Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine

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ABSTRACT Expression of the gene encoding preproglucagon gives rise to different glucagon-related peptides in the pancreas and intestine. Glucagon gene expression is regulated by a protein kinase C-dependent pathway in rat islet cell lines, whereas activation of the adenylate cyclase pathway in islet cell lines is without effect. To elucidate the factors important for the control of proglucagon biosynthesis in the intestine, we have studied proglucagon gene expression and proglucagon biosynthesis in rat intestine. Analysis of intestinal cDNA clones encoding preproglucagon indicated that pancreatic and intestinal glucagon mRNA transcripts were identical. The regulation of proglucagon gene expression in rat intestine differed markedly from that previously observed in islet cell lines. Phorbol esters increased the secretion of glucagon-like immunoreactive peptides (GLI) but had no effect on proglucagon mRNA levels in rat intestinal cells. Bombesin also increased the secretion of GLI without affecting proglucagon mRNA levels or biosynthesis. In contrast, dibutyryl cyclic AMP, forskolin, and cholera toxin increased both proglucagon mRNA levels and GLI biosynthesis and secretion, suggesting that proglucagon gene expression in the intestine is regulated by a cyclic AMP-dependent pathway. These observations suggest that tissue-specific differences in both the regulation of proglucagon gene expression and the posttranslational processing of proglucagon contribute to the diversity of glucagon gene expression.

A variety of different mechanisms contribute to the diversification of peptide biosynthesis, including gene duplication, conservation of functional peptide domains in coding exons, and tissue-specific exon splicing (1). Tissue-specific posttranslational processing of polypeptide precursors provides an additional mechanism for increasing the diversity of gene expression (2). To analyze the molecular determinants of peptide hormone biosynthesis, we have been studying the biosynthesis of glucagon and the glucagon-related peptides.

The glucagon gene is transcribed in a highly tissue-specific manner, being expressed in the A cells of the pancreatic islets, the neuroendocrine L cells of the intestine, and in hypothalamic and brainstem neurons. Analysis of the cDNAs and genes that encode proglucagon (3–6) has shown that this prohormone resembles other polypeptides in that it encodes multiple peptides separated from each other by pairs of basic amino acids characteristic of the sites commonly cleaved by prohormone-processing enzymes. Posttranslational processing of proglucagon is also highly tissue-specific, with the liberation of glucagon in the pancreas and glicentin and the glucagon-like peptides in the intestine (7–9). The glucagon-like peptides appear to be present as larger, incompletely processed forms in the pancreas, whereas processing in the intestine generates authentic GLP-I and GLP-II. The observation that a truncated form of GLP-I released from the gut has potent insulinotropic properties (10–13) has stimulated much interest in the biosynthesis of glucagon and the glucagon-like peptides.

The results of previous studies have shown that transcription of the glucagon gene was stimulated by phorbol esters and sodium butyrate in rat islet cell lines (14, 15). Glucagon gene expression does not appear to be regulated by a cyclic AMP-dependent pathway in rat (14) or hamster (16) islet cell lines, despite the recent detection of a cyclic AMP response element (CRE) in the 5′ flanking region of the rat glucagon gene (17). Moreover, the glucagon gene 5′ flanking sequences, which surround the rat glucagon CRE, have been shown to have a marked inhibitory effect on the cyclic AMP responsiveness of the core 8-base-pair (bp) CRE (18). We now report that transcription of the rat glucagon gene in the intestine produces a mRNA transcript identical to that detected in rat pancreas and that proglucagon gene expression and biosynthesis is regulated by a cyclic AMP-dependent pathway in the intestine.

MATERIALS AND METHODS

Isolation of Proglucagon cDNAs. The phage AGT11 neonatal rat intestinal cDNA library (19) was plated at a density of 3 × 10⁶ plaques per plate, and duplicate nitrocellulose filters were denatured, baked, and hybridized in 1 M NaCl/1% sodium dodecyl sulfate (SDS) with 5 × 10⁶ cpm of radiolabeled rat pancreatic glucagon cDNA per ml at 65°C. Final washing conditions were 0.1 × SSC/0.1% SDS at 65°C (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). Phage were purified by successive replating at lower densities, and cDNA inserts were subcloned into pBluescript plasmids (Stratagene). DNA sequencing was performed as described (20).

Tissue Culture. The method for preparing primary intestinal cultures has been described (21). Briefly, pregnant Wistar rats (19–21 days of gestation) were lightly anesthetized and sacrificed, and intestines from fetal rats were minced and pooled. Tissue culture medium was Dulbecco’s minimal essential medium containing 4.5 g of glucose per liter, 5% (vol/vol) fetal calf serum, and 50 units of penicillin and 50 μg of streptomycin per ml. After two 15-min digestions with collagenase, hyaluronidase, and DNase-I, cells were filtered through cheesecloth, collected by centrifugation, plated in 100-mm culture dishes, and grown at 37°C under 90% air/10% CO₂. After 16–20 hr, intestinal cultures were washed, and peptides or chemical agents were added at 100–1000 times the desired final concentration in medium containing 1 g of glucose per liter, 20 micromolar of insulin per ml, and 0.5% fetal calf serum. We have determined (22) that optimal doses of the test agents, dibutyryl cyclic AMP and phorbol 12-myristate 13-acetate (PMA), were 5 mM and 1 μM, respectively.

Abbreviations: GLI, glucagon-like immunoreactive peptides (immunoreactivity); CRE, cyclic AMP response element; FRIC, fetal rat intestinal cell; PMA, phorbol 12-myristate 13-acetate; CCK, cholecystokinin.

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tively. Lower doses were found to be less active, and higher doses were toxic. All experiments were repeated at least twice (in duplicate) with different preparations of FRIC cultures. Medium and cell peptides were extracted by reversed-phase adsorption techniques as described (21). Recoveries of glucagon and all related peptides were found to be >88% with this methodology (21, 22). Radioimmunoassays for glucagon-like immunoreactivity (GLI) were performed as described (21). Statistical significance was assessed by unpaired Student's t test.

**RNA Analysis.** RNA was isolated from tissues and tissue culture plates as described (23). RNA was size-fractionated through a 1.3% agarose/formaldehyde gel, ethidium-stained to assess the integrity and migration of the RNA, and transferred to a nylon membrane. The RNA was fixed on the membrane by UV irradiation, following which prehybridization was performed overnight in 1x Denhardt's solution (0.02% polyvinilpyrrolidione/0.02% Ficoll/0.02% bovine serum albumin) containing 4x SSC, 200 μg of salmon sperm DNA per ml, 40% deionized formamide, and 0.014 M Tris (pH 7.4). The rat cholecystokinin (CCK) cDNA was obtained from J. Dixon (Purdue University) (24). cDNA probes for rat glucagon, somatostatin, CCK, peptide YY, and chicken actin were labeled with [32P]dATP by the random priming technique (25) to a specific activity of 5 x 10^8 cpm/μg. Hybridization was performed in the same solution with 1 x 10^6 cpm of 32P-labeled cDNA probes per ml for 24 hr at 42°C. Final washing was with 0.1x SSC/0.1% SDS at 65°C. Autoradiography was carried out by using Kodak X-Omat film at −70°C.

**RESULTS**

To determine the sequence of the intestinal mRNA encoding proglucagon, a cDNA library prepared from rat intestine was screened with the rat pancreatic glucagon cDNA as probe. Eleven positive clones were identified after screening 5 x 10^5 phage, and the cDNA inserts were further characterized by restriction mapping and DNA sequencing (Fig. 1). The longest cDNA isolated extended 19 nucleotides 5' to the previously reported rat pancreatic cDNA sequence (26). Restriction mapping and DNA sequence analysis of several intestinal cDNAs showed that the sequences of the rat intestinal and pancreatic mRNAs were identical.

Blot-hybridization analysis of RNA (Northern blot) prepared from rat pancreas and intestine demonstrated the presence of a single glucagon mRNA species of ~1250 bp (Fig. 2A). To examine the regulation of proglucagon gene expression in the intestine, we utilized a primary FRIC culture system (21). FRIC cultures were characterized with respect to peptide hormone gene expression by analyzing total cellular RNA for the expression of regulatory peptide genes commonly transcribed in the endocrine cells of the intestine. Total cellular RNA was prepared from individual subconfluent 100-mm tissue culture dishes containing ~5 x 10^6 cells per dish. Total yield was 25–35 μg of RNA per plate. Northern blot analysis of FRIC RNA showed the presence of mRNA transcripts for glucagon and somatostatin that were indistinguishable in size from those detected in rat pancreatic and intestinal RNA (Fig. 2A). A single peptide YY mRNA transcript in amounts comparable to that obtained for glucagon was also detected in RNA prepared from FRIC cultures (Fig. 2B). This was not unexpected since peptide YY and GLI have been reported to colocalize to the same population of intestinal L cells (27). A single mRNA species of the expected size was also detected with a cDNA probe for CCK (Fig. 2C). These observations suggested that the primary FRIC culture system was at least partially representative of normal intestinal epithelium with respect to regulatory peptide gene expression. Interestingly, the FRIC cultures appear to be at least somewhat enriched for endocrine cells in that all four peptide hormone mRNA transcripts were relatively more abundant in RNA prepared from FRIC cells than in total intestinal RNA (Fig. 2).

The factors important for the regulation of glucagon gene expression in the intestine remain poorly understood. Although glucagon gene transcription has been shown to be stimulated by phorbol esters in a rat islet cell line (14), incubation of FRIC cultures with PMA at doses up to 1 μM resulted in a 1.5-fold increase in GLI secretion into the medium, but no change in peptide biosynthesis (Fig. 3) or glucagon mRNA levels (not shown) was observed. The effects of bombesin on proglucagon biosynthesis were next examined, as bombesin has been shown to exert its effects in part through a PMA-sensitive pathway (28). Although bom-

**Fig. 1.** Schematic representation and restriction map of intestinal proglucagon cDNA. The map shown represents the longest intestinal glucagon cDNA isolated. This cDNA is identical in sequence to the rat pancreatic cDNA previously reported (22), with a 5' extension of the following 19 nucleotides, which are underlined (5'−AGAACAACATCAAAAGTTCCGAAGAAGCTCC−3').

**Fig. 2.** Northern blot analysis of RNA from FRIC cultures. Total cellular RNA (5 μg) from rat pancreas (lanes P), intestinal cell cultures (lanes IC), and rat intestine (lanes I) was hybridized with [32P]-labeled cDNA probes for rat glucagon (G) and somatostatin (S) (A), peptide YY (B), and CCK (C).
be detected. We next studied the effects of agents that exert their effects via a cyclic AMP-dependent pathway. Treatment of RIN 1056A islet cells with 5 mM dibutyryl cyclic AMP had no effect on glucagon mRNA levels (14). Incubation of FRIC cultures with 5 mM dibutyryl cyclic AMP for 12-24 hr resulted in a 2.5- to 3-fold increase in GLI secretion into the medium and a 2.5-fold increase in total GLI biosynthesis (Fig. 3). Analysis of RNA prepared from dibutyryl cyclic AMP-treated and control cells demonstrated an ~2- to 3-fold increase in glucagon mRNA levels after a 24-hr incubation with dibutyryl cyclic AMP (Fig. 4 Upper b). To further define the importance of a cyclic AMP-dependent pathway in the regulation of glucagon gene expression, we tested the effects of forskolin (10 μM), a direct activator of adenylate cyclase, and choleragen (5 ng/ml), a molecule known to exert its effects via an increase in intracellular cyclic AMP, on the levels of glucagon mRNA in FRIC cultures. Both forskolin and cholera toxin increased glucagon mRNA levels 2- to 3-fold after 24-hr incubations (Fig. 4 Lower). In contrast, no changes in the mRNA levels of the housekeeping gene, β-actin, were detected. The observations that dibutyryl cyclic AMP, forskolin, and cholera toxin independently and specifically increased glucagon mRNA levels in FRIC cultures provides strong evidence for the importance of a cyclic AMP-dependent pathway in the regulation of intestinal glucagon gene expression. To further assess the specificity of the cyclic AMP-dependent increase in proglucagon mRNA levels, we examined the effects of dibutyryl cyclic AMP, forskolin, and cholera toxin on the levels of peptide YY and somatostatin mRNAs. Although somatostatin mRNA levels in FRIC cultures were increased after treatment with dibutyryl cyclic AMP (Fig. 5 Upper), no change in peptide YY mRNA levels was noted after treatment of FRIC cultures with forskolin or cholera toxin (Fig. 4 Lower) or dibutyryl cyclic AMP (not shown).

Examination of the time course of glucagon and somatostatin mRNA accumulation after treatment with 5 mM dibutyryl cyclic AMP is shown in Fig. 5. An increase in both mRNA levels was seen as early as 4 hr after which both glucagon and somatostatin mRNA transcripts accumulated in a linear yet quantitatively different fashion (Fig. 5 Lower). In contrast to the observed increases in peptide hormone mRNAs, no significant changes in the levels of actin mRNA after treatment with dibutyryl cyclic AMP were detected. Biosynthesis of the GLI paralleled the changes in glucagon mRNA levels at each time point studied (not shown).

**DISCUSSION**

Analyses of RNA from pancreas and intestine showed the presence of a single glucagon mRNA transcript of ~1250 bp in both tissues. The transcription start site as well as the cDNA sequence of the 5' end of the human pancreatic and intestinal mRNAs has been shown to be identical (29). Our results indicate that the full-length intestinal proglucagon mRNA transcript, including the coding sequence and untranslated regions, is identical to that found in pancreas, thereby suggesting that differences in the patterns of post-translational processing of proglucagon in pancreas and intestine are not due to subtle differences in mRNA structure and hence proglucagon sequence.

To study the intestinal biosynthesis of the glucagon-related peptides, we have established a primary FRIC culture system that contains distinct epithelial cells that produce a number of different peptide hormones. Several lines of evidence suggest that FRIC cultures are representative of normal rat intestine. We have previously reported the chromatographic profile of immunoreactive glucagon-related peptides from this cell culture system (21) and have shown that under basal and stimulated conditions, the cultured intestinal cells stored and

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**Fig. 3.** Secretion and biosynthesis of GLI. Results are expressed as the percentage of paired control values ± SEM for both medium (hatched bars) and cells (solid bars). PMA (1 μM), 1 μM bombesin (Bom), and 5 mM dibutyryl cyclic AMP (DB) were used. Net biosynthesis is the total increase in GLI (medium plus cells) compared with controls. *, P < 0.01; **, P < 0.001 (for change in total-cell-plus-medium GLI).

**Fig. 4.** Northern blot analysis of RNA from FRIC cultures. (Upper a) Five micrograms of total RNA from control (lanes C) or 1 μM bombesin-treated cells (lanes B). (Upper b) Five micrograms of total RNA from control (lanes C) and dibutyryl cyclic AMP-treated cells (lanes DB). Experiments were carried out for 24 hr, after which RNA was prepared and Northern blots were hybridized with cDNA probes for glucagon (g) and somatostatin (s) in Upper a and for glucagon alone in Upper b. (Lower) Five micrograms of total RNA from control (lanes C) or FRIC cultures incubated with 10 μM isobutylmethylxanthine and 10 μM forskolin (lanes F) or cholera toxin at 5 ng/ml (lanes CT) hybridized with cDNA probes for glucagon (G), peptide YY (P), and actin (A).
were expressed only in glucagon-producing islet cell lines (17, 30). Furthermore, a glucagon–simian virus 40 large tumor (T) antigen fusion gene containing 850 bp of rat glucagon gene 5' flanking sequences was expressed in the pancreas and brain but not in the intestine of transgenic mice (31).

Our experiments suggest that the control of glucagon gene expression in the intestine and pancreas may also differ at another level—namely, the cyclic AMP responsiveness of the glucagon gene in these two tissues. Recent studies have shown that a conserved octamer sequence TGACGTCA is found in the 5' flanking region of a number of different genes that are transcriptionally regulated by cyclic AMP (32–34). Analysis of the rat glucagon gene 5' flanking sequences has revealed that this exact octamer CRE is present from −298 to −291 upstream of the transcription start site (17). Interestingly, no such consensus sequence was detected in the 5' flanking region of the human glucagon gene (5). Previously we have attempted to confer cyclic AMP responsiveness to the rat glucagon gene CRE without success. Transfection of fusion genes containing the rat glucagon CRE into cell lines that are known to respond to cyclic AMP has failed to elicit any cyclic AMP responsiveness from the glucagon CRE (D.J.D., unpublished observations). A similar series of experiments, which have examined the apparent lack of cyclic AMP responsiveness of the rat glucagon CRE, has indicated that the 5' and 3' contextual sequences that flank the rat glucagon CRE may play an important role in negating the activity of the 8-bp core cyclic AMP enhancer (18). Our observation that the rat glucagon gene is responsive to cyclic AMP in primary intestinal cell cultures suggests that the cyclic AMP responsiveness of different CREs may be highly tissue- and cell-specific. Further analysis of the control of glucagon gene transcription in pancreas and intestine may provide useful insights into the tissue-specific control of peptide hormone gene transcription.

Little data is available regarding the physiological control of GLI biosynthesis and secretion in the intestine (35). The control of intestinal GLI secretion has been shown to be inhibited and stimulated by somatostatin and bombesin, respectively (36, 37). These regulatory peptides are known to exert their effects through cyclic AMP- and protein kinase C-dependent pathways, respectively (28, 38). A role for cyclic AMP in the control of glucagon secretion has also been suggested by the observation that the glucagon response to hypoglycemia is adrenergically mediated (39) and that GLI secretion in pancreatectomized dogs appears to be adrenergically mediated (40). Furthermore, adrenergic nerve terminals have been identified in close proximity to the GLI-secreting cells in the intestine (41). The observation that adrenergic stimulation increased the levels of cyclic AMP in the intestine (42) suggests that cyclic AMP-dependent glucagon gene expression and biosynthesis may have potential relevance for understanding the physiological control of the biosynthesis and secretion of the proglucagon-derived peptides.

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Fig. 5. Time course of mRNA accumulation following treatment with dibutyryl cyclic AMP. (Upper) Five micrograms of RNA from FRIC cells treated with 5 mM dibutyryl cyclic AMP for 4, 8, 12, 24, 36, and 48 hr was hybridized with cDNA probes for glucagon (G), somatostatin (S), and actin (A). (Lower) Autoradiographic signals were quantitated by scanning with a laser densitometer and the accumulation of glucagon and somatostatin mRNA transcripts was plotted as densitometric units (experimental/control) versus time by dividing the values for the dibutyryl cyclic AMP-treated signals by the appropriate control values for each time point (not shown).
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