascertain whether forskolin induction of PGDP synthesis was mediated in part by an increase in proglucagon gene expression, GLUTag cells were incubated with either PMA or forskolin-IBMX, and the levels of proglucagon mRNA transcripts were determined by Northern blot analysis. No increase in proglucagon mRNA transcripts was detected after exposure of the cells to PMA (data not shown). In contrast, forskolin consistently increased the levels of proglucagon mRNA transcripts, and this increase was maximal by 12 h (Fig. 5A). Furthermore, the levels of CCK and tubulin mRNA transcripts were not significantly increased by forskolin in the same experiment. To ascertain the mechanism for the forskolin induction of proglucagon mRNA, GLUTag cells were incubated with forskolin-IBMX in the presence of the transcriptional inhibitor actinomycin-D. A small decrease in the levels of proglucagon mRNA transcripts was detected after a 12-h incubation with actinomycin-D alone, consistent with the known half-life (20) of proglucagon mRNA transcripts (Fig. 5B). Actinomycin-D almost completely inhibited the forskolin induction of proglucagon gene expression, suggesting that the increase in proglucagon mRNA transcripts was primarily attributable to an increase in proglucagon gene transcription. No induction of actin mRNA transcripts by forskolin was detected in the same experiment (Fig. 5B). To provide more direct evidence for the effect of forskolin on proglucagon gene transcription, nuclear run-on experiments were carried out after treatment of GLUTag cells with vehicle alone or forskolin-IBMX. The results of this experiment (Fig. 6) clearly demonstrated that forskolin increased the transcription rate of the proglucagon gene (6-fold by densitometry) in GLUTag cells, whereas no comparable induction of the transcrip-

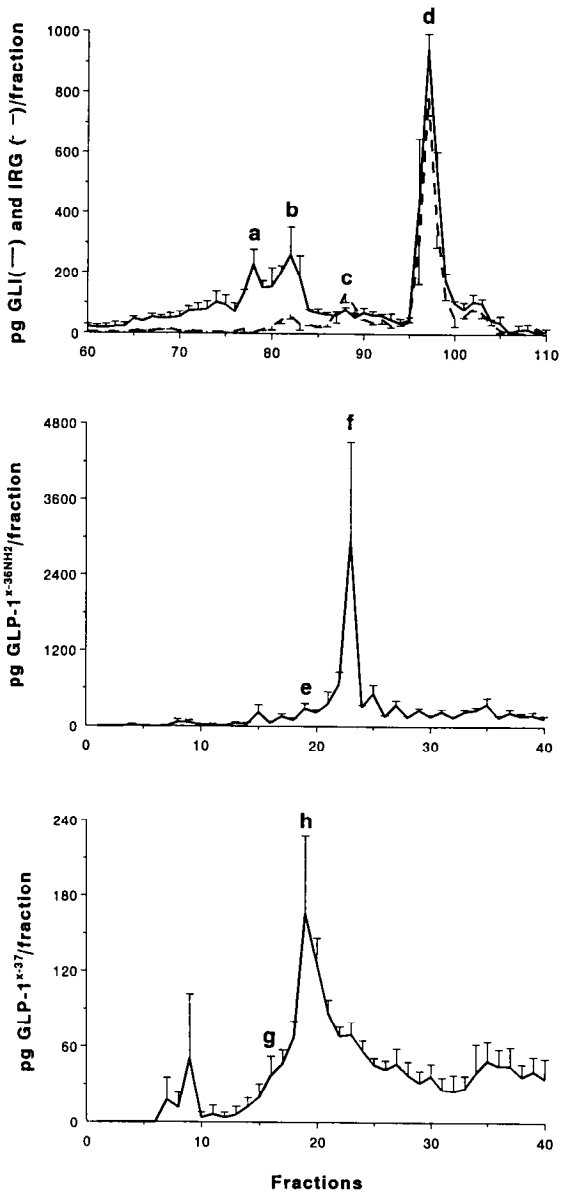


Fig. 4. HPLC Analysis of Immunoreactive PGDPs Extracted from GLUTag Cells after 24-h Treatment under Control Conditions or with 10 μM Forskolin plus 10 μM IBMX or 5 ng/ml Cholera Toxin plus 10 μM IBMX (n = 1 for Each Treatment, Combined to Make n = 3 for Each Profile)

An equivalent amount of cell extract was loaded onto the column (10 ng GLI) for each analysis. a, Glicentin; b, oxyntomodulin; c, 9-kDa peptide; d, glucagon; e, GLP-1-(1–36)NH_2; f, GLP-1-(7–36)NH_2; g, GLP-1-(1–37); h, GLP-1-(7–37). The elution positions of oxyntomodulin, glucagon, and the four GLP-1-related peptides were determined by comparison with the elution positions of synthetic peptide standards, whereas the elution position of glicentin was determined by comparison with the results of previous studies (13, 41, 42, 48). The 9-kDa peptide was tentatively identified on the basis of immunoreactivity and elution position relative to those of the other GLI peptides. For chromatography of GLI peptides, an internal radioactive standard was added to all samples, whereas for chromatography of GLP-1-related peptides, standard peptides were run both before and after samples. The elution times of the internal standard and standard peptides varied by less than 2% between runs.

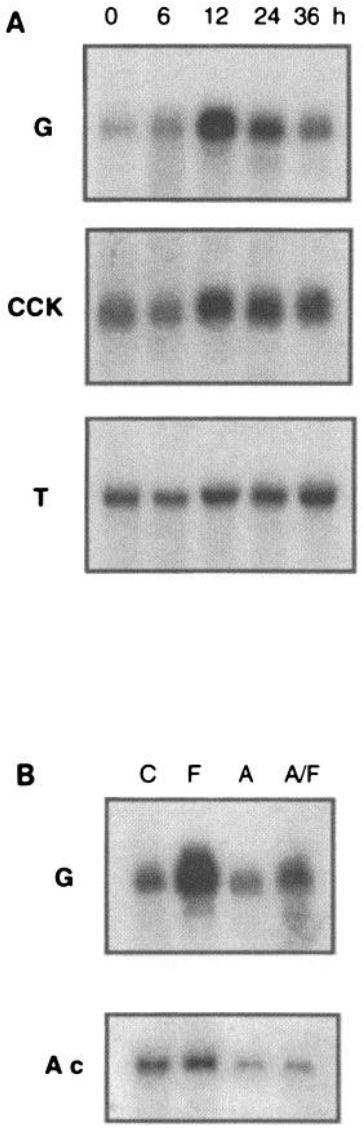


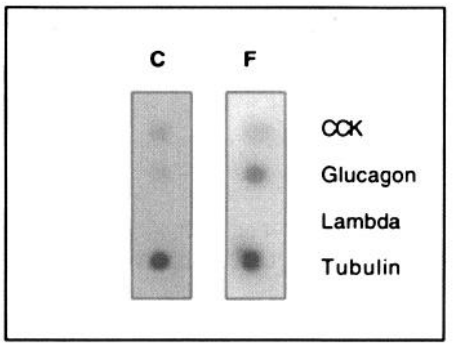
Fig. 5. A, Regulation of Proglucagon Gene Expression

GLUTag cells were treated with 10 μM forskolin and 10 μM IBMX for 6–36 h, and RNA was isolated for Northern blot analysis. G, Proglucagon; T, tubulin mRNA transcripts. B, Effect of actinomycin-D on forskolin induction of proglucagon mRNA. GLUTag cells were incubated for 12 h in DMEM alone (C) or in DMEM supplemented with 10 μM forskolin, 10 μM IBMX (F), 5 μg/ml actinomycin-D (A), or forskolin/IBMX plus actinomycin-D (A/F). G, Glucagon; Ac, actin mRNA transcripts.

tional activity of the CCK or tubulin genes was observed.

DISCUSSION

The four principal epithelial cell types of the intestine, enterocytes, Goblet, Paneth, and enteroendocrine cells, are derived from a common multipotential stem cell (21). The enteroendocrine cell population is highly com-



Nuclear run on analysis of gene transcription in GLUTag cells

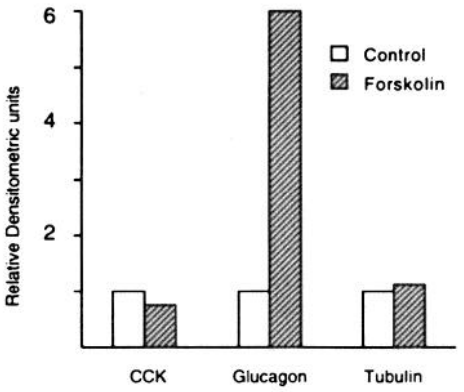


Fig. 6. Nuclear Run-on Analysis of Gene Transcription in GLUTag Cells

Nuclei were isolated from GLUTag cells treated with vehicle alone (C) or from cells treated with 10 μM forskolin plus 10 μM IBMX (F) for 90 min. Immobilized DNAs on the membrane include CCK, glucagon, λ-phage, and tubulin. The graph represents the densitometric quantification of the relative signals after computerized scanning of the images with densitometric software.

plex, exhibiting species- and region-specific differences in distribution as well as in hormone expression throughout the gastrointestinal tract (22). Immunocytochemical studies of enteroendocrine cell differentiation in normal mouse intestine have demonstrated several populations of endocrine cells that contain one or more peptide hormones (22). Enteroglucagon and PYY as well as neurotensin have been shown to be colocalized within the same enteroendocrine cell. The hormone most commonly colocalized with GLP-1 in mouse large intestine is CCK, with 48% of GLP-1-immunoreactive cells containing both immunoreactive GLP-1 and CCK (23). These observations are consistent with our observations that GLUTag cells express both the proglucagon and CCK genes, suggesting that the hormonal phenotype of the GLUTag cell line may be representative of a distinct lineage of mouse enteroendocrine cells.

The majority of studies to date analyzing the control of proglucagon gene transcription have used islet cell lines derived from tumors arising from the endocrine pancreas (10, 20, 24, 25). These experiments have

defined the signal transduction pathways and *cis*-acting DNA sequences important for control of pancreatic proglucagon gene transcription. In contrast, very little is known about the regulation of proglucagon biosynthesis in the intestine, and several lines of evidence suggest that important differences may exist in the control of islet *vs.* intestinal proglucagon gene expression. Studies using the rat islet cell RIN1056A have suggested that pancreatic proglucagon gene transcription may be regulated by a protein kinase-C-dependent pathway (20), whereas PMA had no effect on proglucagon mRNA transcripts in GLUTag cells or in studies using FRIC cultures (12). Furthermore, experiments using transgenic mice to define tissue-specific domains important for proglucagon gene transcription have suggested that the proglucagon gene sequences required for targeting transgene expression to the pancreas and nervous system are not sufficient for expression in the intestine (14, 15). The isolation of a novel proglucagon-producing enteroendocrine cell line represents an important advance for studies of both the regulation and tissue-specific determinants of intestinal proglucagon gene expression.

The observation that proglucagon gene transcription in GLUTag cells is regulated by a PKA-dependent pathway represents the first direct demonstration that cAMP activates proglucagon gene expression at the level of gene transcription. Although proglucagon mRNA transcripts are regulated by a PKA-dependent pathway in primary cultures of rat islet and intestinal cells (12, 26), studies using immortalized islet cell lines have not demonstrated direct cAMP-dependent induction of proglucagon gene transcription (10, 24). Previous studies of proglucagon gene expression using primary cell cultures have shown that proglucagon mRNA was regulated by activators of PKA, but the precise mechanism for this effect (either a prolongation of the mRNA $t_{1/2}$ or transcriptional induction) had not been ascertained. Furthermore, islet cell lines that express proglucagon exhibit a defect in cAMP-mediated gene expression and have not proven useful for analysis of the cAMP-dependent transcriptional control of proglucagon gene expression (24, 27). More recent studies have circumvented this limitation by transfecting islet cell lines with the catalytic subunit of PKA. The results of these experiments have mapped a putative proglucagon gene PKA-response element to a specific cAMP response element sequence in the 5′-flanking region of the rat proglucagon gene from −292 to −298 that binds cAMP response element-binding protein and mediates activation of reporter gene expression by a cotransfected catalytic subunit of PKA (26, 28, 29).

The posttranslational processing of proglucagon in GLUTag cells exhibited both similarities and differences compared with the nonimmortalized intestinal L-cell. Consistent with normal intestine, GLP-1-(7–36)NH_2 was the predominant GLP-1-containing peptide in cell extracts. In contrast, the predominant molecular species derived from the N-terminal end of proglucagon was glucagon, whereas little or no glucagon is usually found in normal L-cells (19, 30). This aberrant pattern of processing was initially observed in the original intestinal tumors in transgenic mice, which contained approximately equivalent amounts of glicentin, oxyntomodulin, and glucagon (15).

The posttranslational processing of proglucagon has also been previously analyzed in a number of different tumor-derived islet and intestinal cell lines. Although mouse α-TC-1 islet cells process proglucagon in a pancreatic-specific manner (25), an aberrant pattern of proglucagon processing, inconsistent with the expected cellular phenotype, was detected in studies of InR1-G9, RIN1056A, and STC-1 cells (10, 24, 31). Aberrant processing in these cell lines was also reflected by the detection of a spectrum of PGDPs that overlapped the expected pancreatic- and intestinal-specific patterns of proglucagon processing. Furthermore, propagation of GLUTag tumors sc *in vivo* is associated with a switch in the pattern of proglucagon processing away from that exhibited by the normal intestinal L-cell (16). The mechanism(s) underlying these cellular switches in the phenotype of proglucagon processing is not known, but is probably attributable to differential expression of the prohormone convertases, enzymes that may be responsible for the tissue-specific patterns of proglucagon processing (32).

Curiously, although Northern blot analysis detected CCK mRNA transcripts in GLUTag RNA, no CCK-immunopositive GLUTag cells were observed using antisera directed against CCK-(1–39). In contrast, the identical antisera consistently detected CCK-immunopositive cells in sections from control mouse intestine. These observations suggest that either the CCK mRNA is not translated in GLUTag cells or, alternatively, the CCK prohormone is aberrantly processed or degraded, precluding recognition by the antisera used here. Evidence for incomplete processing of CCK *in vivo* has been obtained from studies of CCK expression in the pituitary (33). Furthermore, although CCK mRNA is readily detectable in porcine cerebellum, no immunoreactive CCK peptides could be detected in the same tissue using a panel of CCK-specific antisera (34). Taken together, the detection of CCK mRNA, but not immunoreactive CCK, in GLUTag cells suggests that this cell line may also be useful for studies of the control of CCK translation *in vitro*.

Previous transgenic experiments that involved mating two lines of transgenic mice (rat insulin promoter-SV40 *Tag* × rat insulin promoter-polyoma *Tag*) resulted in the establishment of several lines of double transgenics, one line of which developed endocrine tumors of the small intestine, presumably due to an integration effect (35). Although the precise cellular and embryological derivation of these rapidly metastasizing tumors could not be defined, a secretin tumor cell line (designated STC-1) established from these tumors produced large amounts of secretin as well as GLP-1, neurotensin, and pancreatic polypeptide *in vitro* (36). In contrast to the profile of hormone gene expression exhibited by GLUTag cells, the plurihormonal STC-1 intestinal cell

line does not recapitulate the normal pattern of hormone gene expression seen in intestinal enteroendocrine cells and, hence, may not be representative of the L-cell *in vitro* (35). In contrast, the molecular and cellular phenotype of the GLUTag cell line described here suggests that GLUTag cells should be useful for identification of the specific *cis*-acting proglucagon gene sequences and the molecular factors important for intestinal-specific proglucagon gene transcription.

MATERIALS AND METHODS

Cell Culture

GLUTag tumor cells were derived from solid GLUTag tumors propagated in nude mice, as previously described (16). A solid GLUTag tumor was grown *in vivo* for 1 month, excised, and minced into small pieces. After two cycles of agitation at 37 C with trypsin, dispersed cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. After 4 weeks, surviving clones of neuroendocrine cells were pooled and propagated *in vitro*. The GLUTag cell line characterized here was isolated by picking a single isolated clone from a plate of surviving endocrine tumor cells. Cells were trypsinized and passaged every 3–4 days and grown continuously for over 18 months (100 passages) without any change in hormonal phenotype, as assessed by Northern blot analysis and immunocytochemistry.

RNA Isolation and Analysis

Total cellular RNA was isolated using the acid-ethanol precipitation method, as previously described (37). For Northern blotting, RNA was size-fractionated on a formaldehyde-agarose gel, and the gel was stained with ethidium bromide to assess the loading and integrity of the RNA. The RNA was subsequently transferred to a nylon membrane and fixed with UV light, and hybridization and washing were carried out as previously described (12). Nuclear run-on assays were carried out as described previously (20, 38).

Peptide Analysis

For examination of PGDP synthesis and secretion, cells were grown to 90% confluence in 24-well dishes and incubated with test agents in DMEM containing 0.5% (vol/vol) fetal bovine serum. Test agents included DMEM alone (control), 10 μM forskolin plus 10 μM IBMX, 5 ng/ml cholera toxin plus 10 μM IBMX, 5 mM $(Bu)_2$AMP, 5 mM sodium butyrate, 1 μM PMA, and 1 μM phorbol 12,13,20-triacetate (negative control). Cell medium was collected after 2 h and replaced with identical medium, then medium and cells were collected after an additional 22-h incubation. Peptides were extracted from medium and cells by acidification and adsorption to C_{18} silica, as previously described (39–41). Briefly, cell extracts were homogenised twice in 2 ml extraction medium [1 N HCl containing 5% (vol/vol) formic acid, 1% (vol/vol) trifluoroacetic acid (TFA), and 1% (wt/vol) NaCl] and centrifuged at 1300 × *g* for 10 min. Supernatants were collected and passed twice through a C_{18} silica cartridge (C_{18} Sep-Pak, Waters Associates, Milford, MA); adsorbed peptides were eluted with 4 ml 80% (vol/vol) isopropanol-0.1% (vol/vol) TFA and stored at −70 C, as previously described (12, 42–45). These extractions methods have previously been shown to permit greater than 88% recovery of intact PGDPs from tissues (13, 42).

HPLC

PGDPs contained in tissue extracts were separated on the basis of hydrophobicity, using a Waters Associates Liquid Chromatography System and a C_{18} μBondpak column (Waters Associates), as previously described (16, 45). N-Terminally-derived products of the proglucagon precursor were separated using a 45-min linear gradient of 25–62.5% (vol/vol) solvent B [solvent A, 1% (vol/vol) TFA buffered with diethylamine to pH 2.5; solvent B, 80% (vol/vol) acetonitrile], followed by a 10-min purge at 99% (vol/vol) solvent B. The flow rate was 1.5 ml/min, and fractions were collected every 0.3 min. Full-length and truncated forms of GLP-1 were separated using a 30-min linear gradient of 45–68% (vol/vol) solvent D [solvent C, 0.1% (vol/vol) phosphoric acid and 0.3% (vol/vol) triethylamine, buffered with NaOH to pH 7.0; solvent D, 60% (vol/vol) acetonitrile and 40% (vol/vol) solvent C], followed by a 10-min purge at 99% (vol/vol) D. The flow rate was 1.0 ml/min, and fractions were collected every minute.

Assays

Aliquots of cell extracts and HPLC fractions were dried *in vacuo* before assay. Products of N-terminal processing of proglucagon (glicentin, oxyntomodulin, 9-kDa peptide, and glucagon) were analyzed by RIA using two different antisera, as previously described (16, 45). 1) Antiserum K4023 (Biospacific, Emeryville, CA) recognizes the midsequence of glucagon and, therefore, detects the GLI peptides (glicentin, oxyntomodulin, 9-kDa peptide, glucagon, and proglucagon). 2) Antiserum 04A (Dr. R. H. Unger, Dallas, TX) recognizes only the free C-terminal end of glucagon, cross-reacting with glucagon and the 9-kDa peptide, and thereby measures IRG. The detection range of both GLI and IRG assays was 4–400 pg/tube.

C-Terminally processed products of proglucagon, GLP-1-(1–37), GLP-1-(7–37), GLP-1-(1–36)NH_2, and GLP-1-(7–36)NH_2, were detected using two different antisera. 1) The b5 antiserum (a gift from Dr. S. Mojsov, New York, NY) recognizes the free C-terminal end of GLP-1-(x-37) and, therefore, detects both GLP-1-(1–37) and GLP-1-(7–37) (19, 46). 2) C-Terminally amidated forms of GLP-1 were detected using the GLP-1-(7–36)NH_2 antiserum (Affinity Research, Nottingham, United Kingdom), which recognizes both GLP-1-(1–36)NH_2 and GLP-1-(7–36)NH_2. The detection limits for the GLP-1-(x-37) and GLP-1-(x-36)NH_2 assays were 1–160 and 3–800 pg/tube, respectively.

Morphological Studies

Cultured cells were trypsinized, harvested by centrifugation, washed with medium and PBS, and centrifuged into pellets. For light microscopy and immunocytochemistry, the cell pellets were fixed in 10% buffered formalin, dehydrated in graded ethanols, and embedded in paraffin. Sections 4–6 μm thick were stained with hematoxylin and eosin. For immunohistochemistry, the streptavidin-biotin-peroxidase complex technique was used with primary antisera directed against the following antigens and used at the specified dilutions: antisera against insulin (Biomeda), prediluted; glucagon (Biomeda), prediluted; GLP-1 (prepared by D. Drucker), 1:1000; PYY (Peninsula Laboratories, Belmont, CA), 1:1500; somatostatin (Dako, Copenhagen, Denmark), 1:2000; pancreatic polypeptide (Dako), 1:1000; calcitonin (Biomeda), prediluted; bombesin and cholecystokinin (Serotec, Oxon, United Kingdom), 1:2000 and 1:5000, respectively; gastrin (Diagnostic Products Corp., Los Angeles, CA), prediluted; vasoactive intestinal peptide (Zymed), 1:400; secretin (Biogenex), 1:200; ACTH (Dako), 1:600; and CRH (Peninsula Laboratories, Belmont, CA), 1:1000. Monoclonal antibodies were used to localize chromogranin-A (ENZO Diagnostics, New York, NY; 0.48 mg/ml), synaptophysin (Dako; 43 μg/ml), GH-releasing hormone (donated by Dr. T. Sano, University of Tokushima Medical School, Tokushima Japan; 1:30), serotonin (Dako; 1:30), neurofilaments (Sanbio, Uden, Holland; 1:10), and carcinoembryonic antigen (Zymed Laboratories, San Francisco, CA; 1:20). The

specificity of the immunostaining was verified by using both positive and negative controls, as previously described (47).

For electron microscopy, pellets were fixed in 2.5% (wt/vol) glutaraldehyde in Sorensen's buffer, postfixed in 1% (wt/vol) OsO_4 in Millonig's buffer, dehydrated in graded ethanols, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope (Philips, Mahway, NJ).

Statistics

Statistical significance was determined by analysis of variance, using a Statistical Analysis System (SAS, Cary, NC) program for IBM computers. In experiments analyzing PGDP secretion and content, data for each of the four RIAs were analyzed separately and then combined for analysis; the trends were found to be identical, and therefore, only the results of the combined analyses are shown.

Acknowledgments

Received July 18, 1994. Revision received August 23,1 994. Accepted September 21, 1994.

Address requests for reprints to: Dr. D. Drucker, Toronto General Hospital, 200 Elizabeth Street, Toronto, Canada M5G 2C4.

This work was supported by grants from the Canadian Diabetes Association and the Medical Research Council of Canada.

* Supported by a Scientist Award from the Medical Research Council of Canada.

REFERENCES

1. Mojsov S, Weir GC, Habener JF 1987 Insulinotropin: Glucagon-like peptide I (7–37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. J Clin Invest 79:616–619
2. Holst JJ, Orskov C, Nielsen OV, Schwartz TW 1987 Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut. FEBS Lett 211:169–174
3. Kreymann B, Ghatei MA, Williams G, Bloom SR 1987 Glucagon-like peptide-1 7–36: A physiological incretin in man. Lancet ii:1300–1304
4. Lund PK, Goodman RH, Habener JF 1981 Pancreatic preproglucagons are encoded by two separate mRNAs. J Biol Chem 256:6515–6518
5. Bell GI, Santerre RF, Mullenbach GT 1983 Hamster preproglucagon contains the sequence of glucagon and two related peptides. Nature 302:716–718
6. White JW, Saunders GF 1986 Structure of the human glucagon gene. Nucl Acids Res 14:4719–4730
7. Bell GI, Sanchez-Pescador R, Laybourn PJ, Najarian RC 1983 Exon duplication and divergence in the human preproglucagon gene. Nature 304:368–371
8. Heinrich G, Gros P, Lund PK, Bentley RC, Habener JF 1984 Pre-proglucagon messenger ribonucleic acid: Nucleotide and encoded amino acid sequences of the rat pancreatic complementary deoxyribonucleic acid. Endocrinology 115:2176–2181
9. Drucker DJ, Philippe J, Jepeal L, Habener JF 1987 Glucagon gene 5′-flanking sequences promote islet cell-specific glucagon gene transcription. J Biol Chem 262:15659–15665
10. Philippe J, Drucker DJ, Knepel W, Jepeal L, Misulovin Z, Habener JF 1988 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
11. Knepel W, Jepeal L, Habener JF 1990 A pancreatic islet cell-specific enhancer-like element in the glucagon gene contains two domains binding distinct cellular proteins. J Biol Chem 265:8725–8735
12. Drucker DJ, Brubaker PL 1989 Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. Proc Natl Acad Sci 86:3953–3957
13. Brubaker PL, Vranic M 1987 Fetal rat intestinal cells in monolayer culture: A new *in vitro* system to study the glucagon-like immunoreactive peptides. Endocrinology 120:1976–1985
14. Efrat S, Teitelman G, Anwar M, Ruggiero D, Hanahan D 1988 Glucagon gene regulatory region directs oncoprotein expression to neurons and pancreatic alpha cells. Neuron 1:605–613
15. Lee YC, Asa SL, Drucker DJ 1992 Glucagon gene 5′-flanking sequences direct expression of SV40 large T antigen to the intestine producing carcinoma of the large bowel in transgenic mice. J Biol Chem 267:10705–10708
16. Drucker DJ, Lee YC, Asa SL, Brubaker PL 1992 Inhibition of pancreatic glucagon gene expression in mice bearing a sc glucagon-producing GLUTag transplantable tumor. Mol Endocrinol 6:2175–2184
17. 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
18. Baldissera FGA, Holst JJ 1984 Glucagon-related peptides in the human gastrointestinal mucosa. Diabetologia 26:223–228
19. 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
20. 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
21. Cheng H, Leblond CP 1974 Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. Am J Anat 141:537–562
22. Roth KA, Hertz JM, Gordon JI 1990 Mapping enteroendocrine cell populations in transgenic mice reveals an unexpected degree of complexity in cellular differentiation within the gastrointestinal tract. J Cell Biol 110:1791–1801
23. Roth KA, Kim S, Gordon JI 1992 Immunocytochemical studies suggest two pathways for enteroendocrine cell differentiation in the colon. Am J Physiol 263:G174-G180
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. Powers AC, Efrat S, Mojsov S, Spector D, Habener JF, Hanahan D 1990 Proglucagon processing similar to normal islets in pancreatic a-like cell line derived from transgenic mouse tumor. Diabetes 39:406–414
26. 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
27. 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
28. 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
29. Miller CP, Lin JC, Habener JF 1993 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
30. Orskov C, Holst JJ, Poulsen SS, Kirkegaard P 1987

Pancreatic and intestinal processing of proglucagon in man. Diabetologia 30:874–881
31. Ehrlich P, Tucker D, Asa SL, Brubaker PL, Drucker DJ 1994 Inhibition of pancreatic proglucagon gene expression in mice bearing sc endocrine tumors. Am J Physiol Endocrinol Metab in press:
32. Rouillé Y, Westermark G, Martin SK, Steiner DF 1994 Proglucagon is processed to glucagon by prohormone convertase PC2 in aTC1–6 cells. Proc Natl Acad Sci USA 91:3242–3246
33. Rehfeld JF 1986 Accumulation of nonamidated progastrin and preprocholecystokinin products in porcine pituitary corticotrophs. J Biol Chem 261:5841–5847
34. Gubler U, Chua AO, Young D, Fan Z-W, Eng J 1987 Cholecystokinin mRNA in porcine cerebellum. J Biol Chem 262:15242–15245
35. Grant SGN, Seidman I, Hanahan D, Bautch VL 1991 Early invasiveness characterizes metastatic carcinoid tumors in transgenic mice. Cancer Res 51:4917–4923
36. Rindi G, Grant SGN, Yiangou Y, Ghatei MA, Bloom SR, Bautch VL, Solcia E, Polak JM 1990 Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Am J Pathol 136:1349–1363
37. Allinson ET, Drucker DJ 1992 PTH-like peptide shares features with members of the early response gene family: rapid induction by serum, growth factors, and cycloheximide. Cancer Res 52:3103–3109
38. Li X, Drucker DJ 1994 PTH-related peptide is a downstream target for ras and src activation. J Biol Chem 269:6263–6266
39. Brubaker P, Vranic M 1987 Glucagon-like immunoreactive peptides in a rat ileal epithelial cell line (IEC-18). Endocrine Res 13:229–241
40. Brubaker P 1987 Ontogeny of glucagon-like immunoreactive peptides in rat intestine. Regul Peptides 17:319–326
41. Brubaker PL, So DCY, Drucker DJ 1989 Tissue-specific differences in the levels of proglucagon-derived peptides in streptozotocin-induced diabetes. Endocrinology 124:3003–3009
42. Brubaker P 1988 Control of glucagon-like immunoreactive peptide secretion from fetal rat intestinal cultures. Endocrinology 123:220–226
43. Brubaker PL, Drucker DJ, Asa SL, Greenberg GR 1991 Regulation of peptide-YY synthesis and secretion in fetal rat intestinal cell cultures. Endocrinology 129:3351–3358
44. Lee YC, Brubaker PL, Drucker DJ 1990 Developmental and tissue-specific regulation of proglucagon gene expression. Endocrinology 127:2217–2222
45. Brubaker PL, Lee YC, Drucker DJ 1992 Alterations in proglucagon processing and inhibition of proglucagon gene expression in glucagon-SV40 T antigen transgenic mice. J Biol Chem 267:20728–20733
46. 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
47. Asa SL, Henderson J, Goltzman D, Drucker DJ 1990 PTH-like peptide in normal and neoplastic human endocrine tissues. J Clin Endocrinol Metab 71:1112–1118
48. Kervran A, Blache P, Bataille D 1987 Distribution of oxyntomodulin and glucagon in the gastrointestinal tract and the plasma of the rat. Endocrinology 121:704–713