Isolation and in Vitro Translation of the Messenger RNA Coding for Pancreatic Amylase*

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RNA prepared from dog pancreas polysomes or microsomes directs the synthesis of pancreas-specific proteins in heterologous cell-free translation systems. A translation product, approximately 1500 daltons larger than authentic amylase, corresponding to pancreatic amylase was identified by immunoprecipitation with anti-amylase γ-globulin and tryptic peptide analysis. We suggest that this larger form of amylase is an amylase precursor. Using amylase immunoprecipitation of reticulocyte translation reactions as an assay, we have shown that greater than 90% of the mRNA for amylase is associated with polysomes bound to the endoplasmic reticulum. Electrophoresis of pancreatic mRNA preparations in formamide-containing polyacrylamide gels and subsequent translation of the fractions have shown that amylase mRNA is of a discrete size with a mobility equivalent to that of 18 S ribosomal RNA, and therefore significantly larger than required to code solely for the amino acid sequence of the amylase precursor.

The vertebrate exocrine pancreas is readily amenable to analysis of the mechanisms of differential gene expression. Acinar cells are highly specialized for the synthesis and secretion of a limited number of well defined enzymes and proenzymes utilized in digestion (1-4); greater than 80% of the protein synthesis of the gland is required for the synthesis of 12 to 20 proteins. During acinar cell development these proteins accumulate in 104 to 106-fold excess over nondifferentiated levels (2, 5). These pancreas-specific proteins accumulate during a small development interval, but not in a strictly coordinate manner. The accumulation profiles of three pairs of proteins (amylase and chymotrypsinogen, specific lipase and procarboxypeptidase A, trypsinogen and procarboxypeptidase B) appear independently synchronous. Others (ribonuclease and certain avian species since the pancreases of these animals do not produce significant amounts of RNase (7). Indeed, Dickman and Bruenger (8) have shown that intact polysomes capable of incorporating amino acids into protein can be isolated from dog pancreas, and Redman et al. (9) have demonstrated the synthesis of amylase in a reconstituted system containing microsomes from pigeon pancreas. We have found that intact RNA can be isolated from dog pancreas polysomes or microsomes and that this RNA is capable of directing the synthesis of pancreatic amylase in heterologous cell-free systems.

MATERIALS AND METHODS

Isolation of Free and Membrane-bound Polysomal RNA—All glassware and utensils used for the preparation of RNA were either heat treated for 9 h at 180° or treated with 0.5% diethylpyrocarbonate for 1 h. As a further precaution ribonuclease inhibitors were added to buffers as indicated.

Dog pancreas was washed with distilled water, weighed, and finely minced with scissors. The minced tissue was then homogenized in 5 volumes of Buffer A (150 mM KCl, 5 mM MgCl₂, 0.25 mM sucrose, 10 mM sodium iodoacetate, 10 μg/ml of potassium polyvinyl sulfate, 50 mM Tris/Cl, pH 7.4) with five strokes of a Potter-Elvehjem homogenizer, filtered through four layers of sterile gauze, and centrifuged for 10 min at 12,000 × g. The fat layer was removed and 35 ml aliquots of the supernatant were layered over step gradients containing 16 ml of 2.0 and 1.0 M sucrose in Buffer A. The gradients were centrifuged for 10 h at 55,000 × g in a Beckman type 50 rotor. The microsomes which banded at the 1.0 to 2.0 M sucrose interface were removed with a sterile syringe after the solution above the banded microsomes had been aspirated. Free polysomes sedimented through the 2.0 M sucrose cushion and were resuspended in Buffer B (10 mM sodium EDTA, 0.5% SDS, 50 mM sodium acetate, pH 5.0). Beaglase was added to the microsome suspension to achieve concentrations equal to those of Buffer B. One volume of phenol saturated with Buffer B was shaken with the microsome and polysome suspensions for 10 min at room temperature, after which 1 volume of chloroform: isoamyl alcohol (24:1) was added and the suspension was shaken for an additional 10 min. After separation of the phases, the aqueous phase was extracted with an equal volume of phenol:chloroform.

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.
isoamyl alcohol (25:24:1) and was re-extracted twice with chloroform:isoamyl alcohol (24:1).

Oligo(dT)-cellulose (Collaborative Research, Inc., type 2) chromatography was performed as described by Aviv and Leder (10), omitting two washes with 2 M NaCl, 0.1 M Tris.Cl, pH 8.0 containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% sodium deoxycholate. Cell-free Translation - Rabbit reticulocytes were prepared and lysed by the methods of Evans and Lingle (11) and stored in 2-ml aliquots at -70°. The standard 250-μl reticulocyte cell-free reaction mixture contained: 100 μl of freshly thawed lysate, 25 μM concentrations of 10 unlabeled amino acids excluding methionine, 75 to 100 μCi of [35S]methionine (New England Nuclear, 100 to 400 Ci/mmol), 80 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 10 μg/ml of creatine kinase (Warthington), 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM Hepes, pH 7.5. One hundred μl of the 50% isopropyl alcohol with 10% trichloroacetic acid for 0.5 h, stained with 0.05 M 2-mercaptoethanol and 0.3 M sodium bicarbonate, and precipitated with 4 volumes of acetone and stored at -20°.

Electrophoretic Analysis of Cell-free Translation Products - For analysis of translation products by SDS-polyacrylamide gel electrophoresis, reticulocyte assays were terminated by the addition of 30 μg/ml of pancreatic ribonuclease A and 10 μM EDTA, followed by incubation at 37° for 15 min. Samples were then precipitated at 4° with 4 volumes of acetate and stored at -20°.

Discontinuous SDS-polyacrylamide gels were prepared according to Laemml (12). Gels (0.75 mm x 11 cm) containing 4.75% acrylamide stacking gels and 12% acrylamide running gels were cast and run according to the procedure of Laemmli (12). Gels (0.75 mm x 11 cm) were run at 4 mA/tube until the dye traversed the length of the gel. The radioactive immunoprecipitates were dissolved in NCS and counted.

Trypsin Degradation Analysis - Trypsin degradation of the radioactivity was determined by liquid scintillation spectrometry. The results were expressed as percentage of the total radioactivity in the pellet. The samples were adjusted to a constant amount of the y-globulin fraction. Fifty micrograms of the y-globulin preparation optimally precipitated 20 μg of amylase (equivalent to 140 μg of y-globulin protein for the complete precipitation of 1 μg of amylase).

For estimating amylase synthesis by specific immunoprecipitation, 50-μl aliquots of the translation assays which had been terminated with RNAase were diluted with 90 μl of a solution containing 6 to 10 μg of purified amylase, 15 mM NaCl, 2% Triton X-100, 2% sodium deoxycholate, 10 mM l-methionine, 2 mM sodium azide, and 10 mM sodium phosphate, pH 7.5. After mixing, the solution was incubated at 37° for 1 min, and the samples were incubated at 37° for 30 min. Freshly dissolved sodium iodoacetate was then added to 0.1 M and the reaction continued for 30 min at 23°. The carboxymethylated proteins, collected by precipitation with 4 volumes of acetone at 4°, were dissolved in and dialyzed against electrophoresis sample buffer (12).

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molecular weight side of the large absorbance peak in Fig. 1D co-migrated with 18 S rRNA. Two major RNA peaks migrating at 4.5 and 6 cm (Fig. 1D) were clearly enriched by this procedure. The RNA fraction obtained by binding twice to oligo(dT)-cellulose is designated poly(A\(^+\)) RNA.

**Translation of Pancreatic Poly(A\(^+\)) RNA**

**Identification of in Vitro Translation Products - Poly(A\(^+\)) RNA** was assayed for its capacity to direct protein synthesis (messenger RNA activity) in both the wheat germ and reticulocyte cell-free systems. Only the results for translation in the reticulocyte system are reported; similar results were obtained with a cell-free system derived from wheat germ. The translation products were analyzed concurrently with a reticulocyte control (without exogenous poly(A\(^+\)) RNA) by SDS-polyacrylamide gel electrophoresis. The results shown in Fig. 2 demonstrate that the poly(A\(^+\)) pancreas RNA elicited the synthesis of several polypeptide species not observed in the control lysate; these polypeptides could easily be discriminated from the peptides (largely globin and a 65,000-dalton protein) synthesized in the control reticulocyte lysate (Fig. 2A, Column 2). In the presence of dog poly(A\(^+\)) RNA at 20 \(\mu\)g/ml, approximately 30% of the total incorporation was attributable to synthesis of pancreas-specific polypeptides. A polypeptide of \(M_\text{r} = 57,500\), slightly larger than pancreatic amylase (\(M_\text{r} = 56,000\)), is a major translation product. The data in Fig. 2B show that only amylase polypeptides were precipitated from \(^{35}\)S-labeled pancreatic secretory proteins by this \(\gamma\)-globulin preparation. A radioactive polypeptide was observed coincident with authentic purified amylase, the major protein synthesized by the exocrine pancreas. The broad amylase band is due to overloading; as a result, the minor components are emphasized. The minor bands of slightly lower molecular weight present in the immunoprecipitate appear to be proteolytic fragments of amylase rather than other nonspecifically precipitated proteins; these polypeptides do not co-migrate with other secretory proteins, and polypeptides of identical mobility are generated upon storage of purified amylase.

The 57,500-dalton polypeptide synthesized in vitro was identified by specific amylase immunoprecipitation (Fig. 2B). The immunoprecipitates from the reticulocyte lysate consisted pre-
dominantly of one polypeptide with a molecular weight of 57,500, approximately 3% larger than authentic dog amylase. This size difference is clearly shown in Fig. 3, which illustrates the separation of amylase synthesized in vitro (\(^{3}H\)-labeled) and amylase isolated from zymogen granules (\(^{14}C\)-labeled) in the same SDS-polyacrylamide gel. Only small amounts of lower molecular weight amylase polypeptides were immunoprecipitated in the reticulocyte system, and were presumed to be nascent amylase chains, since they were precipitated by anti-

amylase and could be largely removed by sedimentation of polysomes prior to immunoprecipitation. The fragments are not generated by proteolysis of completed amylase chains since \(^{14}C\)-labeled dog amylase is stable when incubated in the cell-free lysates under conditions employed in these translation experiments. As a further precaution, proteinase inhibitors, phenylmethylsulfonyl fluoride (10\(^{-4}\) M) or soybean trypsin inhibitor (0.1 mg/ml), were routinely included in the translation reactions. At high levels of added RNA (20 \(\mu\)g/ml of poly(A\(^{+}\)) or 300 \(\mu\)g/ml of unfraccionated polysomal RNA), amylase polypeptides quantitated by immunoprecipitation accounted for 5 to 13% of the incorporation into total protein and approximately 20% of the incorporation into pancreatic polypeptides.

The methionine-labeled tryptic peptide patterns of authentic amylase (labeled with \(^{3}H\)methionine) and in vitro synthesized amylase (labeled with \(^{35}S\)methionine) are identical, as shown in Fig. 4. The profile represents the separation of six peptides not clearly resolved because of the slicing and counting procedures required to measure the double label. No additional peptides are observed for the larger amylase synthesized in vitro; thus, the additional sequence contains no methionine residues. Devillers-Thiery et al. (30) have demonstrated that methionine is absent from the additional \(NH_{2}\)-terminal sequence found in several pancreatic proteins synthesized in vitro. We conclude that the 57,500 dalton band synthesized in vitro represents amylase chains 1500 daltons larger than amylase isolated from zymogen granules.

**Distribution of Message Activity in Free and Membrane-bound Polysomes**—Comparison of [\(^{35}S\)]methionine incorporation as a function of RNA concentration (Fig. 5) indicates a 70-fold higher level of amylase mRNA in membrane-bound polysomes as compared to RNA prepared from free polysomes. The total incorporation (primarily endogenous globin synthesis) was identical for the lysates containing free or bound polysomal RNA; thus the action of an inhibitor of translation selectively present in free polysomal RNA preparations is excluded as the cause of low amylase synthesis. Resolution of free and membrane-bound polysomes (see Materials and Methods) shows that bound polysomes comprise 80% of the total. Since free polysomal RNA amounts to one-fourth that of membrane bound polysomal RNA and has only one-seventieth the level of amylase mRNA, less than 0.4% of amylase mRNA is present on free polysomes.

**Enrichment of mRNA** by Oligo(dT)-cellulose Chromatography

The data of Fig. 5 describe the enrichment of amylase mRNA activity in poly(A\(^{+}\)) RNA measured in the reticulocyte lysate reaction directed by pancreatic poly(A\(^{+}\)) RNA and (\(^{3}H\)methionine amylase labeled in culture were isolated by preparative SDS-polyacrylamide gel electrophoresis, digested with trypsin, mixed, and subjected to high voltage electrophoresis at pH 3.5 as outlined under "Materials and Methods." (\(^{3}H\)) and (\(^{35}S\))methionine proteins were treated with iodoacetate prior to the isolation of amylase.

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**Fig. 3.** Comparison of the relative sizes of in vitro and in vivo synthesized amylase. Following the in vitro synthesis of \(^{3}H\)-labeled polypeptides in a reticulocyte lysate primed with pancreatic mRNA, authentic \(^{14}C\)amylase was added to the reaction and immunoprecipitated as described under "Materials and Methods." Electrophoresis of the immunoprecipitate was performed twice the normal time in a cylindrical discontinuous SDS-polyacrylamide gel to enhance the separation of in vivo and in vitro synthesized amylase. One-half-millimeter slices of the amylase region of the gel were swollen in TCA and counted. ○—○, \(^{3}H\)-labeled in vitro synthesized amylase; ●—●, \(^{14}C\)-labeled authentic amylase

**Fig. 4.** Thin layer electrophoresis of methionine-labeled tryptic peptides derived from amylase. --- --- ---, (\(^{3}H\)methionine-labeled tryptic peptides of authentic dog pancreas amylase; --- --- ---, (\(^{35}S\)methionine-labeled tryptic peptides of amylase made in the reticulocyte lysate system. Presumptive (\(^{35}S\)methionine amylase synthesized in a reticulocyte lysate reaction directed by pancreatic poly(A\(^{+}\)) RNA and (\(^{3}H\)methionine amylase labeled in culture were isolated by preparative SDS-polyacrylamide gel electrophoresis, digested with trypsin, mixed, and subjected to high voltage electrophoresis at pH 3.5 as outlined under "Materials and Methods." (\(^{3}H\)) and (\(^{35}S\))methionine proteins were treated with iodoacetate prior to the isolation of amylase.

**Fig. 5.** Comparison of the efficiency of amylase synthesis directed by various pancreatic RNA fractions. Unfraccionated RNA from free polysomes (△—△) and RNA from membrane-bound polysomes (unfraccionated RNA ——, poly(A\(^{+}\)) RNA, --- ---, and oligo(dT)-cellulose flow-through RNA, ○—○) were added to reticulocyte cell-free assays at various concentrations. Following incubation, radioactivity incorporated into anti-amylase immunoprecipitates was determined as described under "Materials and Methods."
system. The stimulation of [35S]methionine incorporation per μg of added RNA estimated from the linear region of the plots shown in Fig. 5 is given in Table I. Passage of total membrane-bound polysomal RNA through oligo(dT)-cellulose bound 85% of the amylase message activity as assayed by specific immunoprecipitation in the reticulocyte system. A second passage of the bound RNA enriched the amylase message activity 17-fold relative to total membrane-bound RNA. In this experiment, 3.2% of the total RNA and 87% of the amylase mRNA activity was obtained in the poly(A+) fraction. Thus, a 27-fold purification is predicted, assuming no loss of message activity during the procedure. The discrepancy between the predicted and actual purification values may be due to inactivation of one-third of the amylase RNA during purification, or perhaps to the requirement of a ribosomal RNA for efficient translation as suggested by Kabat (31).

Resolution of Pancreatic mRNA Activities by Formamide-Polyacrylamide Electrophoresis

Resolution of pancreatic mRNAs was performed by electrophoresis in polyacrylamide gels containing formamide to reduce artifacts caused by aggregation and secondary structure. The absorbance profile of poly(A+) RNA after electrophoresis is displayed in Fig. 6. The profile of the RNA is similar when analyzed under nondenaturing conditions (see Fig. 1D). To assay for amylase RNA activity, the gel was sliced, and the RNA was extracted and translated in the reticulocyte system (see "Materials and Methods").

Amylase synthesis directed by fractioned poly(A+) RNA is shown by the histogram in Fig. 6. The amylase mRNA activity, assayed by immunoprecipitation, migrated as a distinct species within the major peak (fraction 12). The amylase mRNA co-migrated with 18 S ribosomal RNA (M, = -7 × 10^6) when mixed with polysomal RNA and analyzed in the same formamide polyacrylamide gels. Woo et al. (32) have pointed out the disparity of ovalbumin message molecular weight estimates by formamide-polyacrylamide gel electrophoresis and other techniques. The most likely source of error is the use of ribosomal RNA molecular weight standards which may retain significant secondary structure under conditions in which the mRNA does not. Thus, this technique is expected to yield maximal estimates for mRNA molecular weights. The equivalent mobility of amylase mRNA and 18 S rRNA indicates that amylase mRNA is at most 700 nucleotides longer than required to code for the amylase amino acid sequence (1440 nucleotides for a polypeptide of M, = 57,500).

**TABLE I**

Amylase mRNA content of dog pancreas RNA fractions

| RNA fraction                     | Amylase synthesis in reticulocyte lysates
|----------------------------------|-----------------------------------------------
| RNA from membrane-bound polysomes | 0.023                                         |
| Oligo(dT)-cellulose flow-through RNA | 0.0037                                         |
| Poly(A+) RNA                     | 0.39                                          |

* Immunoprecipitable incorporation in a control reticulocyte lysate (without pancreatic RNA) was subtracted. The calculation of methionine incorporation includes the dilution of [35S]methionine by methionine in the reticulocyte extract. The final methionine concentration ([35S]methionine plus reticulocyte extract methionine) was estimated as the midpoint of inhibition of 35S incorporation when unlabeled methionine was added over a wide range of concentrations to a series of translation assays. By this method the extract contribution was estimated as 0.5 μM.

**Discussion**

By choosing a vertebrate pancreas with very low levels of ribonuclease, pancreatic RNA could be isolated from free and membrane bound polysomes by straightforward techniques of cell fractionation and phenol/chloroform extraction. In the reticulocyte cell-free translation system, the isolated RNA directed the synthesis of a limited number of discrete polypeptides. The number and relative size of these translation products approximate those of pancreatic secretory proteins isolated from zymogen granules or from pancreatic secretions. We are continuing an investigation to determine whether all major translation products are related to the pancreatic secretory proteins.

Two independent criteria indicate that pancreas mRNA directs the synthesis of amylase in heterologous cell-free translation systems. First, a monospecific antibody prepared against purified dog pancreatic amylase selectively precipitates an in vitro translation product approximately the same size as amylase. Second, the methionine-labeled tryptic peptide profile of the in vitro synthesized product is identical to that of authentic amylase. Although this does not confirm identity of the molecules (since not all tryptic peptides are labeled with methionine), it does indicate extensive homology between the synthetic product and amylase.

The in vitro synthesized product is larger than authentic amylase. Consistent differences in electrophoretic mobility in SDS suggest the synthesized product is approximately 1500 daltons larger. The mobility difference remains apparent...
when both preparations are carboxymethylated prior to electrophoresis. We have not demonstrated a precursor/product relationship; however, the evidence is consistent with the hypothesis that the amylase-like molecule synthesized in vitro is a precursor of the enzyme found in zymogen granules.

The in vitro synthesis of putative dog pancreas secretory protein precursors has also been reported by Devillers-Thiery et al. (30). Larger in vitro translation products have been reported for other secretory proteins in cell-free systems which lack a membrane-bound processing activity. Human placental lactogen (33, 34), immunoglobulin heavy (35, 36) and light (36, 37) chains, proparathyroid hormone (38), prolactin (39, 40), and proinsulin (41, 42), synthesized in vivo by membrane-bound polysomes, are synthesized in vitro as precursors containing additional amino acid sequences. In contrast, translation of immunoglobulin light chain and human placental lactogen in cell-free systems with membrane-bound ribosomes yields translation products of identical size to the secreted proteins (43, 44).

As early as 1958 Siekevitz and Palade (45) proposed that pancreatic secretory proteins are synthesized by polysomes attached to the endoplasmic reticulum. Redman et al. (9) demonstrated that microsomes isolated from pigeon pancreas are capable of synthesizing amylase. The secretion of secretory proteins in several other systems investigated occurs predominantly on membrane-bound polysomes (35, 37, 46, 47). We have used in vitro translation to estimate the distribution of amylase mRNA in free and membrane-bound polysome fractions. The results demonstrate that at least 99% of the amylase mRNA was present in membrane-bound polysomes.

Blobel and co-workers have proposed (48) and recently presented confirmatory evidence (37, 43) that the additional NH₂-terminal sequence of secretory protein precursors functions to bind the nascent chain to the endoplasmic reticulum and commits the polypeptide to transport into the cisternae of the endoplasmic reticulum as a first step to secretion. The NH₂-terminal addition is hydrolyzed during transit into the cisternae (43). The presence of a secretion-specific peptide segment on the nascent chain may explain the specificity of binding to the endoplasmic reticulum of those polysomes synthesizing proteins for export.

We have also obtained evidence that the putative amylase precursor has altered functional properties.* Very small amounts of dog pancreatic amylase can be specifically precipitated by the addition of carrier pig pancreatic amylase and stoichiometric amounts of glycogen as described by Levitzki et al. (49). Amylase molecules and a pigment dextrin of the glycogen, each possessing more than one combining site, form an extensive lattice structure that can be collected by brief centrifugation. The putative amylase precursor is not precipitated under these conditions. This suggests that glycogen binding is blocked by the additional peptide sequence present in in vitro synthesized amylase.

Loss of catalytic activity may be another important biological consequence of the additional sequence in the polypeptide. Thus, the specificity of binding of ribosomes to the endoplasmic reticulum need not be so stringent. If an occasional polypeptide for a secretory protein were not attached to the endoplasmic reticulum, translation would produce a catalytically inactive molecule which would not interfere with intracellular functions. A catalytically inactive precursor would be an obvious biological advantage for such digestive enzymes as amylase, RNase, DNase, and perhaps other secretory molecules not synthesized as inactivezymogens.

The various mRNA species present in the pancreas poly(A⁺) fraction can be partially resolved by electrophoresis in acrylamide gels. Resolution is indicated not only by the dispersion of optical density (Fig. 6), but also by the analysis of translation products of the separated mRNA. In these experiments, there is a direct correlation between the size of the RNA and the size of the translation product. As a consequence of the considerable size range of the pancreatic mRNAs, and the high concentration of the mRNA for amylase, it is feasible to obtain amylase mRNA only slightly contaminated with other mRNAs.

This identification and preliminary characterization of amylase mRNA and further identification of the mRNAs for the remaining secretory proteins will afford a basis for investigations of transcriptional and post-transcriptional control mechanisms operative during acinar cell differentiation. In particular, it is now possible to use the reticulocyte translation system and specific immunoprecipitation to measure the changing levels of amylase mRNA during pancreatic differentiation.

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