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Translational control of anionic trypsinogen and amylase synthesis in rat pancreas in response to caerulein stimulation

(gene expression/regulation of mRNA translation)

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ABSTRACT **Infusion of rats with optimal doses of caerulein for up to 24 hr resulted in divergent changes in protein synthesis in the exocrine pancreas: a 3-fold increase in synthesis of anionic trypsinogen and a 75% decrease in synthesis of amylase. Lipase synthesis showed no change. Rates of total protein synthesis increased 2-fold, while DNA, RNA, and poly(A)$^+$ mRNA concentrations were unchanged during hormonal stimulation. mRNA concentrations for anionic trypsinogen, lipase, and amylase were determined by dot blot hybridization analysis with cDNA and cRNA probes. Despite 12-fold changes in the ratio of synthesis of anionic trypsinogen to amylase at 24 hr of caerulein stimulation, changes in levels of mRNA encoding these two proteins were not observed. The slight decreases observed in amylase mRNA concentrations were found in both hormone and saline-infused animals. *In vitro* pulse–chase experiments after 12 hr of saline or caerulein infusion indicated that differential turnover of anionic trypsinogen and amylase did not occur during hormone stimulation. These data demonstrate that the differential regulation observed in protein synthesis that results from a single period of hormone stimulation is mediated by differential regulation of mRNA translation. The high degree of conservation observed in the 5′ terminal sequences of both amylase and anionic trypsinogen mRNAs between mouse, rat, and dog suggests that sequence-specific mechanisms and secondary structure may play a role in the translational control of these two mRNAs.**

Regulation of gene expression has been studied to the greatest extent in prokaryotic organisms where mechanisms that regulate gene transcription predominate (1). In prokaryotes the half-lives of mRNA are measured in minutes (2). In eukaryotes, the half-lives of mRNA, which represent differentiated products of gene transcription (e.g., mRNAs encoding secretory products), are measured in hours to days (2, 3). mRNAs for pancreatic secretory proteins show half-lives of 3–6 hr (3). It is therefore likely that mechanisms that regulate rapid changes in gene expression in eukaryotic tissues (biological response in minutes to hours) occur at the translational level. However, few examples of translational control have been documented in higher animals. Recently, translational control has been demonstrated for insulin biosynthesis in response to changes in glucose concentrations in the blood circulation in the rat (4), for a 70-kDa protein during the response to heat shock in *Drosophila* (5), and for a group of ≈20 proteins during the early mitotic response in 3T3 cells (6). In the exocrine pancreas, we have reported that the synthesis of anionic but not cationic trypsinogen is regulated at the level of mRNA translation during caerulein stimulation (7–9). We now report that the divergent changes in synthesis of anionic trypsinogen and amylase during a 24-hr period of caerulein stimulation are regulated through mechanisms of translational control.

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MATERIALS AND METHODS

Hormone Infusion. Caerulein, a cholecystokinin analog, was diluted in saline and infused into the tail vein of conscious male rats at a rate of 0.25 $\mu g \cdot kg^{-1} \cdot hr^{-1}$, which resulted in maximal pancreatic secretion of amylase without evidence of pathological changes, as described by Schick *et al.* (7). Control animals received saline alone at a rate of 0.2 $ml \cdot hr^{-1}$ per animal. Rats were fasted during the infusion periods.

Diets. Before and during hormone infusion studies, animals had free access to a standard laboratory diet containing 63% carbohydrate, 22% protein, and 5% lipid. In dietary adaptation experiments, a separate group of animals was fed a low carbohydrate diet containing 2% carbohydrate, 83% protein, and 5% lipid over a period of 14 days. The remainder of the two diets consisted of cellulose and minerals.

Measurement of DNA, RNA, and Poly(A)$^+$ RNA. After hormone stimulation or dietary adaptation, the animals were weighed and then sacrificed by exsanguination during light ether anesthesia. The pancreas was surgically removed and briefly bathed in 0.9% NaCl to remove connective tissue. One-quarter of each pancreas was homogenized in 5 ml of a solution containing 25 mM Tris·HCl (pH 8.9) and 0.1% Triton X-100. DNA content was measured according to Richards (10). RNA content was determined by the orcinol method (11). Poly(A)$^+$ mRNA was determined from total cellular RNA purified through CsCl by absorption to mAP paper (Medac, Hamburg, F.R.G.) as outlined in the manufacturer's protocol.

Analysis of Total and Individual Rates of Protein Synthesis. Pancreatic lobules were prepared from one-half of pancreatic glands as described by Scheele and Palade (12). Lobules were incubated in a Krebs–Ringer Hepes solution containing ^{14}C-labeled amino acids (algal hydrolysate, 50 $\mu Ci/ml$; 1 Ci = 37 GBq; Amersham) for 90 min. Lobules were then washed and homogenized, and rates of total protein synthesis per μg of DNA were determined by the protocol of Rausch *et al.* (13). Proteins contained in homogenates were separated by two-dimensional isoelectric focusing/$NaDodSO_4$ gel electrophoresis as described by Bieger and Scheele (14). Protein spots, identified with Coomassie brilliant blue, were excised and homogenized in 2 ml of H_2O. After adding 1 ml of 5% $NaDodSO_4$ and 10 ml of a toluene-based scintillation liquid, radioactivity was monitored in a liquid scintillation counter. Individual rates of protein synthesis were calculated from fractional incorporation values multiplied by total incorporation values.

Intracellular Stability of Secretory Proteins. Four animals were infused either with caerulein or saline for 12 hr and pancreatic lobules were prepared as described above. The lobules were pulse-labeled with [^{35}S]methionine (15 $\mu Ci/ml$; 1000 Ci/mmol) in Krebs–Ringer Hepes solution for 10 min.

The lobules were washed and the [^{35}S]methionine was replaced by an excess of unlabeled methionine and further incubated for 0 or 90 min. After the given chase periods, the lobules were homogenized and radioactivity present in total protein and individual secretory protein forms was determined. The stability of protein forms was determined by comparing values obtained after 0- and 90-min chase periods.

Isolation of RNA. In separate experiments for mRNA quantification, three-fourths of the pancreas was put into a 30-ml Corex (Corning) centrifuge tube containing 10 ml of 4 M guanidinium thiocyanate in 0.1 M Tris·HCl, pH 7.5/0.14 M 2-mercaptoethanol (15) and was subsequently homogenized with a Turrax homogenizer (Kinematic, Lucerne, Switzerland) at full speed for 30 sec. The homogenate was centrifuged at 6000 rpm for 10 min at 4°C in a Beckman JA-20 rotor. The supernatant was decanted into a 200-ml Erlenmeyer flask and 10 ml of a proteinase K solution (0.5 mg of proteinase K per ml/20 mM Tris·HCl, pH 7.5/0.1 M NaCl/40 μg of polyvinylsulfate per ml/2 mM dithiothreitol) was slowly added within 5 min with gentle agitation. After incubation at 37°C for 15 min, it was mixed with 12 ml of 3.5 M $NaClO_4$ and incubated at 37°C for an additional 5 min with occasional shaking. Then, 120 ml of saturated $NaClO_4$ ($NaClO_4$ saturated in H_2O/$NaClO_4$ saturated in ethanol, 1:4) and 1.8 ml 20% $NaDodSO_4$ were added, mixed well, and incubated at 10°C for 12 min with vigorous shaking. The solution was centrifuged at 5500 rpm for 10 min at 4°C in a Beckman JA-14 rotor. The pellet was resuspended in 40 ml of 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.2% $NaDodSO_4$ and centrifuged for 30 min at 30,000 rpm at 4°C in a Beckman SW 50.2 rotor. After adding 4 M NaCl to a final concentration of 0.2 M and 0.6 vol of isopropanol to the supernatant, the RNA was precipitated at −20°C overnight. The RNA pellet was washed twice with 70% ethanol, dried under vacuum, and dissolved in 3 ml of H_2O. After purification through 5.7 M CsCl (16), the integrity of the RNA was checked by electrophoresis in a 1% denaturing agarose gel containing 2.2 M formaldehyde and by RNA blot hybridization analysis with the three cDNA probes. The gel running buffer contained 10 mM $NaPO_4$ instead of Mops (17).

cDNA Probes. A cDNA clone encoding rat anionic trypsinogen mRNA [0.804 kilobase (kb); ref. 18] was obtained from R. MacDonald (Department of Biochemistry, University of Texas Health Sciences Center, Dallas). Clones encoding rat pancreatic amylase and lipase were isolated from a cDNA library prepared from rat pancreatic poly(A)$^+$ mRNA by S. Pinsky in the laboratory of G.A.S. A cDNA clone encoding a rat amylase (1.5 kb) was identified by use of a mouse pancreatic cDNA clone (19) obtained from U. Schibler (Swiss Institute for Cancer Research, Epalinges, Switzerland). A clone for rat lipase (0.82 kb) was identified by using a dog pancreatic lipase clone (20). The identities of cDNA clones encoding rat pancreatic amylase and lipase were confirmed by restriction enzyme mapping, *in vitro* translation of hybrid-selected mRNA followed by analysis of translation products by polyacrylamide gel electrophoresis in $NaDodSO_4$, and in the case of lipase by partial nucleotide sequence analysis.

Quantification of mRNA. Concentrations of individual mRNAs were determined by dot blot hybridization analysis. The appropriate amount of total cellular RNA purified through CsCl was dissolved in 50 μl of H_2O mixed with 150 μl of a solution of 6.15 M formaldehyde/10× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) and heated for 15 min at 65°C (Schleicher & Schuell, application update, no. 371). Denatured RNA samples (0.5 μg of total RNA in 200 μl) were spotted onto a nitrocellulose filter (BA 85, Schleicher & Schuell) with the dot blot apparatus from Schleicher & Schuell with a continuous flow rate of ≈100 μl per 10–15 sec. To control for interassay variation, each filter contained a standard dilution curve of total pancreatic RNA obtained from uninfused animals. The wells were washed once with 10× SSC before and after application of the sample. The filter was dried at room temperature and then baked at 80°C for 2 hr. The filters were hybridized with ^{32}P-labeled nick-translated (21) cDNA probes. The filters were prewetted in 2× SSC and prehybridized in 50% formamide/5× SSC/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/50 mM $NaPO_4$, pH 6.5/0.1% $NaDodSO_4$/100 μg of tRNA per ml/50 μg of poly(A) per ml at 42°C for 4 hr. Hybridizations were carried out overnight in the prehybridization medium plus 10^6 cpm per ml of radioactive probe (specific activity, ≈2 × 10^8 cpm/μg). After hybridization, the filters were washed once in 2× SSC for 10 min at 42°C, once in 2× SSC for 10 min at 65°C, three times in 2× SSC/0.1% $NaDodSO_4$ at 65°C for 1 hr each, and finally in 0.2× SSC/0.1% $NaDodSO_4$ at 65°C for 30 min. The filters were dried at room temperature, covered with SaranWrap, and subjected to autoradiography for 16 hr. Individual spots were cut out and the radioactivity bound to the filter was measured in a liquid scintillation counter with a toluene-based scintillation liquid.

RESULTS

Fig. 1 shows the changes observed in relative rates of protein synthesis during a 24-hr period of caerulein infusion at 0.25 $\mu g \cdot kg^{-1} \cdot hr^{-1}$. With time, a progressive increase was observed in anionic trypsinogen synthesis to rates that were three times higher than those observed prior to stimulation. From 6–24 hr, progressive decreases were observed in the relative synthesis of amylase forms 1 and 2. Lipase synthesis showed no change with caerulein stimulation. The ratio of anionic trypsinogen to amylase synthesis under these conditions increased 1.25-, 1.5-, 3-, and 12-fold at 3, 6, 12, and 24 hr, respectively.

Concentrations of DNA, RNA, poly(A)$^+$ RNA, and total protein synthesis were determined for the various periods of caerulein infusion studied. Significant changes were not observed in the concentrations of DNA, RNA, or poly(A)$^+$ mRNA. In contrast, rates of total protein synthesis increased ≈2-fold (4.15 to 8.75 dpm per μg of DNA × 10^{-3}) between

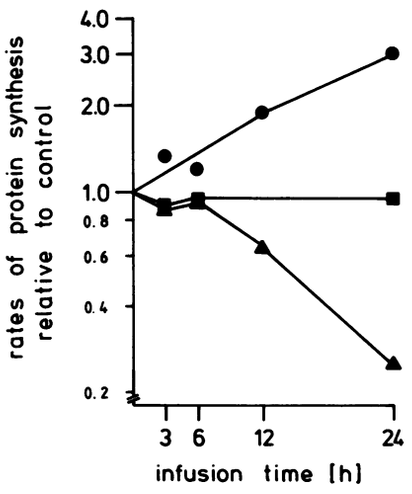

FIG. 1. Kinetic changes in relative rates of protein synthesis in response to optimal caerulein stimulation (0.25 $\mu g \cdot kg^{-1} \cdot hr^{-1}$). Individual rates of protein synthesis were determined for each secretory protein and are expressed as percentage synthesis observed for all secretory proteins. Relative changes in protein synthesis are shown for anionic trypsinogen (●), lipase (■), and amylase forms 1 and 2 (▲). Ordinate, logarithmic scale. Data represent average values derived from four or five animals at each time point.

3 and 24 hr. Absolute rates of protein synthesis were calculated from relative and total rates. Since relative rates of lipase synthesis do not change, absolute rates followed those observed with total protein synthesis. Absolute rates of anionic trypsinogen increased 1.7-, 2.7-, 3.8-, and 6-fold with 3, 6, 12, and 24 hr of caerulein stimulation, respectively. In respect to absolute rates of amylase synthesis, the increases observed in total protein synthesis offset, to some extent, the decreases observed in the relative rates of amylase synthesis. However, absolute rates of amylase synthesis remained decreased at 24 hr of stimulation (50% compared to controls). These results confirm the divergent and anticoordinate changes observed with caerulein stimulation previously reported from our laboratory (7).

Preliminary experiments demonstrated that amylase mRNA concentrations varied 10-fold, related to carbohydrate contained in the diet and in direct proportion to amylase synthesis in the pancreas, as described (22, 23). However, under conditions of a normal laboratory diet (56% carbohydrate), amylase mRNA concentrations remained unchanged with 24 hr of caerulein stimulation despite 75% and 50% decreases in relative and absolute amylase synthesis rates, respectively. This finding suggested that the changes observed in protein synthesis in response to caerulein stimulation might be mediated through mechanisms different from those that mediate changes in response to alterations in dietary substrates. We therefore studied in detail the kinetic changes observed in levels of mRNA encoding pancreatic anionic trypsinogen, amylase, and lipase in response to caerulein stimulation. The levels of mRNA encoding anionic trypsinogen, lipase, and amylase at 0.5, 1, 3, 6, 12, and 24 hr during optimal stimulation with caerulein at 0.25 $\mu g \cdot kg^{-1} \cdot hr^{-1}$ are shown in Fig. 2. Cellular RNA samples extracted from pancreatic glands of three to five animals were studied at each time point by dot blot hybridization analysis as described. For anionic trypsinogen and lipase, mRNA concentrations during hormone stimulation showed no differences from those observed with saline infusion alone and no differences from a group of uninfused control animals. Amylase mRNA levels showed a slight tendency to decrease (≈20%) with either hormone or saline infusion when compared to uninfused controls. However, no differences were observed between hormone-infused and saline-infused animals.

However, the possibility remained that differential turnover of protein forms might account for the changes observed in levels of radioactive proteins. We therefore analyzed the rates of enzyme degradation in pulse–chase experiments by measuring the fractional percentage of radioactivity in individual exocrine proteins after 90-min compared to 0-min chase periods under conditions of both saline and caerulein infusion (Table 1). Under both basal conditions and hormone infusion, fractional rates as a function of time of chase increased for amylase and decreased for the majority of other exocrine enzymes. Progressive enrichment of amylase with increasing times of chase is compatible with our previous observations that the intracellular transport of amylase is asynchronous (slower when compared to other proteins) (24), a finding that is unaffected by secretagogue stimulation (25). Thus, the changes observed in amylase representation did not correlate with hormone stimulation. Furthermore, the increases observed in amylase representation with time of chase did not agree with the decreases observed in amylase synthesis during caerulein infusion. Thus, the results summarized in the table demonstrate no change in enzyme stability under basal or hormone-stimulated conditions. Differential turnover of individual proteins did not occur with hormone stimulation, and therefore such mechanisms cannot account for the differential changes observed in protein synthesis. The changes, an increase and a decrease in the synthesis of anionic trypsinogen and amylase, respectively, are observed in the table both immediately after the pulse and after a 90-min chase period.

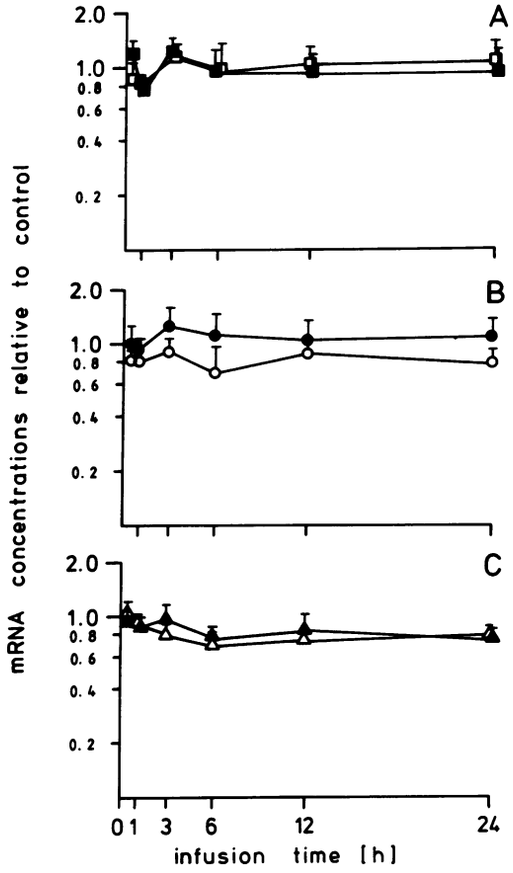

FIG. 2. Concentrations of mRNA encoding rat pancreatic (pro)enzymes. (*A*) Anionic trypsinogen; (*B*) lipase; (*C*) amylase. mRNA concentrations were measured with ^{32}P-labeled cDNA probes on nitrocellulose dot blots. Measurements were made on RNA extracted from pancreatic tissue from hormone-infused (solid symbols) or saline-infused (open symbols) animals. Vertical bars indicate SD. Three or four animals were studied at each time point under both experimental and control conditions. Ordinate, logarithmic scale, and at the zero time point, values represent 600, 200, and 1000 cpm in *A*, *B*, and *C*, respectively. Statistical differences between pairs of curves were not observed at the $p = 0.05$ level.

Table 1. Intracellular turnover of exocrine proteins in the absence and presence of caerulein infusion: Percentage distribution of exocrine proteins after 0- and 90-min chase periods

	% radioactivity			
	Saline control		Caerulein	
	10-min pulse	90-min chase	10-min pulse	90-min chase
Amylase 1 and 2	39.9	49.2	24.8	33.5
Trypsinogen 1 and 2	8.2	7.7	13.1	12.8
Trypsinogen 3	4.4	3.3	4.3	4.0
Lipase	2.5	1.9	3.0	2.8
Procarboxypeptidases A and B	15.1	12.0	16.7	15.6
Chymotrypsinogen 1 / Proelastase 1	22.0	19.1	27.2	23.2
Chymotrypsinogen 2 / Proelastase 2	4.4	3.5	5.4	4.4
Phospholipase A_2	0.3	0.3	0.5	0.4
RNase	3.2	2.9	4.9	3.2

DISCUSSION

Recent studies have described in detail the patterns of divergent or anticoordinate regulation of gene expression observed in the exocrine pancreas (26, 27). Synthesis of three functional groups of secretory (pro)enzymes (protease zymogens, amylase forms 1 and 2, and lipase) appears to be largely regulated in an independent manner by three corresponding classes of dietary substrates (protein, carbohydrate, and lipid, respectively). We (22) and others (23, 28) have shown that differential changes in protein synthesis observed with alterations in dietary components are mediated by changes in mRNA concentrations within pancreatic cells.

In other studies, it has been demonstrated that hormones play an important role in the anticoordinate changes observed in gene expression in the pancreas. Synthesis of the majority of protease zymogens is regulated by cholecystokinin-like hormones including caerulein (7), lipase synthesis is regulated by secretin (13, 29), and amylase synthesis is regulated by insulin (30–33) and glucocorticoids (34). In contrast to the results observed after changes in diets, the findings shown here indicated no change in mRNA levels during 24 hr of caerulein stimulation. In earlier studies, which measured functional concentrations of mRNA by *in vitro* translation in a high-fidelity reticulocyte lysate system (8), no changes in mRNA levels were observed through 6 hr of hormone stimulation. After 12 and 24 hr of hormone infusion, the small changes (25–50%) that were observed in functional mRNA levels could not account for the 3- and 4-fold changes observed in trypsinogen and amylase synthesis, respectively. Because of the presence of RNase in rat pancreas, we consider data derived from dot blot hybridization studies to be more reliable than those derived from *in vitro* translation of mRNA. Thus, despite 12-fold changes in the ratio of synthesis of anionic trypsinogen to amylase at 24 hr of caerulein stimulation, significant changes in levels of mRNA encoding these two secretory enzymes were not observed. These results suggest that the initial effects of hormone stimulation, which result in anticoordinate changes in protein synthesis, are not associated with changes in RNA transcription rates or posttranscriptional processes that affect steady-state levels of mRNA.

In pulse–chase studies under basal or hormone-stimulated conditions, nascent proteins, synthesized in pancreatic lobules during a pulse period of 10 min, remained intact during the 90-min period of analysis. These findings indicate that the changes observed in levels of radioactive proteins after caerulein stimulation, as judged by a 90-min period of protein synthesis under *in vitro* conditions, are due to changes in protein synthesis rates and are not due to the differential intracellular degradation or turnover of nascent proteins. Taken together, the results presented in this paper suggest that the initial effects on protein synthesis observed after caerulein stimulation are controlled at the level of mRNA translation.

Because of the presence of large concentrations of RNase in rat pancreas (200 μg per g of tissue; ref. 36), we were unable to analyze specific mRNAs associated with polysomal and messenger ribonucleoprotein fractions. Consequently, it could not be determined whether the observed differential regulation of mRNA translation is due to differential changes in the efficiency of translation of individual mRNAs or to the differential sequestration of mRNA into nonproductive forms.

Despite detailed knowledge of the mRNA sequences that encode pancreatic digestive enzymes in mouse (19), rat (18, 35), and dog (9, 20), the mechanisms that could mediate differential changes in mRNA translation are not at present understood. Conceivably, sequence-specific mRNA binding

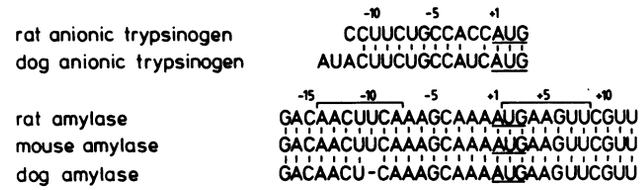

FIG. 3. Conserved sequences at the 5′ termini of mRNAs encoding pancreatic anionic trypsinogen and amylase. Identities are indicated by vertical lines. The dash in the sequence of dog amylase mRNA is introduced as a deletion to maximize sequence alignments. AUG initiation codons are underlined. Brackets show complementary sequences, which may base-pair to form stem–loop structures. Sequences are from the following references: anionic trypsinogen mRNA from the dog (9) and rat (18); amylase mRNA from the dog (K. S. LaForge and G.A.S., unpublished data), mouse (19), and rat (35).

proteins, possibly altered by second messenger-activated pathways, may be responsible for differentially altering initiation of translation of individual mRNAs. Consistent with this possibility is the high degree of conservation observed between the 5′ nontranslated sequences of anionic trypsinogen mRNAs (10/11 bases, 90.9% identity) and between the 5′ terminal sequences of amylase mRNAs (28/29 bases, 96.6%) derived from several species (Fig. 3). Potential hybridization involving 7 contiguous bases (positions −8 to −14 paired with positions +2 to +8) in amylase mRNAs from rat, mouse, and dog is shown in Fig. 4. The high degree of identity observed at the 5′ end of the three amylase mRNAs and the potential secondary structure involving the AUG initiation codon suggest that regulation of secondary structure could be involved in the translational control of amylase mRNA translation.

The observation that cholecystokinin-like hormones differentially regulate the translation of anionic trypsinogen and amylase mRNAs, in this case in opposite directions, represents one of the first examples of translational control in higher animals. The effects of cholecystokinin on discharge of enzymes via exocytosis are believed to be mediated through receptor-mediated processes involving calcium, calmodulin, and calmodulin-dependent protein kinase II and protein kinase C (37). The findings reported in this paper, which dissociate changes in protein synthesis from pre- or posttranslational mechanisms, provide an optimal system to determine whether second messenger systems also regulate the differential translation of individual mRNAs. Should this be the case, the mechanisms responsible for the kinetic differences observed in the biological responses to caerulein [exocytosis responds within seconds to minutes (12, 37);

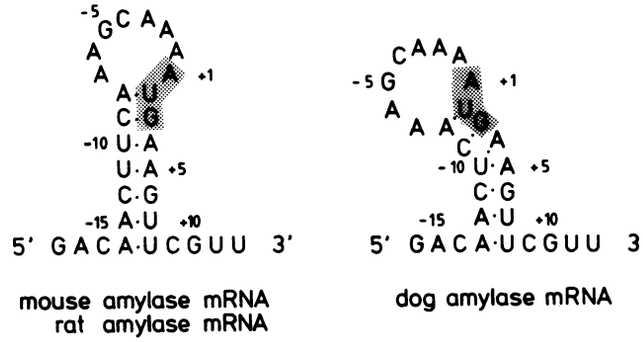

FIG. 4. Potential secondary structure at the 5′ ends of pancreatic amylase mRNAs. Potential base-pairing is indicated by dots in stem–loop structures. In mRNAs from each of the three species, AUG initiation codons are highlighted and UG dinucleotides in these codons show potential base-pairing.

protein synthesis occurs within 1–3 hr (7)] will need to be elucidated.

Two other important findings emerge from our study. The first is the relatively slow response observed in changes in gene expression in the pancreas in comparison with other biological stimulus–response systems. In the pancreas, an exocrine gland responsive to changes in nutritional substrates in the diet, alterations in gene expression occur on the time scale of hours to days. This response is slower than that observed in cases of rapid glucocorticoid induction (response in minutes to hours), where direct activation of gene transcription occurs by interaction of glucocorticoid–receptor complexes with defined nucleotide regulatory sequences. The time course of response is more similar to that observed in cases of delayed glucocorticoid induction (response in hours to days) where the response depends on the synthesis of protein factors (see ref. 38 for a review). Second, results derived from repeated periods of hormone stimulation (39), which lead to changes in mRNA concentrations, are different from those described here for a single period of hormone stimulation, in which mRNA levels show no change. Of importance to the biological regulation of gene expression in this exocrine tissue is the finding that the response mediated by changes in mRNA levels upon repeated stimulation augments and amplifies the response mediated at the level of translational control with a single period of hormone stimulation (27). Thus, the sequential phases of biological response, which can be expected to depend on the integration of divergent mechanisms activated over different periods of time, result in similar patterns of response but reflect an increase in biochemical commitment during repeated hormone stimulation.

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