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Differential Regulation of Trypsinogen mRNA Translation: Full-Length mRNA Sequences Encoding Two Oppositely Charged Trypsinogen Isoenzymes in the Dog Pancreas

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In the absence of changes in functional mRNA levels, stimulation of the pancreas with caerulein, a peptide analog of cholecystokinin, has been previously shown to increase the synthesis of anionic but not cationic trypsinogen. To look for structure-function correlations, a high-yield, full-length cDNA library has been constructed from canine pancreatic poly(A)$^+$ mRNA. Full-length clones coding for the two major trypsinogen isoenzyme forms have been identified by colony hybridization and verified by in vitro translation of hybrid-selected mRNA in the presence of microsomal membranes and an optimal redox potential. Disulfide-bonded translation products were separated and identfied by two-dimensional isoelectric focusing–sodium dodecyl sulfate-gel electrophoresis. Nucleotide sequence analysis allowed us to deduce the amino acid sequences for the anionic and cationic forms of canine trypsinogen, which contain 232 and 231 residues, respectively (77% amino acid identity), and the 15-residue amino terminal signal sequences (53% amino acid identity) associated with the two presecretory forms. Measurements of relative and absolute mRNA levels, when related to relative protein synthesis values, indicated that the translational efficiency of anionic trypsinogen mRNA exceeded that of cationic trypsinogen mRNA by 1.5- to 2.9-fold under basal conditions. Analysis of the 5′ noncoding regions of trypsinogen mRNAs revealed a striking conservation of sequence (10 of 12 bases) between dog and rat anionic trypsinogen forms. This contrasted markedly with the divergence of the 5′ noncoding regions observed between dog anionic and cationic trypsinogen mRNAs.

In the past, studies on regulation of gene expression have focused largely on mechanisms which regulate gene transcription. The majority of these studies have been carried out in procaryotes. Half-lives of mRNA in procaryotes are short, measured in seconds to minutes (5), and thus changes in transcription rates can be expected to have profound and immediate adaptive effects in such organisms. In contrast, half-lives of mRNA in eucaryotes are measured in minutes to hours (2, 5). In differentiated tissues specialized for the production of secretory proteins (e.g., the exocrine pancreas), half-lives of mRNAs coding for exportable proteins vary between 3 and 6 h (2). Under such conditions it would seem that adaptive changes in individual tissues in response to external stimuli might also be mediated by regulation of posttranscriptional events. Although differential regulation of protein synthesis at the translational level and at the level of mRNA stability have been observed in several eucaryotic systems (3), the mechanisms responsible for such changes remain largely obscure.

Recent studies in the rat exocrine pancreas have revealed that synthesis of exportable proteins is differentially regulated by hormonal stimulation (23). Of considerable interest was the finding that synthesis of anionic but not cationic trypsinogen was increased during a 24-h infusion of caerulein, a cholecystokinin-like peptide derived from *Hyla caerulea*. Levels of functional mRNA analyzed by in vitro translation showed small changes relative to those observed in protein synthesis, suggesting that caerulein-induced differential regulation occurs predominantly at the level of mRNA translation (C. Wicker, A. Puigserver, U. Rausch, G. Scheele, and H. Kern, Eur. J. Biochem., in press). To investigate the regulation of trypsinogen synthesis in response to hormonal stimulation, we have cloned cDNAs representing the two major forms of trypsinogen mRNA observed in the canine pancreas. Nucleotide sequence analysis of full-length cDNA clones has allowed us to compare (i) the amino acid sequence for each of the zymogen forms, (ii) the amino-terminal signal sequences involved in translocation of the isozymogen forms across the rough endoplasmic reticulum, and, most importantly, (iii) the noncoding mRNA sequences for structures which may play a role in the regulation of expression of the corresponding genes.

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MATERIALS AND METHODS

Sodium perchlorate and acrylamide were obtained from BDH. Restriction enzymes were obtained from Bethesda Research Laboratories, New England BioLabs, and International Biotechnologies, Inc. (IBI). Reverse transcriptase was purchased from Life Sciences, Inc. Oligo(dT)-cellulose was supplied by Collaborative Research, Inc. The Klenow fragment of DNA polymerase I was obtained from IBI. Deoxynucleoside triphosphates were obtained from P-L Biochemicals, Inc. ^{32}P-labeled nucleotides and [^{35}S]methionine were purchased from New England Nuclear Corp. pSP6 vectors and *Salmonella* RNA polymerase were obtained from Promega Biotech. All other chemicals were of reagent grade.

Construction of a canine pancreatic cDNA library containing full-length transcripts. A full-length cDNA library was prepared from dog pancreatic poly(A)$^+$ mRNA by the method of Heideker and Messing (7) with the following modifications. (i) First-strand cDNA synthesis was carried out in the presence of human placental ribonuclease inhibitor and 2 mM dithiothreitol (20). (ii) Sephadex G100 chromatography was used to eliminate unincorporated nucleotides after the first-strand cDNA synthesis. (iii) Oligo(dC) tails

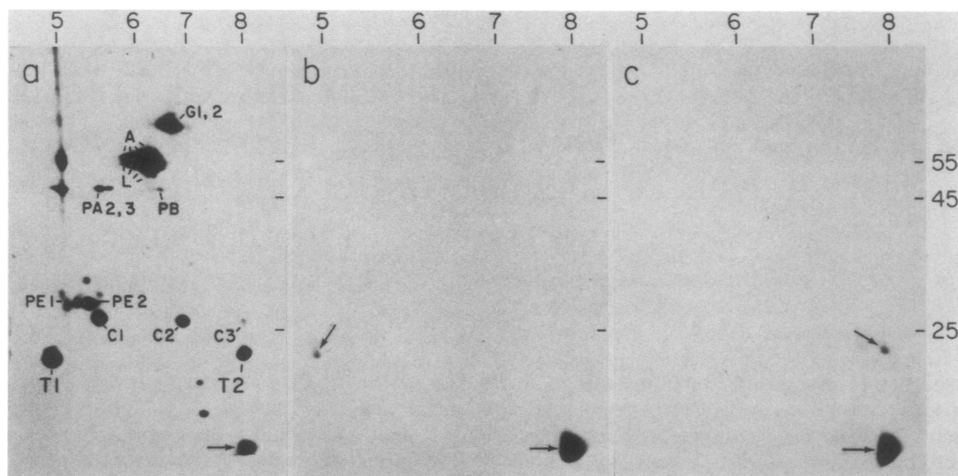


FIG. 1. Translation products separated by two-dimensional isoelectric focusing-SDS-gel electrophoresis and analyzed by fluorography. Products were synthesized in a reticulocyte lysate translation system with dog pancreas poly(A)$^+$ mRNA (a), mRNA hybrid selected to pT1 (b), and mRNA hybrid selected to pT2 (c). All translations were carried out in the presence of micrococcal nuclease-treated microsomal membranes to allow for removal of translocation signal peptides. An optimal redox potential was established by the addition of glutathione to allow for development of the correct sets of disulfide bonds. Proteins are labeled according to their identified biological activities (22). The 7 o'clock arrow points to anionic trypsinogen (T1) and the 4 o'clock arrow points to cationic trypsinogen (T2). In all three panels the 3 o'clock arrows point to globin which is synthesized in the rabbit reticulocyte translation system. The right ordinate shows apparent molecular weight (in thousands). The upper abscissae show isoelectric points.

were attached to the ends of the extended first strands. (iv) To monitor the length of the first-strand transcripts, the alkaline sucrose gradient step was replaced by electrophoresis of oligo(dC)-tailed single-stranded cDNA in a 1% agarose gel containing 30 mM NaOH. Samples were eluted from the gel by electroblotting onto Whatman DE81 paper in 0.1 M Tris borate–2 mM EDTA (pH 8.3). Autoradiography of the paper allowed us to measure the lengths of extended pUC9 strands. The region of the paper containing full-length cDNA transcripts was removed by excision, and DNA was eluted from the paper in 0.5 N NaOH. (v) Extended first strands containing dC tails were circularized by hybridization with an estimated fivefold excess of oligo(dG)-tailed pUC9 in a total volume of 0.5 ml. Hybridization was carried out in the presence of 0.55 M NaCl–20 mM Tris hydrochloride (pH 8.0) for 4 h at 68°C and allowed to equilibrate slowly to 37°C. By virtue of these modifications the full-length character of the mRNA was preserved, the two dialysis steps were avoided, the success of the procedure could be monitored at each step, and the reconstituted *Pst*I site adjacent to the single oligo(dG · dC) tail marked the 5′ end of the insert, allowing rapid sequence assessment of the full-length nature of cloned transcripts.

Characterization of cDNA clones. Colonies were grown on LB agar plates and transferred to Whatman 3MM paper for colony hybridization studies. Probes for anionic and cationic trypsinogen were derived from partial-length cDNAs identified in a previous cDNA library (16). Conditions for hybridization and washing of the filters were as follows. Filters were prewashed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 16 h at 68°C; prehybridization was carried out in 50% formamide–5× SSC–500 μg of carrier DNA per ml–5× Denhardt solution–50 μg of poly(G) per ml for 2 to 5 h at 42°C; hybridization was carried out for 16 h at 42°C in the same solution except that poly(C) was substituted for poly(G) and a nick-translated ^{32}P probe (10^8 cpm/μg;2 ng/ml) was included; and the filters were washed six times each in 1× SSC–0.1% SDS at 68°C for 30 min without agitation. Whatman 3MM filters yielded higher signal-to-background ratios than did nitrocellulose. The coding potentials of clones selected by colony hybridization were verified by in vitro translation of hybrid-selected mRNA as previously described (16) and separation of translation products by two-dimensional isoelectric focusing–SDS-gel electrophoresis (18). The products of hybrid-selected mRNA were compared with authentic secretory products separated by the same procedure.

Nucleotide sequence analysis. Nucleotide sequence analysis of the two cDNA clones pT1 and pT2 was carried out on both strands by the chemical modification procedure of Maxam and Gilbert (15). Primer extension sequence analysis was carried out to determine the extent to which the two clones under investigation, pT1 and pT2, contained complete 5′ noncoding regions. Primers for both clones were restriction fragments labeled at the *Eco*RI site near the 5′ end of each cDNA (see Fig. 2A). Secondary cleavages were at *Hin*FI (base 17 of pT1) and *Xho*II (base 13 of pT2). Primer extensions were carried out with reverse transcriptase and processed as previously described (6, 13).

Measurement of the relative and absolute mRNA levels for dog anionic and cationic trypsinogens. Poly(A)$^+$ mRNA was obtained from the pancreas of a fasting dog. A 26-nucleotide single-stranded DNA probe complementary to the nucleotide sequence which codes for Asp-6 through Tyr-13 and the first two nucleotides of Thr-14 was synthesized by using an Applied Biosystems DNA synthesizer (14). This region is conserved between the anionic and cationic trypsinogen mRNAs. The probe was labeled with ^{32}P at the 5′ terminus and purified by 20% polyacrylamide gel electrophoresis in 8 M urea. Synthetic primer (0.3 μg) was combined with 1 μg of dog pancreas poly(A)$^+$ mRNA in each of four separate tubes. Hybridization was carried out in 0.625 M NaCl–50 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) (pH 6.4)–1 mM EDTA at 40 and 50°C in either the presence or absence of 50% formamide. After hybridization, the samples were ethanol precipitated and suspended for primer extension with reverse transcriptase and deoxynucleoside triphos-

FIG. 2. Restriction enzyme maps and primer extension sequence analysis. (A) Restriction enzyme maps for the two trypsinogen isoenzyme clones pT1 (anionic) and pT2 (cationic). Restriction enzyme sites marked with an a were within the pUC9 plasmid sequence. (B) and (C) Results of primer extension sequence analysis performed on pT1 and pT2, respectively. The *Hin*FI-*Eco*RI restriction fragment used as primer for pT1 and the *Xho*II-*Eco*RI fragment used as primer for pT2 are shown in panel A. Sequence analyses of the fully extended primers revealed that the original cDNA clones were full length in that the primers could not be further extended. The first columns in panels B and C show the amino acid sequences which correspond to the nucleotide sequences shown. Note that the primer extension analyses give the nucleotide sequence complementary to the mRNA sequence.

phates at 42°C under standard conditions. Samples were briefly boiled to hydrolyze the RNA, and the extended DNA strands were resolved on an 8% polyacrylamide gel containing 8.3 M urea. Radioactivity in electrophoretic bands, quantified by both autoradiography and liquid scintillation counting, provided information on relative mRNA levels.

Absolute mRNA levels in poly(A)$^+$ mRNA were measured by dot blot hybridization with labeled synthetic single-stranded DNA probes complementary to the 5′-terminal 30 bases of cationic and anionic trypsinogen mRNAs. To compensate for differences in G+C content between the two probes (anionic, 40%; cationic, 60%), cDNAs representing the two trypsinogen isoenzyme forms were introduced into pSP6 vectors, purified mRNA transcripts were synthesized with *Salmonella* RNA polymerase, and these transcripts were used both to optimize filter washing conditions and to construct standard curves relating the binding of radioactive probe to a quantity of purified mRNA transcripts. Poly(A)$^+$ mRNA and trypsinogen pSP6 mRNA transcripts were bound to nitrocellulose filters in the presence of formaldehyde (27); 5 μg of labeled probe was hybridized to filters in 50% formamide–5× SSC–1× Denhardt solution–20 mM sodium phosphate (pH 6.8)–100 μg of denatured salmon sperm DNA per ml–0.1% SDS in a total volume of 3 ml at 42°C for 18 h. Filters were washed with several changes of 2× SSC–0.1% SDS at 30 and 50°C for the anionic and cationic probes, respectively. Filters were autoradiographed overnight, and the spots of radioactivity were quantitated by Cerenkov counting.

Although measurements of trypsinogen isoenzyme mRNAs could be made under basal conditions in the dog pancreas, reliable measurements could not be made under conditions of hormonal stimulation, since the relationship between hormone infusion and pancreatic response has not been established in vivo in the dog.

RESULTS

The two-dimensional gel patterns in Fig. 1 show the translation products encoded by mRNAs hybrid selected by two cDNA clones, pT1 and pT2. The apparent molecular weight and isoelectric point of each of the native products, which were synthesized in the presence of microsomal

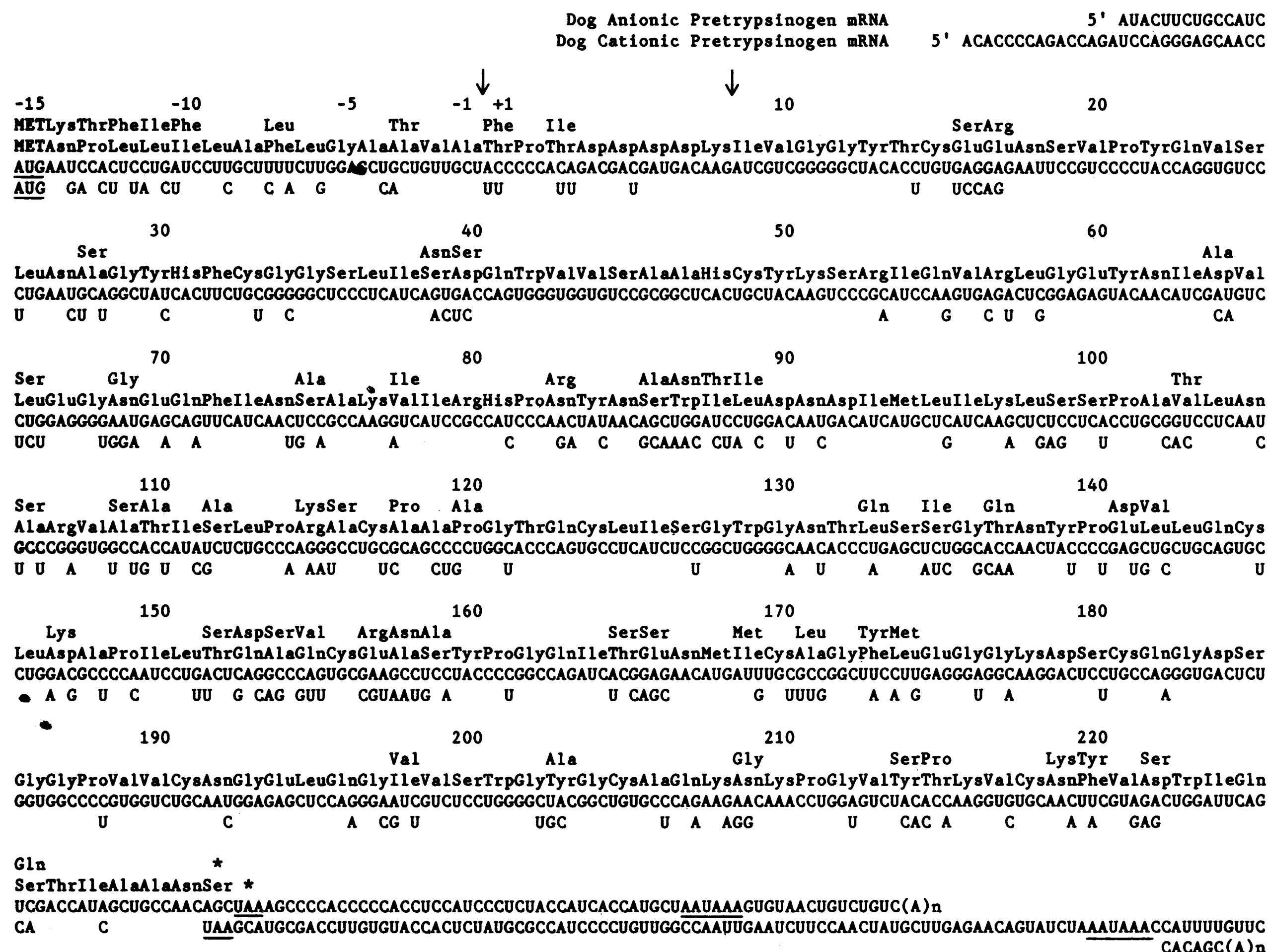


FIG. 3. Nucleotide sequences of canine pancreatic anionic and cationic trypsinogen mRNAs with the deduced amino acid sequences of the two preproenzymes. The complete nucleotide sequence is given for anionic trypsinogen mRNA and the 5' and 3' noncoding regions of both; within the protein-coding regions nucleotide differences are given below for cationic trypsinogen mRNA. $(A)_n$ indicates polyadenylation tracts present at the 3' end of each mRNA. AUG initiation, UAA termination, and AAUAAA polyadenylation signals are underlined. The nucleotide coding sequences for the two trypsinogen mRNAs show 75% identity. The amino acid sequence for anionic pretrypsinogen is given above its nucleotide sequence. Amino acid differences observed in cationic pretrypsinogen are given above the amino acid sequence for anionic pretrypsinogen. Asterisks mark the ends of the amino acid sequences, which show 80% identity. Anionic trypsinogen comprises amino acid residues 1 through 232; cationic trypsinogen comprises residues 1 through 231. Transport signal peptides comprising residues −1 through −15 begin with AUG initiation codons. The two vertical arrows indicate sites where proteolytic processing occurs. Proteolytic cleavage of the transport signal sequence occurs at the Ala-−1-Thr-+1 bond for the anionic form and the Ala-−1-Phe-+1 for the cationic form (1). Zymogen activation, which depends on Asp-184, occurs by enterokinase or tryptic cleavage of the Lys-8–Ile-9 bond in both mature proteins (9). Catalytic activity is determined by the ability of His-48 to transfer protons from Asp-92 to Ser-185. Large areas of sequence surrounding these three amino acid residues are conserved in each of the antipodal canine trypsinogen forms (Gln-41–Ile-63, Asn-91–Ala-102, and Glu-175–Gly-197). Glu-60, Asn-62, Val-65, and Glu-67 and Glu-70, which provide six ligands for the binding of one calcium ion, are conserved. Four of five tryptophan residues and a number of hydrophobic segments which confer thermodynamic stability to the molecule are conserved as well as 12 cysteine residues which are known to form six disulfide bonds (15–145, 33–49, 117–218, 124–191, 156–170, and 181–205) in bovine trypsinogen (8).

membranes and an optimal redox potential, indicated that the cDNAs encoded the two major isoenzyme forms of trypsinogen synthesized in the exocrine pancreas. The anionic (T1) and cationic (T2) forms in the dog have isoelectric points of 4.7 and 8.1, respectively (22).

Plasmids pT1 and pT2 contained inserts with 810 and 875 nucleotides, respectively, including the complete coding sequences of the two presecretory trypsinogen forms and nontranslated sequences at both the 5' and 3' ends of each mRNA. The coding sequences for the two trypsinogen mRNAs showed 75% nucleotide identity. By use of a computer program which allows for insertions and deletions (24), the 3' nontranslated regions of pT1 and pT2 demonstrated homologous regions indicative of an evolutionary relationship. The 5' nontranslated regions showed no detectable similarity. Sequence analysis of both clones revealed a long poly(A) tract at their 3' ends. This finding and data derived from primer extension sequence analysis of T1 (Fig. 2B) and T2 (Fig. 2C) mRNA indicated that pT1 and pT2 contained full-length cDNA inserts.

The nucleotide sequences presented in Fig. 3 reveal single-amino-acid reading frames which code for anionic and cationic pretrypsinogens with 247 (calculated molecular weight, 26,421) and 246 (molecular weight, 26,168) amino acids, respectively. Each of the pretrypsinogen forms has a 15-residue amino-terminal signal peptide, and the mature

zymogens comprise 232 and 231 residues, respectively. Amino acid residues known to be required for zymogen and enzyme function in trypsinogen forms are conserved in each of the canine trypsinogen sequences (9). Within the coding region of the two mature canine zymogens the salient change is the appearance of a net charge of −9 and +3 in the anionic and cationic forms, respectively.

Length differences were observed between the mRNAs in both the 5′ and 3′ noncoding regions. In each case the cationic trypsinogen mRNA was longer than its anionic counterpart: 29 versus 15 nucleotides in the 5′ noncoding region and 105 versus 61 nucleotides in the 3′ noncoding region [not including the poly(A) tails]. In both forms of trypsinogen mRNA an A was observed at position −3 upstream of the AUG initiation codon. A purine residue occupies this position in 97% of mRNAs previously examined (adenosine and guanosine in 79 and 18% of mRNAs, respectively; 10, 11).

Figures 4 and 5 show the results of two independent determinations of the ratio of anionic to cationic mRNA in the canine pancreas. Figure 4 shows the electrophoretic separation of cDNAs extended from a single primer complementary to a 26-base region conserved between the two trypsinogen mRNAs. The difference in length between the 5′ noncoding regions is reflected by the distance between the bands and agrees with the primer extension sequence analyses shown in Fig. 2B and C. The accompanying densitometric scans of the autoradiogram revealed a 2.1 and 2.3 ratio for anionic to cationic trypsinogen mRNAs in hybridizations performed in the absence and presence of 50% formamide, respectively. Data obtained by liquid scintillation counting after excision of the two radioactive bands corroborated the scanning results, giving ratios of 2.0 and 1.7 in the absence and presence of formamide, respectively. The mean value for the ratio of anionic to cationic trypsinogen mRNAs was 2.0.

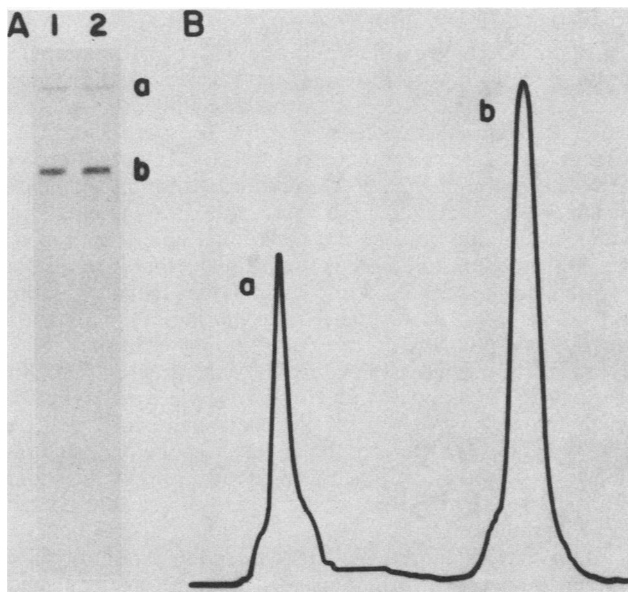


FIG. 4. Measurement of the relative mRNA levels for dog anionic and cationic trypsinogens by primer extension analysis. The autoradiogram shown in Fig. 1A shows two radioactive bands. The band labeled a represents the extended fragment derived from cationic pretrypsinogen mRNA; the band labeled b is derived from anionic pretrypsinogen mRNA, which in its 5′ nontranslated region is 15 nucleotides shorter than cationic trypsinogen mRNA. The intense band representing the 26-base primer which was present in vast excess was eliminated from the figure. Tracks 1 and 2 show the results obtained when hybridization was conducted in 50% formamide at 40 (track 1) and 50°C (track 2). The results obtained with hybridization in the absence of formamide were similar. Part B shows the densitometric scanning profile of track 1 in part A. Peak labels correspond to the bands labeled in part A. Ratios were obtained by integrating the areas under the peaks. The bands were then excised from the dried gels for liquid scintillation counting.

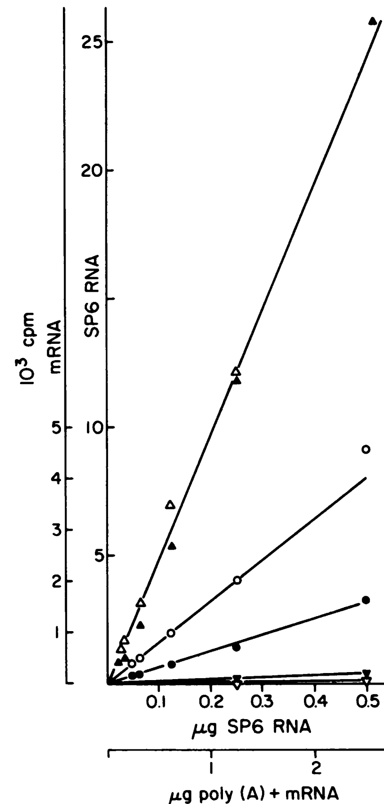


FIG. 5. Measurement of absolute mRNA levels for dog anionic and cationic trypsinogens by dot blot hybridization. Purified mRNA transcripts were synthesized with *Salmonella* RNA polymerase after cloning of anionic and cationic trypsinogen cDNAs into pSP6 vectors. Individual samples of dog pancreatic poly(A)$^+$ mRNA and the purified mRNA transcripts were bound to nitrocellulose after formaldehyde denaturation. Hybridizations were carried out with single-stranded ^{32}P-labeled synthetic DNA probes complementary to the 5′-terminal 30 nucleotides of each trypsinogen mRNA under conditions described in Materials and Methods. The temperature during the posthybridization washing procedure was adjusted to compensate for the differential G+C content of the two probes: the anionic probe (40% G+C) was washed at 30°C; the cationic probe (60% G+C) was washed at 50°C. Symbols: △, anionic oligonucleotide probe:anionic mRNA transcript; ▲, cationic oligonucleotide probe:cationic mRNA transcript; ▽, anionic oligonucleotide probe:cationic mRNA transcript; ▼, cationic oligonucleotide probe:anionic mRNA transcript; ○, anionic oligonucleotide probe:total poly(A)$^+$ mRNA; ●, cationic oligonucleotide probe:total poly(A)$^+$ mRNA.

Because of the potential for secondary structure to influence either hybridization or primer extension efficiency, a second approach was used in which mRNA levels were measured in poly(A)$^+$ mRNA by dot blot hybridization with labeled synthetic probes complementary to the 5′ terminal 30 bases of anionic and cationic trypsinogen mRNAs. Cross-

reactivity studies (Fig. 5) indicated that the two probes were specific for their respective trypsinogen isoenzyme mRNAs. The results for the two purified mRNA standard curves indicated that the two different posthybridization washing conditions which we chose compensated for the differences in G+C content. The two standard curves allowed us to relate bound radioactivity to micrograms of specific mRNA and provided absolute values for each mRNA level. On the basis of a value of 60 pg of RNA per cell (25), we calculated that the number of anionic and cationic trypsinogen mRNA molecules per cell were 30,000 and 11,700, respectively. This represents a ratio of 2.56 for anionic to cationic mRNA, in close agreement with the results of the experiment described above.

DISCUSSION

Studies in the past have shown that the majority of higher organisms, including the rat and the dog, contain two major forms of pancreatic trypsinogen characterized by widely differing isoelectric points. The antipodal nature and widespread occurrence of these isoenzymic forms suggest that their charge complementarity serves an important physiological function (19). Schick et al. (23) have recently shown in the rat that the two major forms of trypsinogen respond differentially to stimulation with caerulein, a synthetic analog of the peptide hormone cholecystokinin. The synthesis of anionic trypsinogen (pI, 4.3) was observed to increase within 2 h of hormone stimulation and showed further progressive increases to levels 4.3-fold higher than that of the controls after 24 h of hormone stimulation. Over the same period, the synthesis of cationic trypsinogen (pI, 8.0) showed no change. Wicker et al. (in press) have investigated the mechanism by which caerulein regulates, in a differential manner, the synthesis of rat trypsinogen isoenzyme forms. They used a high-fidelity rabbit reticulocyte translation system (22) to measure functional levels of mRNA derived from the pancreatic glands of rats stimulated with optimal doses of caerulein. The results indicated no change in mRNA levels in the exocrine pancreas during the first 6 h of hormone stimulation, despite the changes observed in protein synthesis. At 12 and 24 h, mRNA levels were observed to change, in a direction parallel to that of the changes in protein synthesis, but the magnitude of the changes in mRNA levels was small in comparison with those observed at the level of protein synthesis. The data indicate that the changes observed in the synthesis of anionic and cationic trypsinogen are regulated, within the first 6 h, at the level of mRNA translation and that such a regulatory process persists throughout the entire 24-h period of study.

Differential regulation of mRNA translation apparently requires that regulatory elements reside in the mRNA sequence 5′ to the protein coding region. To determine the 5′ nontranslated nucleotide sequences of these mRNAs, we improved methods for the production of a full-length cDNA library and the identification of individual cDNA transcripts. The modifications we introduced in the preparation of the library improved the yield of full-length cDNA clones 10- to 100-fold over the original method. Methods previously developed by Scheele and co-workers for proteolytic processing (22) and conformational maturation (21) of secretory proteins synthesized in vitro and their separation by two-dimensional gel electrophoresis have permitted us to precisely identify cDNA clones coding for secretory isoenzyme forms. As previously described (21), the two-dimensional comigration of nascent translation products with mature secretory proteins synthesized under in vivo conditions indicates the presence of a functional signal transport sequence which mediates the translocation of the nascent chain across the microsomal membrane and into the cisternal space, where the presence of a protein disulfide isomerase acts in the presence of an optimal redox potential to catalyze formation of the correct set of disulfide bonds. In the absence of proteolytic removal of the transport peptide or the correct formation of disulfide bonds, nascent translation products do not comigrate with authentic secretory proteins and largely disappear from the two-dimensional gel owing to heterogeneity introduced by nonspecific protein-protein interactions (21). In a previous paper we showed that clones coding for the isoenzymic forms of chymotrypsinogen could be readily isolated and identified (16). Here we show that this analysis works equally well for the full-length anionic and cationic trypsinogen cDNA clones.

In the present study we measured mRNA levels for the two trypsinogen isoenzymes under basal conditions. By use of synthetic DNA probes, independent aproaches were used to measure relative and absolute levels of mRNA. Primer extension analysis achieved with a 26-nucleotide primer common to both forms of trypsinogen mRNA provided measurments for relative mRNA levels. Dot blot hybridization analysis of poly(A)$^+$ mRNA, together with known quantities of purified mRNA transcripts synthesized from pSP6 constructs, allowed absolute measurements of mRNA levels. By use of these two methods, ratio measurements of anionic to cationic trypsinogen mRNA were in close agreement: 2.0 by primer extension analysis and 2.5 by dot blot hybridization. In contrast, previous measurements of protein synthesis rates by incorporation of [^{35}S]methionine or a mixture of 15 ^{14}C-amino acids into isoenzyme forms in canine pancreatic tissue slices indicated that the ratios of synthesis of anionic to cationic trypsinogen were 3.5 and 6.5, respectively (22). In combination, mRNA levels and protein synthesis rates allow the calculation of the efficiencies of translation of individual mRNAs. The data indicate that, under basal conditions, the translational efficiencies of the two trypsinogen mRNAs is not equal: that for anionic trypsinogen is 1.5- to 2.9-fold higher than that for cationic trypsinogen mRNA. A similar observation has been made in the reticulocyte, where the translational efficiency of β-globin mRNA is approximately 1.7-fold greater than that observed for α-globin mRNA (for a review see reference 12). Furthermore, on the basis of our previous results in the rat pancreas (23; Wicker et al., in press), the translational efficiency of anionic trypsinogen mRNA shows additional increases relative to that of the cationic form during hormone stimulation.

The structural differences observed in the 5′ nontranslated regions of the two trypsinogen isoenzyme mRNA forms may explain the differences observed in translation efficiencies under basal conditions and conditions of hormonal stimulation. One obvious difference between the two 5′ noncoding sequences is length: 14 and 29 nucleotides for anionic and cationic trypsinogen mRNAs, respectively. Consequently, the shorter 5′ noncoding sequence associated with anionic trypsinogen mRNA may be responsible for an increased efficiency of mRNA translation under basal conditions.

Additional mechanisms, however, may be necessary to explain these results, especially those observed with hormonal stimulation. A comparison of the 5′ noncoding sequences for dog anionic and cationic trypsinogen mRNAs and rat anionic trypsinogen mRNA (13) (Fig. 6) provides provocative insight into potential mechanisms for differential regulation of mRNA translation in response to changes in the

cellular environment, including hormonal stimulation. The 5′ nontranslated regions of both dog and rat anionic trypsinogen mRNAs are short (14 and 12 nucleotides, respectively) and show a conserved region of nine contiguous bases, seven of which are pyrimidines. The presence of this conserved sequence represents a dramatic finding when compared with the considerable evolutionary drift observed in the 3′ noncoding regions of these mRNAs and in the 5′ noncoding region of cationic trypsinogen mRNA (Fig. 3 and 6). The drift observed in these sequences reflects more accurately the estimated evolutionary distance (70×10^6 years) between dogs and rats.

Figure 6C shows that 5′ noncoding regions of anionic and cationic trypsinogen mRNAs in the dog contain sequences which are complementary to overlapping regions of eucaryotic 18S ribosomal RNA. These overlapping regions are of interest in regard to the recent review by Van Knippenberg et al. (26), which indicates that the RNA of small ribosomal subunits from all biological sources shows extensive identity at their 3′ ends, specifically within a region of 41 bases downstream of a site in which *Escherichia coli* 16S RNA is cleaved by colicin. In addition, this region contains, in every case, a 9-base-pair potential stem structure containing methylated bases in the loop. Nuclease studies conducted at the 3′ end of rabbit and wheat 18S rRNA indicate that the potential stem loop structure exists in the base-paired configuration under nondenaturing conditions (4). Immediately downstream of this stem structure is a conserved single-stranded RNA sequence: 3′-ACUAGG-5′. Because of the predicted secondary structure in 18S RNA (Fig. 6B), we have highlighted the accessible and cryptic (potential double-stranded) sequences by an open box labeled a and a shaded box labeled c, respectively. A comparison of Fig. 6B and C indicates that dog cationic trypsinogen mRNA contains a pentanucleotide sequence complementary to the a region of 18S rRNA. Potential binding of the 5′ noncoding sequence of cationic trypsinogen mRNA to the accessible region of 18S rRNA may serve to offset the negative effects of its longer flanking sequence through the realignment of mRNA in a manner which may promote the functional translation of this mRNA. Fig. 6C suggests that, in a linear arrangement, the alignments of anionic and cationic trypsinogen mRNAs along the 18S rRNA as a result of potential RNA-RNA interactions place the AUG initiation codons in similar positions.

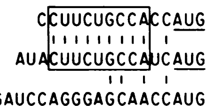
In contrast, anionic trypsinogen mRNA shows a sequence which is largely complementary to the c region in 18S rRNA. The physiological importance of this sequence and its potential interaction with the c region is suggested by the conserved nature of the anionic trypsinogen nonanucleotide sequence shared between dogs and rats. Within this conserved sequence, seven of the bases show perfect complementarity to bases in 18S RNA, one of which appears in the a region and six of which appear in the c region of the stem structure. The finding of a dramatic increase in the translational efficiency of anionic trypsinogen mRNA in the rat with maximal hormonal stimulation suggests that the biochemical events which mediate hormone action might destabilize the stem structure in 18S rRNA and expose G and C nucleotides in the c sequence shown by the shaded box in Fig. 6B and C. Exposure of nucleotides in the c region will have little effect on the functional binding of cationic trypsinogen mRNA to the small ribosomal subunit. In contrast, exposure of this domain may facilitate the functional binding of anionic trypsinogen mRNA in close proximity to (4 nucleotide separation [Fig. 6C]) and in optimal alignment with the

A 5′ Noncoding sequences

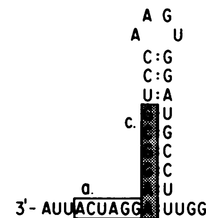
RAT TRYPSINOGEN (pI=4.3) CCUUCUGCCACCAUG

DOG TRYPSINOGEN (pI=4.7) AUACUUCUGCCAUCAUG

DOG TRYPSINOGEN (pI=8.1) ACACCCCAGACCAGAUCCAGGGAGCAACCAUG



B Potential stem-loop structure in 3′ end of 18s rRNA



C Potential hybridization of 5′ noncoding sequences to 18s rRNA

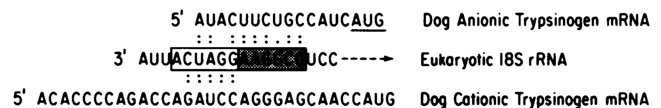


FIG. 6. 5′ noncoding nucleotide sequences in mRNAs coding for dog anionic and cationic trypsinogens and rat anionic trypsinogen (13). (A) 5′ noncoding sequences. pIs are given for each of the secretory trypsinogens encoded by individual mRNAs. Initiation codons are underlined. Vertical bars indicate nucleotide identity in adjacent pairwise comparisons. The box highlights the conserved nucleotide sequence of nine residues observed in the 5′ noncoding regions of the two anionic trypsinogen mRNAs but not the cationic trypsinogen mRNA. (B) Stem loop structure in eucaryotic 18S rRNA (4, 26). (C) Complementarity between the 5′ noncoding sequences of dog anionic and cationic trypsinogen mRNAs and the 3′ end of eucaryotic 18S rRNA. In panels B and C, colons indicate potential base pairs and single dots show potential G · U base pairs. Accessible and cryptic (base-paired) regions are indicated by the open and shaded boxes labeled a and c, respectively.

purine initiator UAC anticodon. Under such conditions the efficiency of translation of anionic but not cationic trypsinogen mRNA may increase, resulting in the observed increase in the synthesis of anionic, but not cationic, trypsinogen during hormone stimulation.

However, in the absence of direct evidence that the double-stranded stem structure in the 3′ terminus of 18S rRNA is destabilized during hormone stimulation, alternative explanations must be considered. For example, the conserved 5′ noncoding sequence present in both rat and dog anionic trypsinogen mRNA may directly bind or facilitate the binding of initiation factors involved in the functional attachment of the mRNA to the 40S preinitiation complex, and hormonal stimulation may promote such interactions. A recent publication showed that the addition of purified eIF4A to an in vitro protein synthesis system resulted in differential stimulation among both capped and uncapped reovirus mRNAs, a result which suggested that special nucleotide sequences or structures, other than the 5′ cap, may be involved (17).

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