Preparation of pancreatic mRNA: cell-free translation of an insulin-immunoreactive polypeptide.

Peter T. Lomedico and Grady F. Saunders.

The University of Texas System Cancer Center, The University of Texas Graduate School of Biomedical Sciences, Houston, TX 77025, USA.

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SUMMARY

Total nucleic acid extraction and selection of poly A-containing molecules yield preparative quantities of undegraded mRNA from adult and fetal pancreas. Using a stringent immunoassay, this mRNA is found to direct the synthesis of an immunoreactive insulin polypeptide in the wheat germ translation system. On sodium dodecyl sulfate polyacrylamide gels, this polypeptide (12,000-13,000 daltons) is larger than proinsulin (9,000 daltons).

INTRODUCTION

Previous attempts to identify proinsulin mRNA have been concerned with the identification of proinsulin-synthesizing polysomes (1,2,3). These approaches have emphasized the numerous technical problems that appear to hinder the eventual purification of proinsulin mRNA: (a) only about 1% of the adult pancreas is endocrine (60-80% ß-cells); (b) islets of Langerhans can be isolated away from the exocrine pancreas, but the quantity of tissue obtained is severely limited by current methodology using rodent pancreas; (c) ribonuclease is present in massive quantities in the adult pancreas and prevents the isolation of intact microsomes. We have developed techniques for the extraction of preparative quantities of undegraded mRNA from both adult and fetal pancreas. Cell-free translation of this mRNA reveals the synthesis of an immunoreactive insulin polypeptide. The evidence suggests that this cell-free translation product may be the precursor of proinsulin.
Pancreata from adult dogs were obtained during surgery for pharmacological and physiological research. Fetal bovine pancreata were collected at a local slaughterhouse from cows dead less than 30 minutes. All tissue was rapidly frozen in dry ice and maintained at -70°C until use.

Total nucleic acid was extracted from dry ice pulverized tissue (4) at 4°C. Powdered tissue (20 - 40 grams) was mixed with 400 ml of buffer (0.2M Tris, pH 8.8, 25 mM EDTA, 0.1M LiCl, and 1% SDS) and 200 ml of buffer saturated phenol-chloroform-isoamyl alcohol (50:48:2, made to 0.1% 8-hydroxyquinoline) and immediately homogenized for 5 minutes at low speed in a 1 gallon Waring blender. Following centrifugation (5,000xg for 10 minutes) the aqueous phase was extracted repeatedly with phenol-chloroform-isoamyl alcohol until no protein remained at the interphase. The nucleic acid was precipitated at -20°C with 2 volumes of 95% ethanol, collected by centrifugation, dried under vacuum, and dissolved in 10mM Tris, pH 7.4. Oligo-dT-cellulose chromatography was performed according to Aviv and Leder (5) except that the 0.1M KCl wash was eliminated. The material eluting with 10mM Tris was ethanol precipitated with 1/10 volume of 2M LiCl and 2 volumes of 95% ethanol, dissolved in distilled water, and stored at -20°C.

Cell-free translation using the wheat germ system was performed as described by Roberts and Paterson (6) with [14C] leucine (6.7 μM) or [3H] leucine (5 μM). With [14C] leucine, the entire 50 μl assay was precipitated with TCA; with [3H] leucine, duplicate 5 μl aliquots were precipitated in the presence of 200 μg of bovine serum albumin. The method for immunological analysis of cell-free translation products involved a double antibody reaction for insulin (7,8), where identical immunoprecipitates were formed in the absence and presence of an excess of unlabeled insulin as competitor. The difference in radioactivity associated with the two immunoprecipitates defined the specific immunoreactive insulin (IRI). All immunological reactions were performed in
buffer I: 1% sodium deoxycholate, 1% Triton X-100, 10mM sodium phosphate buffer, pH 7.4, 0.15M NaCl, and 50 μM leucine. 50 μl of guinea pig anti-bovine insulin serum (1/50 in buffer I), 50 μl of goat anti-guinea pig IgG (1/2 in buffer I) ± 10 μl of bovine insulin (1 mg/ml in buffer I) were mixed in a Microfuge tube (Beckman), incubated at 37°C for 2 hours, and stored at 4°C for a minimum of 4 hours before use. The precipitate formed in the absence of excess insulin will bind approximately 60 ng of bovine insulin; the 10 μg of bovine insulin will compete off greater than 99% of any specifically bound material. The amount of goat anti-guinea pig IgG necessary for quantitative precipitation of the guinea pig antibodies was empirically determined using [125I] insulin as tracer. The [3H] labeled translation products were treated with RNase A (20 μg/ml) for 30 minutes at room temperature, an equal volume of 2 x buffer I was added, and the mixture centrifuged for 5 minutes in the Microfuge at 4°C. The pellet from this centrifugation was discarded (typically this contained 3-5% of the TCA precipitable radioactivity). The supernatant (or [125I] proinsulin) was added to the immunoprecipitates, mixed and kept at 4°C overnight. The entire reaction mixture was layered over 150 μl of 1M sucrose in buffer I (9) and centrifuged for 5 minutes in the Microfuge at 4°C. The precipitate was washed 3 times with cold buffer I and the radioactivity counted in 1 ml of NCS (9). A modified SDS-urea polyacrylamide (15%) gel system using 4M urea in the gel (10) was used to analyze immunoprecipitates. Immunoprecipitates were mixed with 10 μg of porcine proinsulin (as an internal marker) in 50 μl of 10mM Tris (pH 8.0), adjusted to 1% SDS, 2% 2-mercaptoethanol and heated for 3 minutes at 100°C, cooled and applied to a 0.6x10cm gel. Following electrophoresis, the gels were stained, sliced, and the position of the proinsulin marker noted before solubilization with NCS (1 ml of a 9:1, NCS:water mixture per gel slice) at 50°C for 24 hours. Protein standards, on parallel gels, included bovine serum albumin (68,000 daltons), ovalbumin (46,000 daltons), trypsin (23,000 daltons),
lysozyme (14,000 daltons) and porcine proinsulin (9,000 daltons).

Porcine proinsulin was iodinated with $^{125}$I as reported (11). Goat anti-guinea pig IgG was prepared in male goats with monthly injections of Freund's adjuvant-solubilized guinea pig IgG.

RNA was electrophoresed on 2.4% polyacrylamide gels as described by Loening (12). RNA was fractionated on 5-20% linear sucrose gradients (10mM Tris, pH 7.5, 0.1M LiCl, and 1mM EDTA) centrifuged at 25,000 rpm at 4°C for 30 hours in a Beckman SW 27 rotor. RNA (in distilled water) was held at 70°C for 3 minutes and quenched in ice immediately before application to the gels and gradients. E. coli tRNA and rRNA served as markers on parallel gels and gradients.

Materials were obtained from the following: [14C] leucine (300 Ci/mole) and [3H] leucine (40 Ci/mmol) from Schwarz Mann, guinea pig anti-bovine insulin serum and guinea pig IgG from Miles, bovine insulin from Sigma, NCS and $^{25}$I from Amersham, and oligo-dT-cellulose (T2) from Collaborative Research. Porcine proinsulin was the generous gift of Dr. Chance, Eli Lilly & Co., Indianapolis, Indiana.

RESULTS

Total nucleic acid yields from the adult dog pancreas approach 2% of the wet weight. The oligo-dT-cellulose chromatography selects ribonuclease sensitive poly A-containing material which appears largely undegraded (figure 1). Thus, the rapid SDS-phenol-chloroform deproteinization seems to control the massive quantities of pancreatic ribonuclease. Translation of this RNA in the wheat germ system is characteristic with respect to dose response, kinetics and Mg$^{++}$ dependence (Figure 2). Translation experiments are routinely run at 3mM Mg$^{++}$, 100mM K$^+$, 40 μg/ml RNA, for 2½-3 hours at room temperature.

The insulin immunoassay involves the use of an excess of anti-bovine insulin serum which is capable of reacting with certain antigenic determinants
Figure 1. RNA Gel Electrophoresis of Poly A-Containing RNA from the Adult Dog Pancreas. 20 μg of RNA was electrophoresed on a 2.4% polyacrylamide gel and scanned at 260 nm. The relative migration of *E. coli* tRNA and rRNA standards is designated.

Figure 2. Translation Characteristics of Poly A-Containing RNA from the Adult Dog Pancreas.

A. Response to Increasing Quantities of RNA. Each point represents the TCA precipitable material in a 50 μl assay using [14C] leucine, 3mM Mg++, 100mM K+, at room temperature for three hours with varying quantities of RNA.

B. Kinetics. Each point represents the TCA precipitable material in a 50 μl assay using [14C] leucine, 3mM Mg++, 100 mM K+, 40 μg/ml RNA, at room temperature for varying lengths of time. Each reaction was stopped by the addition of TCA.

C. Mg++ Sensitivity. Each point represents the TCA precipitable material in a 50 μl assay using [3H] leucine, 100mM K+, 40 μg/ml RNA, at room temperature for three hours with varying quantities of Mg++. 

385
(13) on any related insulin/proinsulin molecule in a quantitative fashion. An excess of unlabeled insulin (bovine) competes off this specific immunoreactive insulin (IRI) radioactivity (Figure 3). Hence in analyzing unknown mixtures, the difference in radioactivity associated with the two precipitates defines the specific reactivity and subtracts out any non-specific binding.

No immunoreactivity was detectable among the translation products of total dog pancreatic mRNA. However, translation of 6-9S fractions of this RNA yields translation products which include a minor IRI component (Figure 4). Translation of unfractionated mRNA from fetal (early third trimester) bovine pancreas yields IRI which constitutes 0.1% of the total translation products.

Figure 5 shows that on SDS-polyacrylamide gels this IRI migrates as a single polypeptide (12,000-13,000 daltons) and is larger than the proinsulin marker. A portion of the radioactivity (around 24,000 daltons) is present in both precipitates and presumably represents material binding non-specifically. If
Figure 4. Sucrose Gradient Sedimentation of Poly A-Containing RNA from the Adult Dog Pancreas. 1 mg of adult dog pancreatic poly A-containing RNA was centrifuged on a 37 ml 5-20% sucrose gradient as described in the methods. Each gradient was aspirated from the top, monitored at 260 nm, and collected into 36 drop fractions. Equivalent fractions from four parallel gradients were pooled, ethanol precipitated, and the RNA dissolved in distilled water. 40 μg of RNA from each fraction (numbers 6-17) was translated and assayed for insulin immunoreactivity as described above. %IRI = (Immunoprecipitate(-Insulin) - Immunoprecipitate(+Insulin))/TCA Precipitable Input x 100.

Figure 5. SDS-Urea Polyacrylamide Gel Electrophoresis of Immunoprecipitable Translation Products. mRNA from early third trimester fetal bovine calves was translated with [3H] leucine at 40 μg/ml RNA, 3 mM Mg++, 100 mM K+, for three hours at room temperature. 255,000 cpm of the [3H] translation products (in buffer I) was added to identical immunoprecipitates formed in the absence (- - ) and presence (——) of 10 μg of unlabeled bovine insulin. Precipitates were processed as described in the methods and electrophoresed with 10 μg of porcine proinsulin as an internal marker. The relative migration of protein standards on parallel gels is designated by an arrow with the corresponding molecular weight in daltons.
the immunoprecipitate is washed more extensively, much of this non-specific binding material is removed (Figure 6). Gel electrophoresis of fractionated adult dog mRNA immunoprecipitates gave a similar result.

**Figure 6.** SDS-Urea Polyacrylamide Gel Electrophoresis of Immunoprecipitable Translation Products. mRNA from early third trimester fetal bovine calves was translated with \(^{3}H\) leucine at 40 \(\mu\)g/ml RNA, 3mM Mg\(^{2+}\), 100mM K\(^{+}\), for three hours at room temperature. 640,000 cpm of the \(^{3}H\) translation products (in buffer I) was added to identical immunoprecipitates formed in the absence (---) and presence (---) of 10 \(\mu\)g of unlabeled bovine insulin. Precipitates were processed as described in the methods, except that two successive sucrose step gradients were used, and electrophoresed with 10 \(\mu\)g of porcine proinsulin as an internal marker. The exact position of the internal proinsulin marker is noted (PI).

**DISCUSSION**

The major findings of this work indicate (a) undegraded mRNA can be prepared from pancreatic tissue and (b) a portion of this mRNA can be translated into an insulin immunoreactive polypeptide. Based upon the size of the poly A-containing material, its translation characteristics, the size of its translation products (data not shown), and the synthesis of immunoreactive
insulin, it appears that total nucleic acid extraction and selection on oligo-dT-cellulose yield relatively undegraded pancreatic mRNA. The competitive-immunoassay used in this work accurately differentiates non-specific binding from specific immunoreactivity. This sensitivity is required when analyzing radioactive protein mixtures where the unknown component may comprise less than 1% of the total. The fact that no IRI was found among the translation products of adult dog pancreatic mRNA was expected considering the rate of protein synthesis in the exocrine pancreas: certainly proinsulin mRNA is a small fraction of the total mRNA population in the whole pancreas. The presence of IRI (molecular weight 12,000-13,000 daltons) among the translation products of 6-9S mRNA correlates with the expected size of proinsulin (84 amino acids) mRNA. Pancreatic mRNA from early third trimester calves also codes for a polypeptide (12,000-13,000 daltons) that is immunologically related to insulin. Experiments are in progress to identify the nature of this IRI material and define its relationship to proinsulin.

Many proteins have been correctly synthesized using the wheat germ translation system (6,14-20). Early work with the wheat embryo system demonstrated accurate polypeptide chain initiation using a viral RNA (21,22). However, there is a precedent for the cell-free synthesis of larger molecular weight precursors in various translation systems: immunoglobulin light chain (23-27), pro-parathyroid hormone (18), and placental lactogen (19). These proteins are destined for extra-cellular transport (as is insulin) and may fit the hypothesis (23) that the extra amino acids at the amino terminal end of a nascent protein, \textit{in vivo}, directs the ribosome-mRNA-nascent protein complex to the endoplasmic reticulum.

The use of total nucleic acid extraction in the preparation of poly A-containing mRNA has numerous advantages. Most mRNA isolation schemes involve the preparation of polysomes, followed by RNA extraction and selection for poly A-containing molecules. When used for the separation of membrane bound
from free polysomes, this cell-fractionation may enrich for a particular mRNA species. However, there are features which are much less desirable: (a) in preparing post-mitochondrial supernatants, often there is a loss of microsomes in the low speed pellets; (b) in preparing polysomes there is a loss of mRNA not engaged on ribosomes; (c) often broad spectrum RNase inhibitors are required for the isolation of intact polysomes from different tissues; (d) the long time required and many manipulations involved in polysome isolation create many opportunities for RNA degradation. Total nucleic acid extraction circumvents these problems. The speed and improved yield achieved with SDS-phenol-chloroform extractions of frozen tissue insure minimal RNA damage and simplify quantitative mRNA isolation. Not requiring additional RNase inhibitors, successful extraction is independent of the amount or type of RNase present in a particular tissue. A similar isolation technique for ovalbumin mRNA had been used successfully (20).

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Abbreviations used: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; IRI, immunoreactive insulin.

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