Human glucagon gene promoter sequences regulating tissue-specific versus nutrient-regulated gene expression

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Nian, Min, Jun Gu, David M. Irwin, and Daniel J. Drucker. Human glucagon gene promoter sequences regulating tissue-specific versus nutrient-regulated gene expression. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R173–R183, 2002; 10.1152/ajpregu.00215.2001.—The glucagon-like peptides (GLPs) are synthesized and secreted in a nutrient-dependent manner in rodents; however, the factors regulating human GLP-1 and GLP-2 biosynthesis remain unclear. To understand how nutrients regulate human proglucagon gene expression, we studied the expression of a human proglucagon promoter-growth hormone (GH) transgene in 1.6 human glucagon-GH transgenic mice. Fasting-refeeding significantly decreased and increased the levels of circulating mouse insulin and transgene-derived hGH ($P <$ 0.05 fasting vs. refeeding) and decreased and upregulated, respectively, the levels of endogenous mouse proglucagon RNA in the ileum but not in the jejunum or colon. High-fiber feeding significantly increased the levels of glucose-stimulated circulating hGH and upregulated levels of mouse intestinal proglucagon gene expression in the jejunum, ileum, and colon ($P <$ 0.05, 0 vs. 30% fiber diet). In contrast, neither fasting-refeeding nor a high-fiber diet upregulated the expression of the human proglucagon promoter-hGH transgene. These findings demonstrate that human proglucagon gene regulatory sequences specifying tissue-specific expression in gut endocrine cells are not sufficient for recognition of energy-derived signals regulating murine glucagon gene expression in enteroendocrine cells in vivo.

nutrition; gut; glucagon-like peptide 1; glucagon-like peptide 2; diet

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THE MAMMALIAN PROGLUCAGON gene is expressed in the α-cells of pancreatic islets, enteroendocrine L cells of the small and large intestine, and neuronal cell bodies in the brain stem (14, 26). Tissue-specific posttranslational processing of proglucagon results in the liberation of a distinct profile of proglucagon-derived peptides (PGDPs) in each tissue. Whereas 29-amino acid glucagon is the principal bioactive PGDP generated in the pancreas, glicentin, oxyntomodulin, and two glucagon-like peptides (GLP), GLP-1 and GLP-2, are produced in the intestine and central nervous system (13).

The actions of the PGDPs converge on the regulation of nutrient assimilation and energy homeostasis. Glucagon is released from islet α-cells in response to a decrease in blood glucose and promotes restoration of circulating blood glucose via effects on hepatic glycogenolysis and gluconeogenesis in both normal subjects and in patients with diabetes (12). GLP-1 is a potent glucose-dependent insulinotropic hormone that regulates nutrient intake and disposal via effects on feeding behavior, gastrointestinal motility, and stimulation and inhibition of insulin and glucagon secretion, respectively (13, 21). GLP-2 acts more proximally in the control of energy absorption via effects on gastric emptying (43), intestinal hexose transport (10), mucosal barrier function (3), and both proliferation and apoptosis in the gastrointestinal epithelium (13, 15, 42).

The therapeutic potential of the intestinal PGDPs, specifically GLP-1 and GLP-2 for the treatment of diabetes and intestinal diseases, has engendered considerable interest in the mechanisms regulating control of intestinal PGDP synthesis and secretion. Nutrients, vagal nerve innervation, and peptide hormones constitute key components of the physiological circuits regulating PGDP secretion from rodent and human enteroendocrine L cells (23, 33–36, 44). Of these factors, nutrients consistently stimulate secretion of PGDPs from both rodent and human intestinal L cells; however, the intracellular signaling mechanisms that mediate endocrine cell recognition of luminal nutrient content remain poorly understood (8).

Although nutrients upregulate the levels of proglucagon mRNA transcripts in specific regions of the rat gastrointestinal tract, the molecular factors regulating intestinal proglucagon gene transcription in the basal and nutrient-stimulated state remain poorly understood. Furthermore, the majority of studies examining the control of proglucagon gene expression have focused on analysis of the rodent proglucagon gene promoter (29). Although rat proglucagon gene 5′-flanking sequences that target reporter gene expression to enteroendocrine L cells in mice have been identified (24), the pathways and molecules coupling nutrient intake to activation of intestinal proglucagon gene expression have not been elucidated. Furthermore, even less is known about the regulation of human proglucagon

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gene expression in the basal and nutrient-stimulated state.

To understand the mechanisms regulating human GLP-1 and GLP-2 biosynthesis, we initiated studies directed at characterizing mechanisms important for regulation of human proglucagon gene expression. Remarkably, although the human and rat proglucagon gene promoters exhibit significant sequence conservation and identity within the proximal 5′-untranslated and 5′-flanking region (20, 27), the activities of the rat and human promoters exhibit significant functional divergence after transfection of islet and intestinal cell lines in vitro and in analysis of tissue-specific expression patterns in transgenic mice in vivo (27).

These observations emphasize that the DNA sequences and transcription factors regulating expression of the rat proglucagon gene promoter may not be directly applicable to understanding the transcriptional control of human proglucagon gene expression. Moreover, the lack of human intestinal endocrine cell lines that produce human GLP-1 and GLP-2 further complicates efforts focused on studies of human proglucagon gene transcription. As a first step toward the analysis of human proglucagon gene expression in differentiated enteroendocrine cells, we generated 1.6 human glucagon-growth hormone (hGLU-GH) transgenic mice that express the hGH reporter gene under the control of human proglucagon gene 5′-flanking sequences. These mice express hGH mRNA transcripts in the gastrointestinal tract, with hGH immunoreactivity localized to enteroendocrine L cells in the epithelial mucosa (27). To identify factors regulating the physiological control of human proglucagon gene transcription, we have now studied the expression of both the endogenous murine proglucagon gene and the hGH transgene after manipulation of the nutrient content of the murine gastrointestinal tract in vivo.

MATERIALS AND METHODS

Reagents. Reagents and chemicals were purchased from Bioshop Canada (Burlington, Ontario, Canada), Caledon (Georgetown, Ontario, Canada), Difco Laboratories (Detroit, MI), Pharmacia (Baie d'Urfe, Quebec, Canada), Sigma Chemical (St. Louis, MO), and Canadian Life Technologies (Burlington, Ontario, Canada). Taq DNA polymerase, $10\times$ PCR buffer, and deoxynucleotide triphosphates were obtained from Roche (Roche Diagnostics, Montreal, Quebec, Canada). Oligonucleotide primers were synthesized by ACGT (Toronto, Ontario, Canada).

Animals and experimental protocols. All experimental protocols were reviewed and carried out in accordance with the experimental ethical guidelines established by the Animal Care Committee of the University Health Network. Mice were housed in microisolator cages, five mice per cage under a 12:12-h light-dark cycle with lights off at ~5 PM. Control wild-type mice (The Jackson Laboratory, Bar Harbor, ME.) were acclimatized to the Toronto General Hospital Animal facility for at least 2 wk before each experiment. *Experiment 1*, the fasting and refeeding study, comprised three groups ($n = 5$ or 6 mice per group) of randomly selected 7- to 10-wk-old male C57B6/SJL 1.6hGLU-GH transgenic mice (27) and 7- to 10-wk-old wild-type male C57B6/SJL control mice (the genotype corresponding to the transgenic mice).

Group 1 mice were allowed unrestricted access to standard rodent chow and euthanized by CO_2 at ~10:00 AM; *group 2* mice were deprived of all food for 20 h (from 2:00 PM to 10:00 AM the next day) and euthanized by CO_2 at 10:00 AM. *Group 3* mice were fasted for 20 h (from 2:00 PM to 10:00 AM the next day) followed by unrestricted access to standard rodent chow for 24 h (from 10:00 AM to 10:00 AM the following day) and euthanized by CO_2 at 10:00 AM. *Experiment 2*, the fiber diet study, comprised two groups of randomly selected 7- to 10-wk-old male 1.6hGLU-GH transgenic mice and 7- to 10-wk-old wild-type male C57B6/SJL control mice. Each group contained five or six mice that consumed either a fiber-free elemental diet (ICN Pharmaceuticals Mississauga, Ontario, Canada) or an isocaloric elemental diet supplemented with 30% fiber (ICN Pharmaceuticals) for 16 days. The elemental diet contains 178.5 g/kg of amino acid mix, 100 g/kg of corn oil, 443 g/kg of glucose, 221.5 g/kg of sucrose, 50 g/kg of mineral mix, 7 g/kg of vitamin mix, and 17.5 kJ/g of digestible energy. At the end of the 16-day experiment, mice were fasted for 20 h and glucose (1.5 mg/g of body wt) was administered by oral gavage as described previously (1, 2). Thirty minutes later, mice were euthanized by CO_2.

Weights and histology of intestinal sections. Intestinal segments for weights and histology were taken from duodenum (10 cm distal to the gastroduodenal junction), midjejunum (13–23 cm distal to the gastroduodenal junction), ileum (10 cm from the ileocecal junction), and colon (10 cm from the anus). Ten-centimeter segments from each region were used for wet weight analysis, and 5 cm were subsequently used for histological analyses. The wet weights of each intestinal section were normalized to the total body weight. Tissues were fixed in 10% buffered neutral formalin for 24 h and embedded in paraffin. Four- to six-micrometer cross sections were cut from each intestinal segment, nine sections per slide, and stained with hematoxylin and eosin as described previously (5, 6, 27). Intestinal micrometry was performed on a minimum of five or six well-oriented sections for each mouse with a Leica Q500MC image analysis system.

RNA isolation and semiquantitative RT-PCR. Total cellular RNA was isolated from various intestinal sections using the TRIzol reagent (Canadian Life Technologies). cDNA was synthesized by reverse transcription using a cDNA synthesis kit (Amersham Pharmacia Biotech). Oligonucleotide primers for semiquantitative RT-PCR are listed in Table 1. The optimal conditions for linear PCR amplification of hGH, mouse proglucagon, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using a range of input RNA concentrations and different cycle numbers. Semiquantitative RT-PCR analysis of hGH mRNA was performed using 60 ng of total RNA for 35 cycles. For mouse proglucagon and

Table 1. *Oligonucleotide primers for semiquantitative RT-PCR*

Genes	Primers	Sizes of PCR Products
hGH	5′-GAAGAAGCCTATATCCCAAAG-3′ 5′-GTAGTGCGTCATCGTTGTG-3′	378 bp
Mouse Proglucagon	5′-TGAAGACCATTTACTTTGTGG-3′ 5′-CCTGGTGGCAAGATTGTCCAGAAT-3′	492 bp
Mouse GAPDH	5′-CATTGACCTCAACTACATGG-3′ 5′-CACGTCAGATCCACGACGGA-3′	630 bp

The predicted size of the PCR products obtained with each primer set is shown in base pairs. hGH, human growth hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

GAPDH, 60 ng of total RNA was analyzed by multiplex RT-PCR for 24 cycles. All PCR reactions were carried out in a Minicycler (MJ Research, Watertown, MA) at 94°C for 1 min, 55°C for 1 min, and extension at 72°C for 1 min. PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. The gel was then denatured in 1.5 M of NaCl and 0.5 M of NaOH for 2 h, DNA products were transferred onto a nylon membrane (Nytran Plus; Schleicher and Schuell, Keene, NH) by capillary transfer, and the membrane was neutralized in 1.5 M NaCl and 0.5 Tris·HCl (pH 7.5) for 30 min. The DNA was then fixed onto the membrane by exposure to ultraviolet (UV) light in a Stratalinker UV crosslinker (Stratagene, La Jolla, CA) and prehybridized in buffer containing 15% formamide, 0.5 M sodium phosphate (pH 7.8), 0.005 M EDTA, 0.1 g of bovine serum albumin, and 7% SDS and hybridized in the same solution plus 3 million counts·min^{-1}·ml^{-1} [α-^{32}P]dCTP randomly labeled cDNA probes at 60°C. Blots were washed with 30 mM sodium phosphate (pH 7.8) and 0.1% SDS. The membranes were exposed on the phosphor screen overnight, and intensity of the PCR products was quantified densitometrically using a STORM 840 PhosphorImager (Molecular Dynamics) and ImageQuant software (version 5.0, Molecular Dynamics).

Insulin and hGH ELISAs. Blood samples were obtained by cardiac puncture at the time of death and collected into tubes containing 10% (vol/vol) anticoagulant TED (500,000 IU/ml trasylol, 1.2 mg/ml EDTA, and 0.1 mM Diprotin A). Plasma was prepared by centrifugation at 4°C for 10 min. Ten microliters of plasma was used for insulin ELISA using a rat insulin ELISA kit (CRYSTAL CHEM, Chicago, IL) with mouse insulin as a standard. One hundred twenty-five microliters of plasma was used to measure plasma hGH using an hGH ELISA kit (Roche Diagnostics). The levels of mouse plasma GLP-1 and GLP-2 were not assessed in the same studies due to the larger volumes of plasma required for these additional assays.

Blood glucose. Blood was drawn from a tail vein, and glucose levels were measured by the glucose oxidase method using a One Touch Basic Glucometer (Lifescan, Burnaby, British Columbia, Canada) as described previously (2, 37).

Statistical analysis. Differences between treatments were determined by Student's *t*-test or one-way ANOVA (InStat version 1.12). Statistical significance is defined as $P \leq 0.05$.

RESULTS

Manipulations designed to vary the levels of specific nutrients in the gastrointestinal tract, such as fasting followed by refeeding or exposure to diets containing fermentable fiber, have been shown to modulate expression of the endogenous proglucagon gene in rat enteroendocrine L cells (31, 32). To ascertain whether the murine gastrointestinal tract exhibits a comparable response to variations in nutrient exposure, we first examined intestinal weights and histology after fasting and refeeding in wild-type and 1.6hGLU-GH transgenic mice.

Fasting reduced and refeeding significantly increased intestinal wet weights in the duodenum and jejunum of both control and transgenic mice (Fig. 1, *A* and *B*; $P < 0.05$ for duodenum and jejunum, fasting vs. refeeding). In contrast, no significant differences in intestinal weights were observed after fasting and refeeding in the ileum and colon in the same experiments (Fig. 1, *A* and *B*). Analysis of histological sections from these same

intestinal regions demonstrated comparable changes in crypt plus villus height after fasting and refeeding in the duodenum and jejunum from control and transgenic mice ($P < 0.05$ for changes in crypt plus villus height in fasted vs. refed wild-type and transgenic mice; Fig. 1, *C* and *D*). In contrast, no significant change in ileal crypt plus villus height or mucosal thickness of the colon was detected after fasting and refeeding in wild-type or 1.6hGLU-GH transgenic mice (Fig. 1, *C–E*).

As fasting and refeeding are associated with reductions and subsequent increases in the levels of PGDPs and insulin secretion from the enteroendocrine L cell and islet β-cell, respectively, we hypothesized that GH produced in the enteroendocrine L cells of 1.6hGLU-GH transgenic mice should also be secreted in a nutrient-dependent manner. We first assessed the effects of fasting and refeeding on levels of plasma insulin in wild-type and transgenic mice. Fasting significantly reduced and refeeding significantly increased the levels of circulating insulin in both wild-type (889 ± 145 vs. 384 ± 62 vs. 1,485 ± 274 pg/ml; normal feeding vs. fasting vs. refeeding, respectively, $P < 0.05$) and 1.6hGLU-GH transgenic mice (919 ± 170 vs. 185 ± 54 vs. 2,326 ± 342 pg/ml; normal feeding vs. fasting vs. refeeding, respectively, $P < 0.05$; Fig. 2, *A* and *B*). A comparable profile of hGH excursion was detected, with a significant reduction of hGH in fasted mice followed by a significant increase with refeeding (91 ± 10 vs. 29 ± 8 vs. 107 ± 14 pg/ml; normal feeding vs. fasting vs. refeeding, respectively, $P < 0.05$; Fig. 2*C*). These findings demonstrate that a transgenic protein targeted to the murine enteroendocrine L cell responds appropriately to acute changes in nutrient intake in vivo.

As fasting and refeeding decrease and increase the levels of intestinal proglucagon mRNA transcripts in the rat small bowel (19), we wished to determine whether sequences contained within the 1.6-kb fragment of the human proglucagon promoter were sensitive to changes in ambient nutrient intake. We first established semiquantitative conditions for analysis of mRNA transcripts for hGH and mouse proglucagon and GAPDH over a linear range of RNA concentrations (Fig. 3). No significant changes in proglucagon mRNA transcripts were detected in mouse jejunum or colon of wild-type mice (data not shown) and 1.6hGLU-GH transgenic mice (Fig. 4, *A* and *C*) after fasting and refeeding. In contrast, proglucagon mRNA transcripts were significantly reduced after fasting and increased after refeeding in the ileum of transgenic mice (Fig. 4*B*). To ascertain whether the human proglucagon promoter-hGH transgene was also regulated by changes in nutrients, we analyzed the levels of hGH mRNA transcripts after fasting and refeeding. No significant changes in the levels of hGH mRNA transcripts were observed in the jejunum, ileum, or colon of 1.6hGLU-GH transgenic mice after fasting-refeeding (Fig. 4).

High-fiber feeding in rats results in increased secretion of intestinal PGDPs and upregulation of intestinal
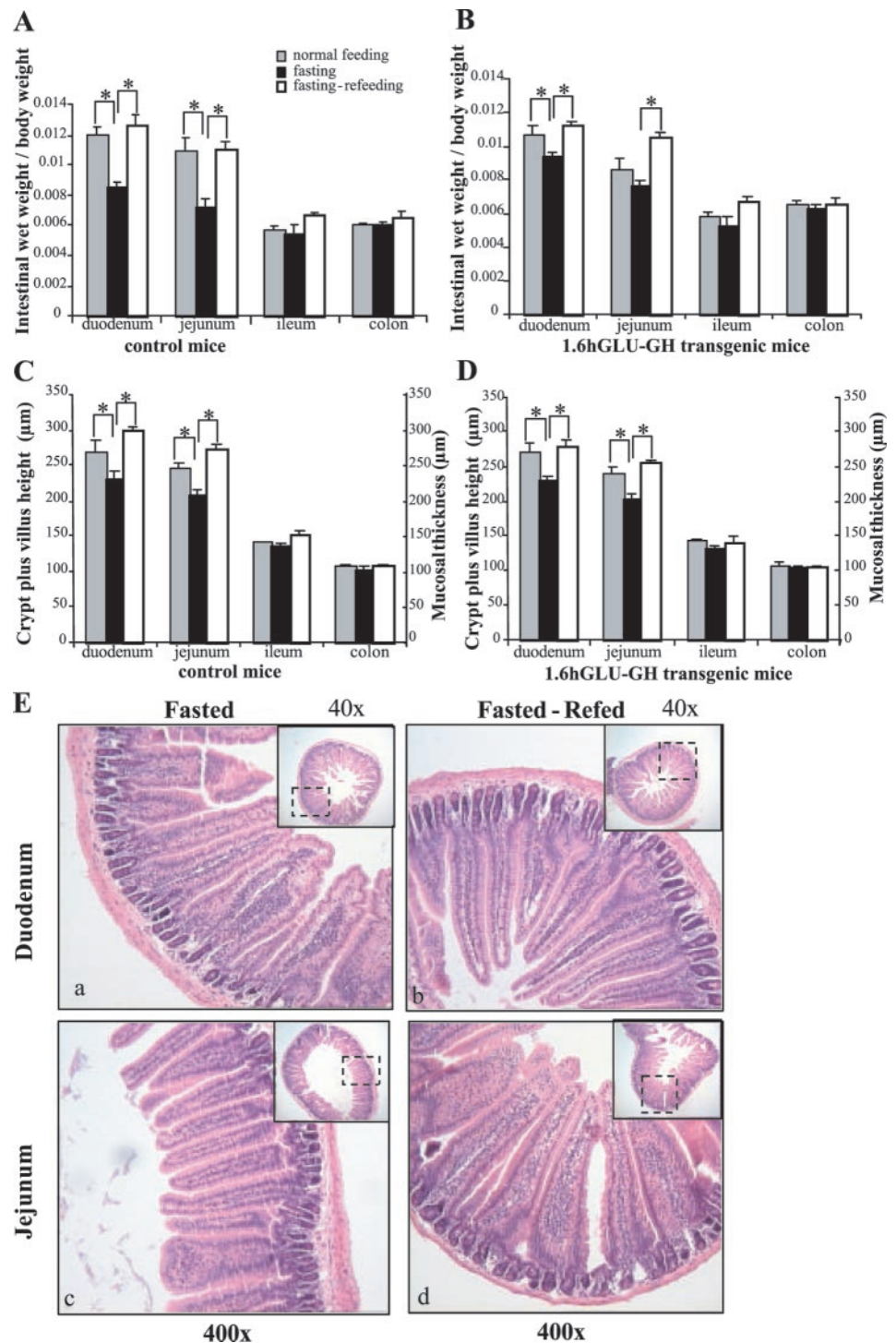
HUMAN GLUCAGON GENE EXPRESSION

Fig. 1. Intestinal weights and histology after fasting and refeeding in mice. Values for *A–D* are expressed as means ± SE; $n = 5$ or 6 mice/group. *$P \leq 0.05$. *A* and *B*: wild-type control mice (*A*) or 1.6 human glucagon-growth hormone (hGLU-GH) transgenic mice (*B*) were fed normally (normal feeding), fasted for 20 h (fasting), or fasted for 20 h followed by refeeding for 24 h (fasting-refeeding). Weight from intestinal segments are normalized to individual mouse body weights. *C* and *D*: crypt plus villus height (small bowel) or mucosal thickness (large bowel) was assessed in duodenum, jejunum, ileum, and colon of wild-type (*C*) and transgenic (*D*) mice after normal feeding, fasting, or fasting-refeeding. Twenty-five longitudinally oriented villi (small bowel) or regions of intact mucosa (colon) were assessed per intestinal region per animal. *E*: representative photomicrographs of histological sections from duodenum (*a, b*) and jejunum (*c, d*) sections from either fasted or fasted-refed transgenic mice. ×40 and ×400 denote the magnitude of the larger photomicrograph and the *inset*, respectively.

proglucagon gene expression (31). To determine whether the mouse proglucagon gene and the 1.6hGLU-GH transgene are similarly upregulated after high-fiber feeding, we fed control and transgenic mice for 16 days with either an elemental diet containing no fiber or the identical elemental diet supplemented with 30% fiber. To stimulate transgene expression, mice were challenged with oral glucose before death. No significant differences in body weight or blood glucose were observed in transgenic or wild-type mice fed with the different diets (data not shown). Similarly, no differences in the wet weights of the duodenum, jejunum, or ileum were detected in control or 1.6hGLU-GH transgenic mice after high-fiber feeding (Fig. 5). In contrast, colon wet weights were significantly increased after 30% fiber feeding in both wild-type and transgenic mice (Fig. 5; $P < 0.05$ for 0 vs. 30% fiber diet in both groups of mice).

To determine the compartments of the murine intestinal tract that are sensitive to high-fiber feeding, we
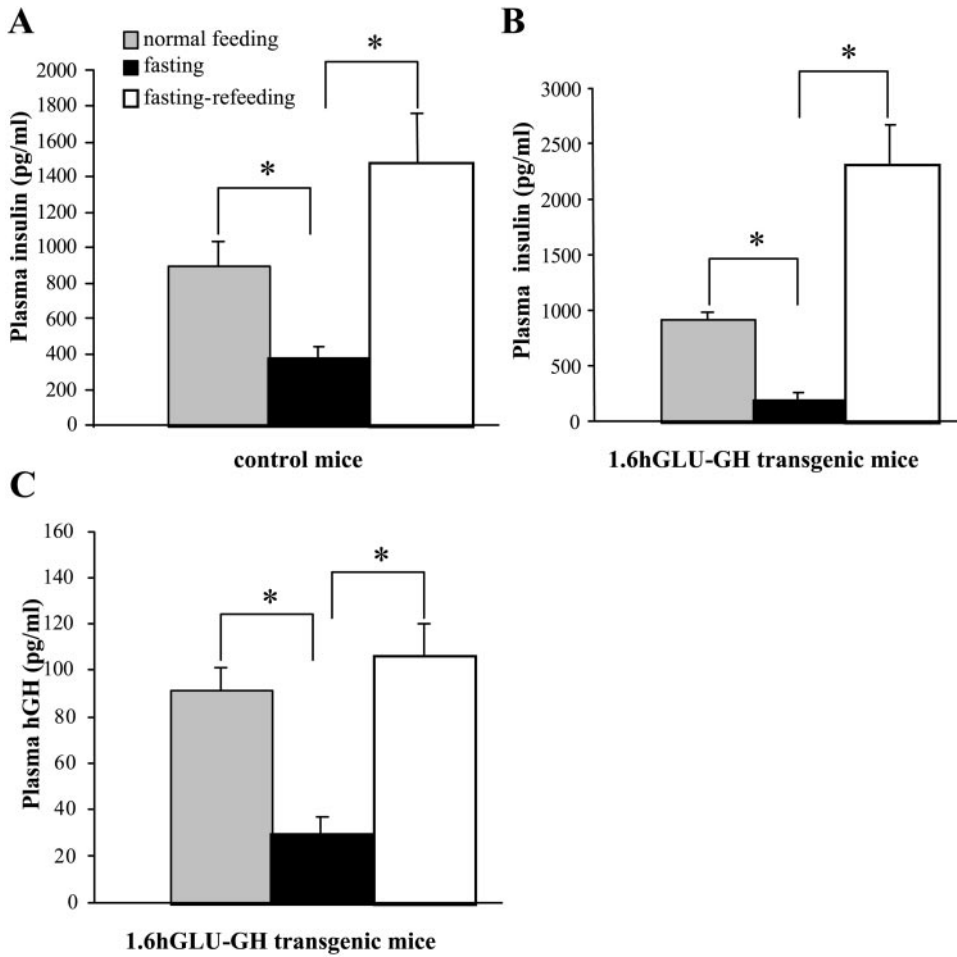
Fig. 2. Plasma levels of insulin and hGH in mice. Values are expressed as means ± SE; $n = 5$ or 6 mice/group. *$P \leq 0.05$. *A* and *B*: plasma levels of insulin in wild-type control (*A*) and transgenic (*B*) mice and levels of hGH (*C*) in transgenic mice were determined by ELISA as described in MATERIALS AND METHODS.
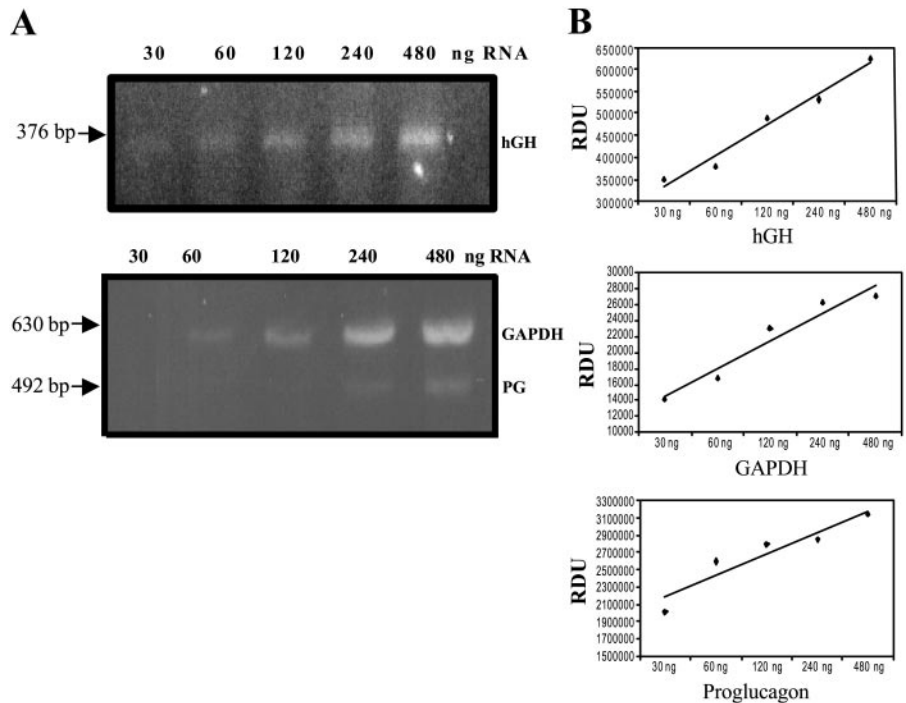

Fig. 3. Semiquantitative RT-PCR analysis of RNA transcripts for hGH, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and proglucagon (PG). *A*: representative RT-PCR analyses for hGH, PG, and GAPDH using different amounts of input RNA. *B*: linear relationship of the amounts of RT-PCR products obtained as a function of varying input RNA concentrations. RDU, relative densitometric units.
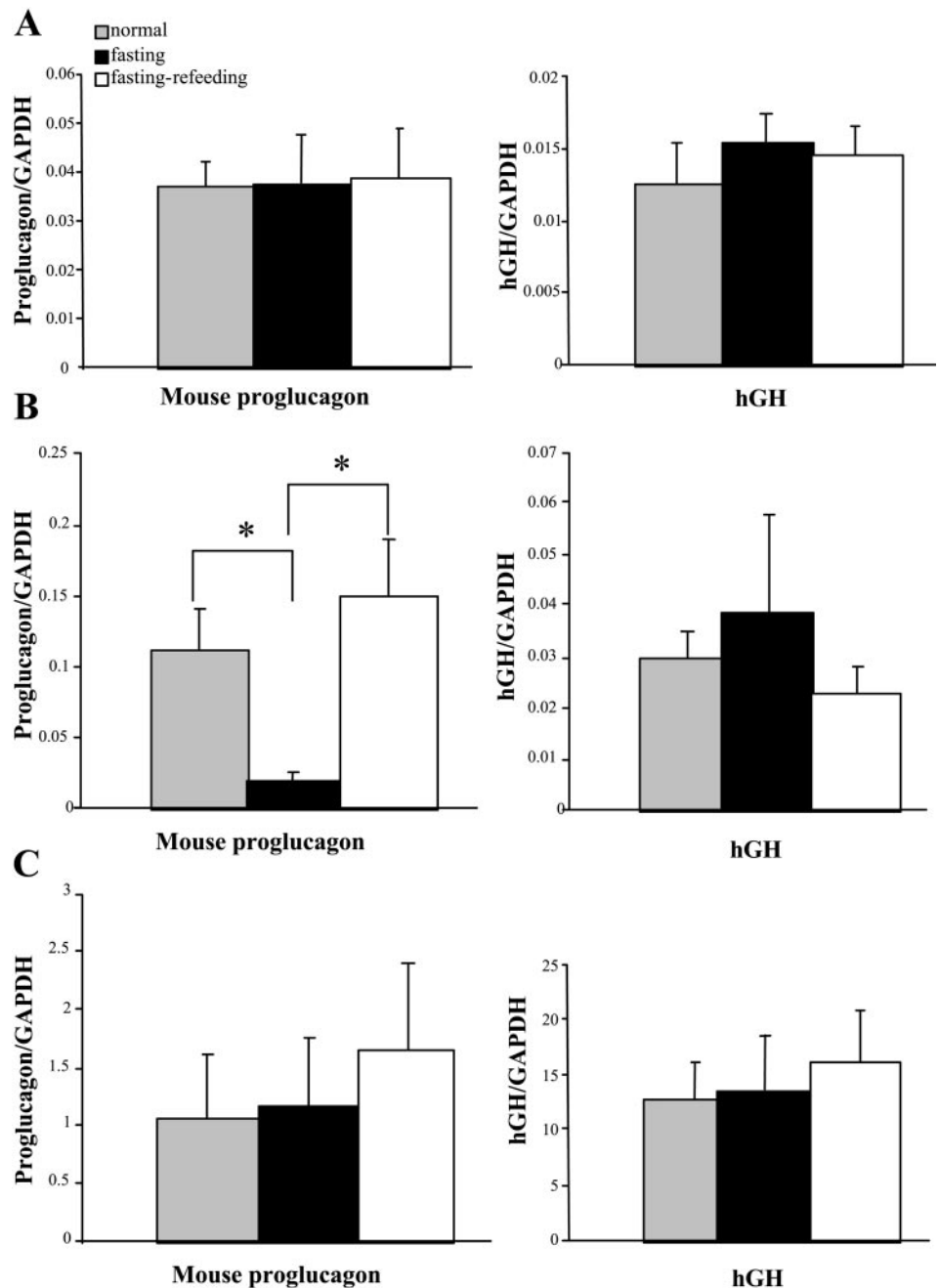
Fig. 4. Analysis of mouse PG and hGH mRNA transcripts in 1.6hGLU-GH transgenic mice after fasting and refeeding. Values are expressed as means ± SE; $n = 5$ or 6 mice/group. *$P \leq 0.05$. *Right*: the relative mRNA abundance expressed as the ratio of hGH/GAPDH mRNA transcripts, in various intestinal segments of 1.6hGLU-GH transgenic mice. *Left*: the data, expressed as a ratio of PG/GAPDH RNA transcripts, from the same intestinal segments in 1.6hGLU-GH transgenic mice. *A*: jejunum. *B*: ileum. *C*: colon.

assessed crypt plus villus height in the small bowel and mucosal thickness in the large bowel in histological sections from duodenum, jejunum, ileum, and colon after 0 or 30% fiber feeding in wild-type and transgenic mice. Consistent with the findings from analyses of wet weights, no differences in crypt plus villus epithelial height were observed in the duodenum, jejunum, or ileum of wild-type or transgenic mice after exposure to the elemental diet without fiber vs. the 30% high fiber-supplemented diet (Fig. 5, *C* and *D*). In contrast, the mucosal thickness of the colon was significantly increased in mice after the 30% fiber diet (Fig. 5, *C–E*; 114 ± 5 vs. 183 ± 6 and 113 ± 7 vs. 184 ± 7 μm, 0 vs.

30% fiber, for control and transgenic mice, respectively, $P < 0.05$).

The results of these analyses established that the mouse gastrointestinal tract also exhibits a trophic response to high-fiber feeding. As rats exposed to high-fiber feeding exhibited increased levels of circulating insulin (31), we assessed levels of glucose-stimulated plasma insulin in wild-type and transgenic mice exposed to 0 or 30% fiber diets. Both wild-type and 1.6hGLU-GH mice exhibited significant increases in glucose-stimulated insulin after high-fiber feeding (Fig. 6, *A* and *B*; 199 ± 65 vs. 388 ± 77 for wild-type and 281 ± 69 vs. 619 ± 70 pg/ml for transgenic mice, 0
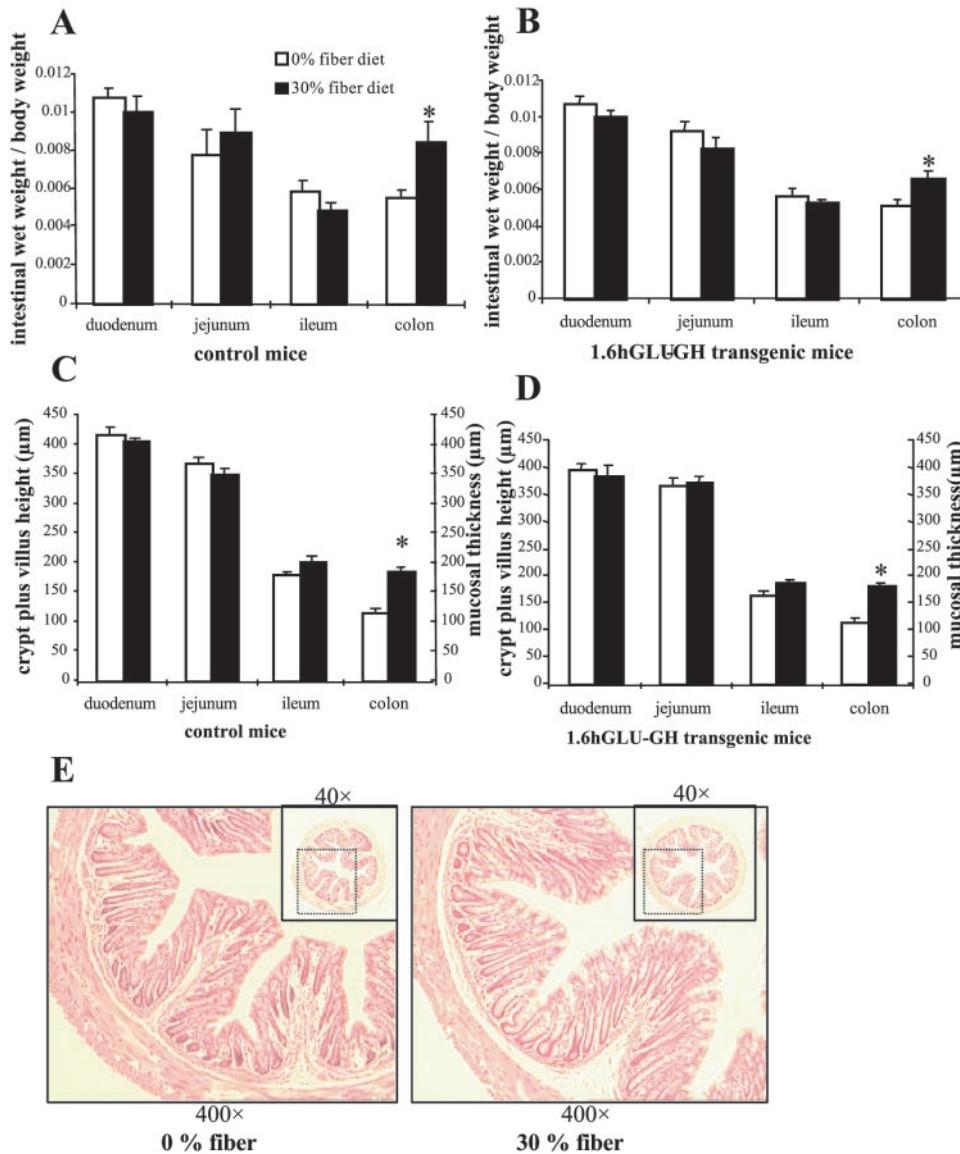
Fig. 5. Intestinal weights and histology in mice after a low- or high-fiber diet. Values are expressed as means ± SE; $n = 5$ or 6 mice/group. *$P \leq 0.05$. *A* and *B*: normal control mice (*A*) or 1.6hGLU-GH transgenic mice (*B*) were fed an elemental diet with either 0 or 30% fiber for 16 days. Mice were killed 30 min after an oral glucose gavage and intestinal wet weights, normalized to body weights, are shown in *A* and *B*. *C* and *D*: crypt plus villus height (small bowel) or mucosal thickness (large bowel) was assessed in duodenum, jejunum, ileum, and colon of wild-type (*C*) and transgenic (*D*) mice after exposure to 0 or 30% fiber-supplemented diets. Twenty-five longitudinally oriented villi (small bowel) or regions of intact mucosa (colon) were assessed per intestinal region per animal. *E*: representative photomicrographs of histological sections from the colon of transgenic mice after 16 days of a 0 or 30% high-fiber diet. ×40 (*inset*) and ×400 refer to the magnification of the different photomicrographs.

vs. 30% fiber feeding, $P < 0.05$ for both wild-type and transgenic mice, respectively).

As increased levels of insulin in fiber-fed rats have been attributed in part to enhanced L cell secretion of GLP-1 (25, 31), we hypothesized that increased secretory activity of the intestinal L cell in fiber-fed mice might lead to increased circulating levels of transgene-derived hGH. Consistent with this hypothesis, plasma levels of glucose-stimulated hGH were significantly increased after high-fiber feeding (57 ± 5 vs. 103 ± 9 pg/ml, 0 vs. 30% high-fiber feeding, $P < 0.05$; Fig. 6*C*).

High-fiber feeding increases intestinal proglucagon gene expression in normal and diabetic rats (31, 32). RT-PCR analyses demonstrated significantly increased levels of proglucagon mRNA transcripts in the jejunum, ileum, and colon of wild-type (data not shown) and 1.6hGLU-GH transgenic mice (Fig. 7; $P < 0.05$ for 0 vs. 30% fiber for jejunum, ileum, and colon). To ascertain whether the signals activating the endogenous murine proglucagon gene were also capable of

increasing hGH transgene expression under the control of the human proglucagon promoter, we assessed levels of hGH mRNA transcripts in the same experiments. In contrast to the upregulation of endogenous mouse proglucagon mRNA transcripts in the 30% fiber group, no change in the levels of hGH mRNA transcripts was detected in the small or large intestine (Fig. 7).

DISCUSSION

In the present study, we investigated the effects of fasting, refeeding, and dietary fiber on regulation of endogenous murine proglucagon and hGH transgene expression in the small and large bowel of 1.6hGLU-GH transgenic mice. A 14-day exposure to a 30% fiber diet in rats led to significantly increased levels of proglucagon mRNA transcripts in the ileum, in association with increased levels of circulating GLP-1 and insulin after oral glucose challenge (31). In contrast,
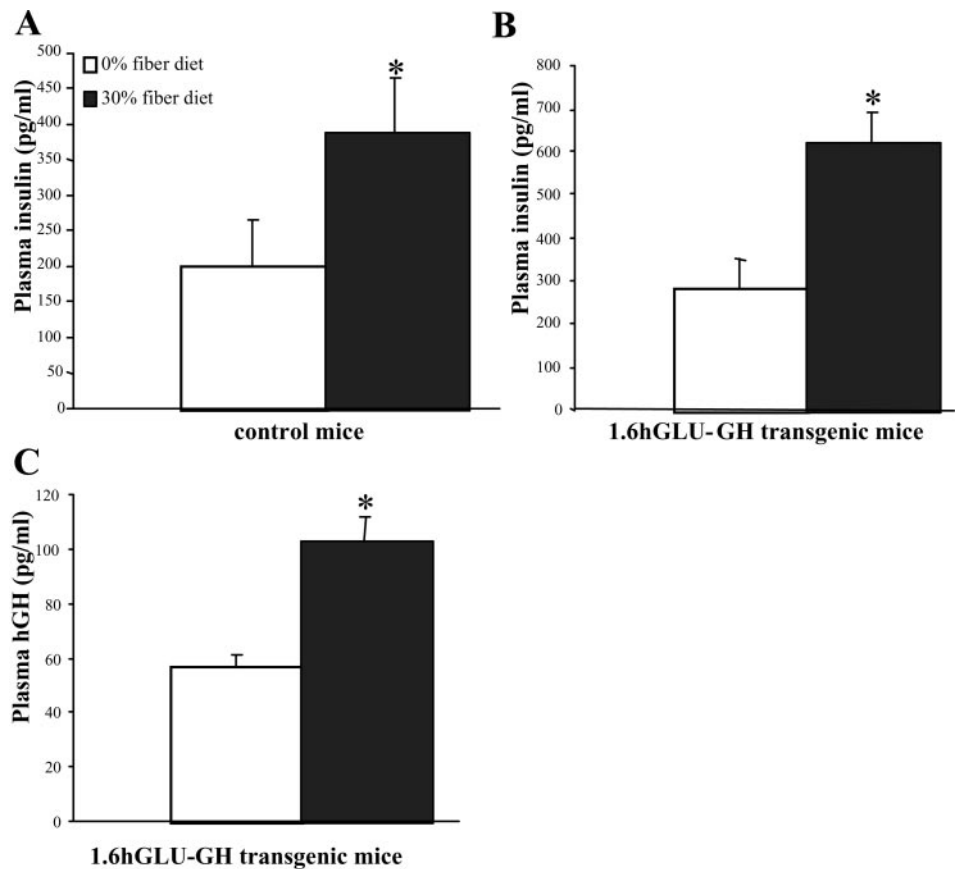
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Fig. 6. Plasma levels of insulin and hGH in mice after low- or high-fiber diet. Values are expressed as means ± SE; n = 5 or 6 mice/group. *P ≤ 0.05. A and B: plasma levels of insulin in wild-type control (A) and transgenic (B) mice and levels of hGH (C) in transgenic mice. Plasma samples were obtained 30 min after oral glucose challenge for analysis by ELISA as described in MATERIALS AND METHODS.

wild-type and transgenic mice fed an identical 30% fiber-supplemented diet in this study exhibited significantly increased mucosal thickness in the colon, in association with significant increases in the levels of endogenous murine proglucagon mRNA transcripts not only in the colon but also in the jejunum and ileum. Whether these region-specific differences in upregulation of murine intestinal proglucagon gene expression reflect the longer treatment period (16 vs. 14 days), or a species-specific difference in mice vs. rats, remains unclear.

Fiber-enriched diets increase the levels of gut proglucagon-derived peptides in rats (16, 17, 38), likely due to fiber fermentation in the colon and generation of short-chain fatty acids (SCFAs; 31, 40, 41). The proliferative effects of SCFAs are not always confined to the colon, as supplementation of parenteral nutrition with fatty acids reverses the small bowel atrophy seen in rats fed with parenteral nutrition alone (22) and increased RNA and DNA content in the rat ileum (40). SCFAs also increased the abundance of ileal proglucagon RNA transcripts as well as the plasma levels of circulating GLP-2 in rats (39, 40). Furthermore, GLP-2 exerts potent trophic effects on the small bowel epithelium of mice and rats, and coinfusion of GLP-2 prevents the parenteral nutrition-associated mucosal atrophy in the jejunum of rats (9). Hence, our findings of increased proglucagon gene expression in both the small and large bowel of fiber-treated mice are consis-

tent with a role for fiber diet-derived humoral mediators, perhaps SCFAs, as potential mediators of the upregulation of proglucagon gene expression along the murine gastrointestinal tract.

Fasting is associated with decreased circulating levels of the intestinal PGDPs in rodents and humans, and nutrient ingestion rapidly increases the secretion of glicentin, GLP-1, and GLP-2 (7, 18, 34, 44). In contrast, the effects of nutrient deprivation and refeeding on intestinal proglucagon gene expression have been less extensively examined. A 72-h fast decreased levels of jejunal proglucagon RNA by 40% in rats, whereas refeeding increased levels back to normal nonfasted controls (19). In contrast, a much smaller (20%) fasting-related decrease in proglucagon RNA was observed in the rat ileum, and ileal proglucagon mRNA transcripts did not increase significantly after refeeding (19). Our data in wild-type and 1.6hGLU-GH transgenic mice demonstrate that even a much shorter period of fasting (20 h) was associated with a significant decrease and rebound increase in the levels of ileal proglucagon mRNA transcripts after fasting and refeeding, respectively. In contrast to the findings in rats (19), we did not observe significant changes in proglucagon gene expression in the jejunum or colon after fasting or refeeding in wild-type or transgenic mice.

Although the levels of endogenous mouse intestinal proglucagon gene expression were upregulated in re-
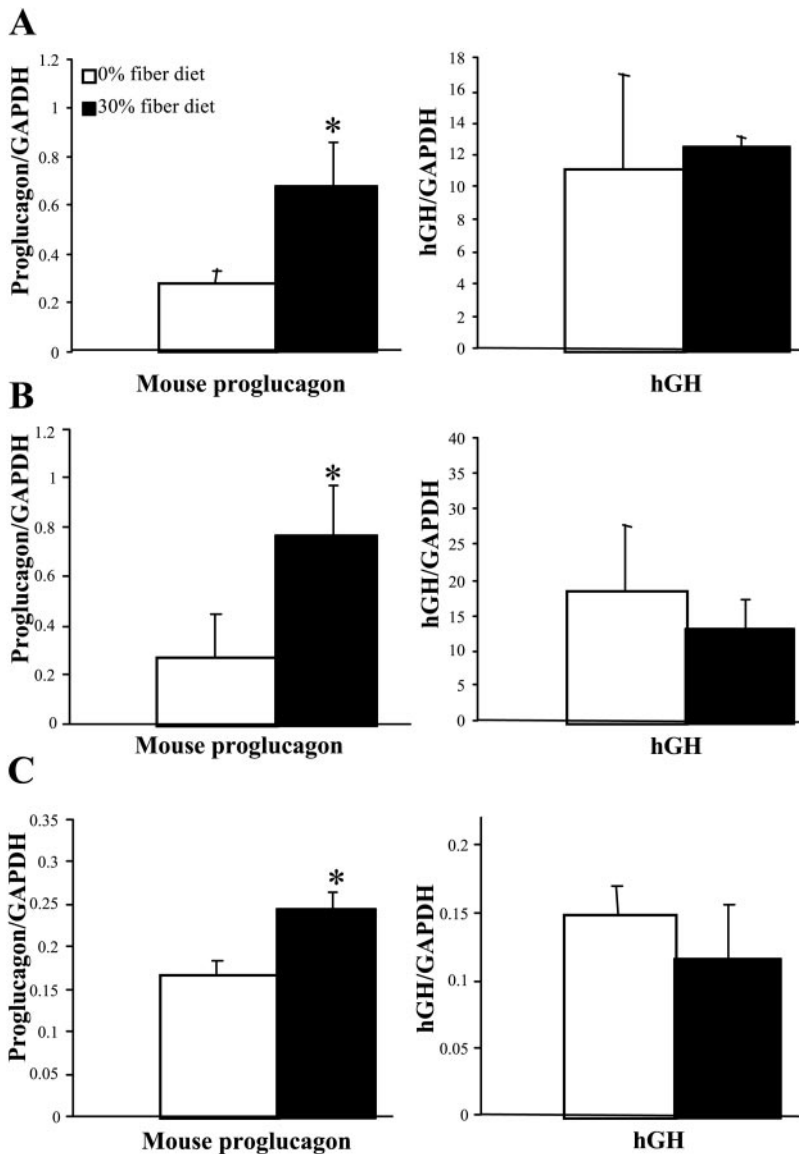
Fig. 7. Analysis of mouse PG and hGH mRNA transcripts in 1.6hGLU-GH transgenic mice after a low- or high-fiber diet. Values are expressed as means ± SE; $n = 5$ or 6 mice/group. *$P \leq 0.05$. *Right*: the relative mRNA abundance, expressed as the ratio of hGH/GAPDH mRNA transcripts, in various intestinal segments of 1.6hGLU-GH transgenic mice. *Left*: the relative mRNA abundance, expressed as a ratio of PG/GAPDH mRNA transcripts, from the same intestinal segments in 1.6hGLU-GH transgenic mice. *A*: jejunum. *B*: ileum. *C*: colon.

sponse to the high-fiber diet and modulated appropriately after fasting and refeeding, we did not observe corresponding changes in the levels of hGH mRNA transcripts in the same experiments. These findings imply that the putative nutrient-response elements(s) that mediate nutrient-regulation of the endogenous murine proglucagon gene may not reside within the 1.6 kb of human proglucagon promoter sequences present in our transgene. Alternatively, in contrast to mice and rats, the human proglucagon gene may not be as sensitive to changes in nutrient intake in the small and large intestine. Furthermore, it remains possible that nutrient-regulation of intestinal proglucagon gene expression is regulated at the level of mRNA stability, perhaps via specific sequences in the proglucagon gene 3′-untranslated region that are not present in the 1.6hGLU-GH transgene. Accordingly, the design of our transgene may limit conclusions about the relative contribution of human proglucagon promoter se-

quences to the integrated levels of transgene mRNA transcripts in different cell types.

At present, it has not been determined whether the nutrient regulation of rodent intestinal proglucagon gene expression in vivo resides at the level of gene transcription and/or modulation of RNA stability. Although the SCFA sodium butyrate activates rat proglucagon gene transcription in an islet cell line in vitro (30), a specific "butyrate-response element" has not yet been identified in the proglucagon gene promoter. Analysis of the mechanisms used by protein hydrosylates for upregulation of proglucagon gene expression in immortalized STC-1 cells demonstrated that peptones increased proglucagon gene transcription and sequences within the first several hundred base pairs of the rat proglucagon gene 5′-flanking region were sufficient for conferring peptone stimulation to a heterologous reporter gene (11). Similarly, peptones stimulated intestinal CCK gene transcription via binding of

transcription factors to the cAMP response element in the CCK gene promoter (4). Hence the available data suggest that nutrient-derived signals may activate proglucagon gene transcription in murine enteroendocrine cells.

The contribution of transgene-derived hGH to proliferative or metabolic effects in the small bowel and colon in 1.6hGLU-GH transgenic mice is unlikely to be significant due to the low levels of circulating GH observed in our experiments. GH concentrations range from 10 to 100 ng/ml in normal mice, whereas mice expressing a metallothionein promoter hGH transgene exhibited circulating basal levels of hGH ranging from 100 to 64,000 ng/ml, with values exceeding 100,000 ng/ml after induction of transgene expression (28). In contrast, the maximal levels of plasma hGH observed in our transgenic mice reached ~120 pg/ml after 16 days of high-fiber feeding. This level is several orders of magnitude lower than values observed in the metallothionein-hGH transgenic mice, likely accounting for our failure to observe any increase in somatic growth in 1.6hGLU-GH transgenic mice (27).

Our findings demonstrate that the mouse L cell appropriately senses and transmits signals from acute nutrient loading and high-fiber feeding to the endogenous mouse proglucagon gene and to the secretory machinery regulating secretion of a transgenic human protein. Nevertheless, human proglucagon gene regulatory sequences specifying tissue-specific expression in murine enteroendocrine cells are not sufficient, in the context of a proglucagon-hGH transgene, for recognition of energy-derived signals regulating intestinal glucagon gene expression.

Perspectives

Human proglucagon-derived peptides play essential roles in nutrient absorption (GLP-2) and nutrient assimilation (GLP-1). Consistent with these findings, enteral nutrient intake is a major regulator of PGDP synthesis and secretion. We established a model for studying the simultaneous regulation of endogenous murine proglucagon gene expression and human proglucagon promoter-directed transgene expression in vivo. We show here when using 1.6hGLU-GH transgenic mice that DNA sequences sufficient for targeting transgene expression to nonimmortalized murine L cells are not sufficient for conferring nutrient regulation of gene expression to a heterologous transgene. Given the interest in stimulating the synthesis and secretion of human GLP-1 and GLP-2 for the potential treatment of diabetes and intestinal insufficiency, respectively, further characterization of the factors regulating human proglucagon promoter activity in enteroendocrine L cells of hGLU-GH transgenic mice appears warranted.

REFERENCES

1. **Baggio L, Adatia F, Bock T, Brubaker PL, and Drucker DJ.** Sustained expression of exendin-4 does not perturb glucose homeostasis, β cell mass or food intake in metallothionein-pre-proexendin transgenic mice. *J Biol Chem* 275: 34471–34477, 2000.
2. **Baggio L, Kieffer TJ, and Drucker DJ.** GLP-1 but not GIP regulates fasting and non-enteral glucose clearance in mice. *Endocrinology* 141: 3703–3709, 2000.
3. **Benjamin MA, McKay DM, Yang PC, Cameron H, and Perdue MH.** Glucagon-like peptide-2 enhances intestinal epithelial barrier function of both transcellular and paracellular pathways in the mouse. *Gut* 47: 112–119, 2000.
4. **Bernard C, Sutter A, Vinson C, Ratineau C, Chayvialle J, and Cordier-Bussat M.** Peptones stimulate intestinal cholecystokinin gene transcription via cyclic adenosine monophosphate response element-binding factors. *Endocrinology* 142: 721–729, 2001.
5. **Boushey RP, Yusta B, and Drucker DJ.** Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. *Am J Physiol Endocrinol Metab* 277: E937–E947, 1999.
6. **Boushey RP, Yusta B, and Drucker DJ.** Glucagon-like peptide (GLP)-2 reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor. *Cancer Res* 61: 687–693, 2001.
7. **Brubaker PL, Crivici A, Izzo A, Ehrlich P, Tsai CH, and Drucker DJ.** Circulating and tissue forms of the intestinal growth factor, glucagon-like peptide 2. *Endocrinology* 138: 4837–4843, 1997.
8. **Buchan AM.** Nutrient tasting and signaling mechanisms in the gut. III. Endocrine cell recognition of luminal nutrients. *Am J Physiol Gastrointest Liver Physiol* 277: G1103–G1107, 1999.
9. **Chance WT, Foley-Nelson T, Thomas I, and Balasubramaniam A.** Prevention of parenteral nutrition-induced gut hypoplasia by coinfusion of glucagon-like peptide-2. *Am J Physiol Gastrointest Liver Physiol* 273: G559–G563, 1997.
10. **Cheeseman CI.** Upregulation of SGLT-1 transport activity in rat jejunum induced by GLP-2 infusion in vivo. *Am J Physiol Regulatory Integrative Comp Physiol* 273: R1965–R1971, 1997.
11. **Cordier-Bussat M, Bernard C, Levenez F, Klages N, Laser-Ritz B, Philippe J, Chayvialle JA, and Cuber JC.** Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene. *Diabetes* 47: 1038–1045, 1998.
12. **Drucker DJ.** The glucagon-like peptides. *Diabetes* 47: 159–169, 1998.
13. **Drucker DJ.** The glucagon-like peptides. *Endocrinology* 142: 521–527, 2001.
14. **Drucker DJ and Asa S.** Glucagon gene expression in vertebrate brain. *J Biol Chem* 263: 13475–13478, 1988.
15. **Drucker DJ, Ehrlich P, Asa SL, and Brubaker PL.** Induction of intestinal epithelial proliferation by glucagon-like peptide 2. *Proc Natl Acad Sci USA* 93: 7911–7916, 1996.
16. **Gee JM, Lee-Finglas W, and Johnson IT.** Fermentable carbohydrate modulates postprandial enteroglucagon and gastrin release in rats. *Br J Nutr* 75: 757–766, 1996.
17. **Gee JM, Lee-Finglas W, Wortley GW, and Johnson IT.** Fermentable carbohydrates elevate plasma enteroglucagon but high viscosity is also necessary to stimulate small bowel mucosal cell proliferation in rats. *J Nutr* 126: 373–379, 1996.
18. **Ghatei MA, Uttenthal LO, Christofides ND, Bryant MG, and Bloom SR.** Molecular forms of human enteroglucagon in tissue and plasma: plasma responses to nutrient stimuli in health and in disorders of the upper gastrointestinal tract. *J Clin Endocrinol Metab* 57: 488–495, 1983.
19. **Hoyt EC, Lund PK, Winesett DE, Fuller CR, Ghatei MA, Bloom SR, and Ulshen MR.** Effects of fasting, refeeding and intraluminal triglyceride on proglucagon expression in jejunum and ileum. *Diabetes* 45: 434–439, 1996.
20. **Irwin DM.** Molecular evolution of proglucagon. *Regul Pept* 98: 1–12, 2001.

21. **Kieffer TJ and Habener JF.** The glucagon-like peptides. *Endocr Rev* 20: 876–913, 1999.
22. **Koruda MJ, Rolandelli RH, Bliss DZ, Hastings J, Rombeau JL, and Settle RG.** Parenteral nutrition supplemented with short-chain fatty acids: effect on the small-bowel mucosa in normal rats. *Am J Clin Nutr* 51: 685–689, 1990.
23. **Kreymann B, Ghatei MA, Williams G, and Bloom SR.** Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet* ii: 1300–1304, 1987.
24. **Lee YC, Asa SL, and Drucker DJ.** 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, 1992.
25. **Massimino SP, McBurney MI, Field CJ, Thomson AB, Keelan M, Hayek MG, and Sunvold GD.** Fermentable dietary fiber increases GLP-1 secretion and improves glucose homeostasis despite increased intestinal glucose transport capacity in healthy dogs. *J Nutr* 128: 1786–1793, 1998.
26. **Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, and Habener JF.** Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J Biol Chem* 261: 11880–11889, 1986.
27. **Nian M, Drucker DJ, and Irwin D.** Divergent regulation of human and rat proglucagon gene promoters in vivo. *Am J Physiol Gastrointest Liver Physiol* 277: G829–G837, 1999.
28. **Palmiter RD, Norstedt G, Gelinas RE, Hammer RE, and Brinster RL.** Metallothionein-human GH fusion genes stimulate growth of mice. *Science* 222: 809–814, 1983.
29. **Philippe J.** Structure and pancreatic expression of the insulin and glucagon genes. *Endocr Rev* 12: 252–271, 1991.
30. **Philippe J, Drucker DJ, Chick WL, and Habener JF.** Transcriptional regulation of genes encoding insulin, glucagon, and angiotensinogen by sodium butyrate in a rat islet cell line. *Mol Cell Biol* 7: 560–563, 1987.
31. **Reimer RA and McBurney MI.** Dietary fiber modulates intestinal proglucagon messenger ribonucleic acid and postprandial secretion of glucagon-like peptide-1 and insulin in rats. *Endocrinology* 137: 3948–3956, 1996.
32. **Reimer RA, Thomson AB, Rajotte RV, Basu TK, Ooraikul B, and McBurney MI.** A physiological level of rhubarb fiber increases proglucagon gene expression and modulates intestinal glucose uptake in rats. *J Nutr* 127: 1923–1928, 1997.
33. **Roberge JN and Brubaker PL.** Regulation of intestinal proglucagon-derived peptide secretion by glucose-dependent insulinotropic peptide in a novel enteroendocrine loop. *Endocrinology* 133: 233–240, 1993.
34. **Roberge JN and Brubaker PL.** Secretion of proglucagon-derived peptides in response to intestinal luminal nutrients. *Endocrinology* 128: 3169–3174, 1991.
35. **Rocca AS and Brubaker PL.** Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. *Endocrinology* 140: 1687–1694, 1999.
36. **Rocca AS and Brubaker PL.** Stereospecific effects of fatty acids on proglucagon-derived peptide secretion in fetal rat intestinal cultures. *Endocrinology* 136: 5593–5599, 1995.
37. **Scrocchi LA, Brown TJ, MacLusky N, Brubaker PL, Auerbach AB, Joyner AL, and Drucker DJ.** Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide receptor gene. *Nat Med* 2: 1254–1258, 1996.
38. **Southon S, Gee JM, and Johnson IT.** The effect of dietary protein source and guar gum on gastrointestinal growth and enteroglucagon secretion in the rat. *Br J Nutr* 58: 65–72, 1987.
39. **Tappenden KA, Drozdowski LA, Thomson AB, and McBurney MI.** Short-chain fatty acid-supplemented total parenteral nutrition alters intestinal structure, glucose transporter 2 (GLUT2) mRNA and protein, and proglucagon mRNA abundance in normal rats. *Am J Clin Nutr* 68: 118–125, 1998.
40. **Tappenden KA and McBurney MI.** Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. *Dig Dis Sci* 43: 1526–1536, 1998.
41. **Tappenden KA, Thomson AB, Wild GE, and McBurney MI.** Short-chain fatty acids increase proglucagon and ornithine decarboxylase messenger RNAs after intestinal resection in rats. *J Parenter Enteral Nutr* 20: 357–362, 1996.
42. **Tsai CH, Hill M, Asa SL, Brubaker PL, and Drucker DJ.** Intestinal growth-promoting properties of glucagon-like peptide 2 in mice. *Am J Physiol Endocrinol Metab* 273: E77–E84, 1997.
43. **Wojdemann M, Wettergren A, Hartmann B, and Holst JJ.** Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. *Scand J Gastroenterol* 33: 828–832, 1998.
44. **Xiao Q, Boushey RP, Drucker DJ, and Brubaker PL.** Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. *Gastroenterology* 117: 99–105, 1999.