where M is the unfolded inactive monomer, M′ is the refolded inactive monomer, M* is the active monomer, M*M* is the dimer not yet in the native configuration, whose specific rate constant, i.e. the rate constant relative to each copper ion, is similar to that of M*, and D is the dimer in the final native configuration, where the specific rate constant is about twice that of M*. On the basis of our measurements we can estimate $k_1 = 0.3\ min^{-1}$, $k_2 = 0.2\ min^{-1}$, $k_3 \gg k_4$, $k_4 = 1.4 \times 10^{-4}\ min^{-1}$, $k_{-4} \ll k_{-3}$, k_{-2} . . . .

It is clear that these results cannot be used to generalize in terms of the influence of the quaternary structure on the activity of all Cu-Zn superoxide dismutase. Nevertheless, they represent further evidence that interaction between subunits is relevant to the regulation of the catalytic mechanism in this class of enzymes, as already suggested in the case of the bovine enzyme (Fielden et al., 1974), though the observed facts do not offer a direct explanation for the half-site mechanism proposed by these authors. It should, however, be kept in mind that the situation of isolated monomers investigated in the present report is clearly different from that of dimers where intersubunit interactions prevent one active site from functioning while the other site is catalytically working.

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Isolation and Partial Purification of Catfish Pancreatic Islet Messenger RNA†

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ABSTRACT: Poly(a)-rich mRNA has been isolated from catfish pancreatic islet total nucleic acid. Cell-free translation of the mRNA by wheat germ extracts yielded a protein of 11 000-12 000 molecular weight, estimated by sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis. This peptide is larger than catfish proinsulin, but contains tryptic peptides of proinsulin. Its synthesis comprises up to 23% of the cell-free product, depending on the conditions of cell-free synthesis. Synthesis is inhibited by 7-methylguanosine 5′-monophosphate suggesting the presence of a 7-methylguanosine cap on the 5′ end of catfish proinsulin mRNA. Sucrose gradient centrifugation of the islet poly(A)-rich mRNA yielded 8S and 12S peaks. These fractions were translated with wheat germ extracts and it was determined that over 60% of the islet mRNA-dependent protein from the 8S fraction was preproinsulin. The 8S mRNA fraction was electrophoresed on 3% agarose-6 M urea gels and demonstrated to be several bands, ranging from 100 000-200 000 molecular weight.

Proinsulin is the most abundant protein synthesized by pancreatic islet cells, comprising as much as 25% of the newly synthesized protein under optimal conditions (Morris and Korner, 1970; Steiner et al., 1972; Permutt and Kipnis, 1972a). Proinsulin mRNA[1] should constitute a major fraction of the total islet mRNA. Unfortunately, mammalian islet tissue is embedded within the exocrine pancreas and comprises only about 1% of total pancreas (Falkmer and Patent, 1972). In contrast, islet tissue in teleost fish exists as a single relatively large gland, up to 100 mg (Bencosme et al., 1965), with less than 25% exocrine tissue in many species (Moule, 1972). Total poly(A)-rich mRNA has been extracted from catfish (Permutt et al., 1976), carp (Rapoport et al., 1976), angler fish and sea raven (Shields and Blobel, 1977), and translated in wheat germ and ascites cell-free protein synthesizing systems. Cell-free products included proteins of approximately 12 000-14 000 mol wt, but nothing as small as proinsulin (9000 mol wt). Anglerfish and sea raven cell-free products (Shields and Blobel, 1977) contain 23-25 extra amino acids on the amino terminus of proinsulin. The structure of the amino-terminal extension of these fish preproinsulins was shown to be very similar to that

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[1] Abbreviations: mRNA and rRNA, messenger and ribosomal ribonucleic acids, respectively: mol wt, molecular weight; poly(A), poly(adenylic acid); dT, deoxythymidine; $NaDodSO_4$, sodium dodecyl sulfate; m^7pG, 7-methylguanosine 5′-monophosphate; m^7G, 7-methylguanosine; PPO, 2,5-diphenyloxazole.

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of rat preproinsulin (Chan et al., 1976), the product of cell-free translation of rat islet mRNA.

We are attempting to purify catfish proinsulin mRNA to synthesize cDNA. The cDNA will be used for studying the structure and control of expression of the fish proinsulin gene. In this report we have confirmed that one of the products of cell-free translation of catfish islet mRNA is a protein of 11 500 daltons, which contains tryptic peptides of catfish proinsulin. Characteristics of the cell-free system were explored for optimal translation, and proinsulin mRNA has been partially purified by sucrose gradient centrifugation and analyzed by agarose–urea gel electrophoresis.

Experimental Procedures

Materials

[^{35}S]Cysteine (20 Ci/mmol), [^{35}S]methionine (200 Ci/mmol), and ^{14}C-labeled L-amino acid mixture (102–485 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.); phenol was obtained from Fisher and redistilled before use; $NaDodSO_4$ and protein standards for gel electrophoresis were from Sigma Chemical (St. Louis, Mo.); sucrose (ribonuclease-free) and urea were from Schwarz/Mann (Orangeburg, N.J.); oligo(dT)-cellulose was from Collaborative Biochemical Corp. (Waltham, Mass.); m^7G and m^7pG were from P-L Biochemical Co. (Milwaukee, Wis.). Porcine proinsulin was kindly supplied by Dr. Ronald Chance, Eli Lilly Co. (Indianapolis, Ind.), and guinea pig anti-insulin serum (lot no. 526) were provided by Dr. Peter Wright (Indiana University). Guinea pig anti-chicken (no. 2/4H) and anti-codfish insulin serum (no. 3) were supplied by Dr. Strath Wilson, Connauht Laboratories (Ontario, Canada). Live channel catfish were supplied by the Lambrich Brothers Live Fish Co. (Imperial, Mo.).

Methods

Nucleic Acid Extraction. All glassware and reagents were either sterilized or rinsed with 1% diethylpyrocarbonate prior to use. Because of the high ribonuclease content of pancreatic islets, attempts to isolate RNA from islet cell fractions such as a post-nuclear supernate, or polyribosomes, yielded completely degraded RNA. For this reason, total nucleic acid was extracted in the following manner. Pancreatic islets were removed from 100–200 lb of channel catfish, dropped into liquid nitrogen, and stored at −70 °C until extraction. Frozen tissue (1–2 g) was crushed in a tissue pulverizer and extracted in 3–10 volumes of extraction buffer containing 0.1 M NaCl, 0.01 M Tris (pH 7.5), 0.01 M EDTA, 1% $NaDodSO_4$, 0.05% heparin, and mixed with an equal volume of buffer saturated phenol–chloroform–isoamyl alcohol (1:1:0.1, v/v). This mixture was shaken vigorously by hand for 5 min at room temperature, cooled to 4 °C, and centrifuged at 1500*g* for 20 min. The aqueous phase was removed and extracted twice more with extraction buffer until no visible interface remained. The aqueous phase was made 0.2 M in NaCl, 2 volumes of 95% ethanol added, and the RNA precipitated overnight at −20 °C. RNA was collected by centrifugation at 30 000*g* for 10 min, washed with 95% ethanol, and dissolved in sterile H_2O.

Oligo(dT)-Cellulose Affinity Chromatography. Total nucleic acid was chromatographed on oligo(dT)-cellulose by the method of Aviv and Leder (1972). Approximately 400 A_{260} units of RNA was applied to a 10-mL column. The adsorbed poly(A)-rich mRNA comprised 1–2% of the total RNA applied. Rechromatography of the nonadsorbed fraction yielded less than 0.3% adsorbed.

Cell-Free Protein Synthesis. Cell-free extracts from wheat germ were prepared as described by Roberts and Paterson (1973), and protein synthesis assays performed essentially as described by Aviv et al. (1971). A typical reaction was performed in a final volume of 50 μL and contained 24 mM Hepes buffer, pH 7.0, 1 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatinine phosphokinase, 64 mM KCl, 1.7 mM $MgCl_2$, 30–50 μM of 19 amino acids excluding methionine, cysteine, or leucine where indicated, 10 μL of wheat germ S-30 extract, 0.5–3.5 μg of mRNA, and 400 μM spermidine. Incubation was at 31 °C for 90 min and terminated by the addition of pancreatic ribonuclease. Reactions were scaled up tenfold to prepare the cell-free product for tryptic peptide analysis.

$NaDodSO_4$ Slab Gel Electrophoresis. Up to 10 μL of the cell-free reaction mixture was mixed with an equal volume of sample buffer (Laemmli, 1970) and heated to 100 °C for 2 min prior to electrophoresis. To optimize detection of small peptides in the 2000–20 000 mol wt range, $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by a modification of the method of Swank and Munkres (1971). Slab gels were prepared of 17.5% acrylamide (Bio-Rad Laboratories, Richmond, Calif.) with 8 M urea and samples electrophoresed with the discontinuous buffer system of Laemmli (1970). Protein standards of known molecular weight (Weber and Osborn, 1969) were detected by staining with Coomassie Blue [ovalbumin (mol wt 43 000); chymotrypsinogen (25 700); myoglobin (17 200); cytochrome *c* (12 300); proinsulin (9000); and insulin (5700)]. Radioactive proteins labeled with ^{35}S were detected by autoradiography as previously described (Permutt et al., 1976), whereas those proteins labeled with ^{3}H- or ^{14}C-labeled amino acids were detected by impregnating the gels with PPO, drying the gel (slab gel dryer, Hoefer Scientific Instruments, San Francisco, Calif.), and exposing it to Kodak XR5 film at −70 °C in a cassette according to the method of Bonner and Laskey (1974). Exposure was 4–9 days.

Labeling and Extraction of Fish Islet Proteins. One islet was sliced into four pieces and incubated in 0.5 mL of Kreb's bicarbonate buffered media with glucose (50 mg/dL) and ^{14}C-labeled L-amino acids (100 μCi/mL) for up to 24 h at 18 °C. The flasks were gassed with 95% O_2–5% CO_2. After the incubation was completed, the islet was homogenized in 10% trichloroacetic acid and the insoluble fraction extracted in acid–alcohol as previously described (Permutt and Kipnis, 1972a,b). The extracts were concentrated by evaporating the alcohol under a stream of nitrogen.

Gel-Filtration Chromatography. Extracts (0.5 mL) of labeled proteins were made 2.5 M in propionic acid, mixed with cytochrome *c* (1 mg) and bovine insulin (3 mg) which serve as internal standards, and chromatographed on a Bio-Gel P-30 column (1 × 60 cm, 400 mesh, Bio-Rad Laboratories) in 2.5 M propionic acid (Chan et al., 1976). The column effluent was monitored at 280 nm on an LKB Uvicord monitor. Bovine insulin contains a high molecular weight contaminant used to indicate the void volume, and 3–5% proinsulin which is readily visible as a distinct peak migrating in between the cytochrome *c* and insulin peaks (Nolan et al., 1971). One-milliliter samples were collected, and 50 μL of each was counted in 5 mL of Instagel (Packard).

Analysis of Tryptic Peptides. ^{3}H-labeled 11 500 mol wt protein synthesized by wheat germ extract in the presence of a mixture of ^{3}H-labeled amino acids was extracted in acid–alcohol and separated from the other translation products by gel filtration on a Bio-Gel P-30 column (Figure 1A) and rechromatography (Figure 1B). [^{14}C]proinsulin, produced by incubation of fish islet slices in a mixture of ^{14}C-labeled amino acids, was similarly extracted and chromatographed on a

TABLE I: Cell-Free Protein Synthesis Directed by Fish Islet Poly(A)-Rich mRNA.[a]

RNA (μg)	[35S]Methionine incorp into protein (cpm)
Poly(A)-rich mRNA	
None	9 500
0.75 μg	31 000
1.00 μg	38 000
1.60 μg	72 000
Total RNA	
1.6 μg	15 000

[a] Assays for cell-free protein synthesis with wheat germ extracts were performed as described in Methods. RNA was added as indicated, and incorporation of [35S]methionine into trichloroacetic acid insoluble material determined. Each result is the average of duplicates which differed by less than 5%.

Bio-Gel P-30 column (Figure 1C). Approximately 50 000 cpm of the purified ^{3}H-labeled cell-free product was added to 20 000 cpm of [^{14}C]proinsulin with 1 mg of carrier bovine insulin, reduced and aminoethylated as described (Neumann and Humbel, 1969), and then desalted on a Bio-Gel P-2 column (10 mL). The void volume was collected, lyophilized, dissolved in 1 mL of 0.1 M ammonium bicarbonate (pH 8.5), and digested with TPCK-trypsin (Worthington Biochemicals, Freehold, N.J.) at an enzyme:substrate ratio of 1:50 for 4 h at 37 °C. ^{3}H-labeled amino acid labeled globin was synthesized by wheat germ extracts with rabbit globin mRNA obtained from rabbit reticulocytes by Peter Yalow, and similarly treated with TPCK-trypsin in the presence of [^{14}C]proinsulin.

The mixture of ^{3}H- and ^{14}C-labeled tryptic peptides was applied to a column (0.9 × 20 cm) of Beckmann AA-15 cation-exchange resin equilibrated with 0.05 N pyridine acetate, pH 2.5, at 50 °C, washed with 2 column volumes and eluted with a gradient of up to 2 N pyridine (total volume 250 mL) by the method of Bradshaw et al. (1969). Two-milliliter fractions were collected and counted in 15 mL of ACS (Amersham/Searle) for ^{14}C and ^{3}H.

Immunoprecipitation Studies. Reaction mixtures up to 50 μL were diluted tenfold with a solution containing 0.15 M NaCl, 0.015 M NaH_2PO_4, 0.5% Triton X-100, and bovine serum albumin 0.5% (w/v). One hundred microliters of anti-insulin serum (diluted 1:100) was added, and after a 30-min incubation at 37 °C, 100 μL of goat anti-guinea pig serum (1:10) was added. The reaction mixtures were incubated overnight at 4 °C. The immunoprecipitates were washed twice with immunoprecipitation buffer and either analyzed by Na-DodSO_4-urea polyacrylamide gel electrophoresis or dissolved in 1 mL of NCS (Amersham/Searle) and counted in 10 mL of Instagel.

Anti-catfish insulin antibodies were prepared in guinea pigs using up to 500 μg of purified catfish insulin by the method of Wright et al. (1971).

RNA Analysis. Poly(A)-rich mRNA was centrifuged on 5–20% sucrose gradients as described previously (Permutt and Kipnis, 1972b) and in the legend of Figure 5. RNA was electrophoresed on 3% agarose–6 M urea slab gels as described by Rosen (1976). These gels resolve the 4–18S RNA region with no evidence of aggregation. The gels were stained with Stainsall overnight and destained with water. Between 5–50 μg of RNA was electrophoresed in each sample.

Results

I. *Translation of Fish Islet mRNA.* Poly(A)-rich mRNA extracted from fish islets produced a concentration-dependent stimulation of protein synthesis in the cell-free translation system with wheat germ extracts (see Table I). Addition of 1.6 μg of mRNA produced an approximate 8-fold stimulation of incorporation into protein compared to that in the absence of mRNA. An equal amount of total fish islet RNA slightly stimulated protein synthesis.

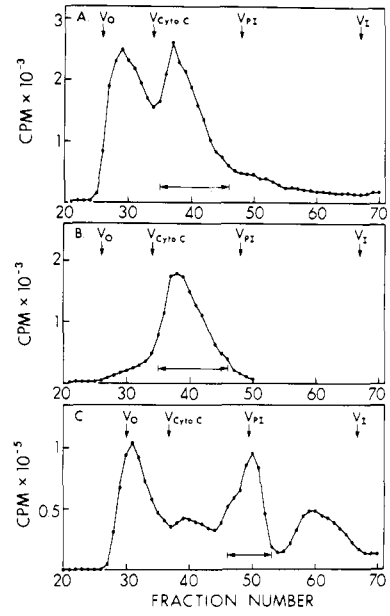

FIGURE 1: Comparison by gel filtration chromatography of fish islet mRNA stimulated cell-free products with those synthesized by fish islet slices. (A) Products of translation with wheat germ extracts directed by fish islet mRNA, labeled with ^{3}H-labeled amino acids, were precipitated with trichloroacetic acid, extracted with acid–alcohol, and chromatographed with carrier proteins on a Bio-Gel P-30 column in 2.5 M propionic acid as described in Methods. The fractions indicated by the bar (35–45) were pooled, lyophilized, and rechromatographed as illustrated in B. The rechromatographed peak in B was used for tryptic peptide analysis in Figure 3. (C) Products of fish islet incubation for 24 h with a mixture of ^{14}C-labeled amino acids (see Methods), extracted and chromatographed as described for A. V_0 represents the void volume, and $V_{cyto\text{-}c}$, V_{PI}, and V_I, the volume of distribution of cytochrome *c*, bovine proinsulin, and insulin, respectively.

II. *Analysis of the Products of Cell-Free Translation of Islet mRNA.* A. Gel-Filtration Chromatography. Proteins synthesized by the wheat germ system in the presence of fish islet mRNA were chromatographed on a Bio-Gel P-30 column and two major peaks observed (Figure 1A). The protein peak in fractions 35–45 was smaller than cytochrome *c* (12 300 daltons) but larger than bovine proinsulin. Nothing was observed in the region of proinsulin or insulin standards, however. Rechromatography of fractions 35–45 gave a single peak (Figure 1B) with estimated mol wt of 11 000–12 000.

Fish islet slices incubated with ^{14}C-labeled amino acids synthesized proteins of higher mol wt as well as major peaks comigrating with bovine proinsulin (Figure 1C, fractions 46–53) and slightly larger than bovine insulin (fractions 55–65). Fish insulin migrates as a slightly larger molecule than bovine or porcine insulin by gel-filtration chromatography (Moule and Yip, 1973). Oxidation of the presumed insulin peak produced two peaks of labeled protein migrating with A and B chains of beef insulin on a Bio-Gel P-6 column (Albert and Permutt, submitted for publication). Identification of the peak comigrating with bovine proinsulin as catfish proinsulin was accomplished first by rechromatographing fractions 46–53 of Figure 1C. Oxidation of this peak produced no change in its migration on the Bio-Gel column. Brief trypsinization (30 min)

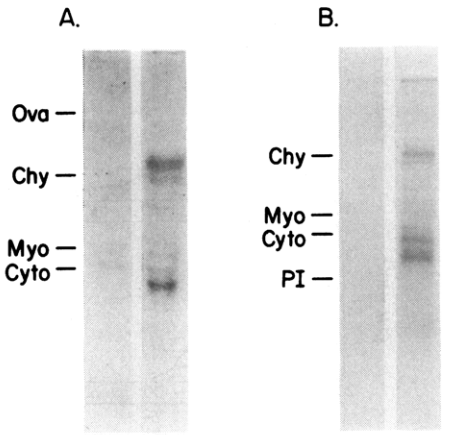

FIGURE 2: NaDodSO$_4$-urea polyacrylamide slab gel electrophoresis of products of cell-free translation. (A) [^{35}S]Cysteine labeled cell-free products in the absence (lane 1) and presence (lane 2) of fish islet mRNA. Approximately 5000 cpm was applied to lane 1, and 10 000 cpm to lane 2, and electrophoresis and autoradiography were performed as described in Methods. Mol wt standards were detected by Coomassie Blue staining. Ova (ovalbumin, 43 000), Chy (chymotrypsinogen, 25 700), Cyto (cytochrome *c*, 12 300), PI (porcine proinsulin, 9000). (B) [^{35}S]Methionine labeled cell-free products in the absence (lane 1) and presence (lane 2) of fish islet mRNA.

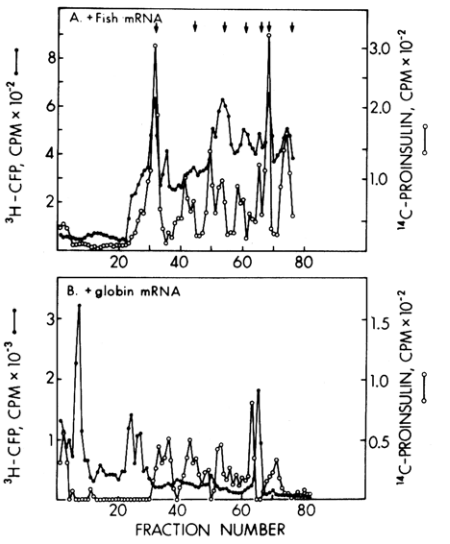

FIGURE 3: Tryptic peptide analysis of the 11 500-dalton cell-free product compared to authentic catfish proinsulin. Approximately 50 000 cpm of ^{3}H-labeled cell-free protein was mixed with 20 000 cpm ^{14}C-labeled catfish proinsulin. The mixture was trypsinized, applied to a Beckman AA-15 column, eluted, and counted for ^{3}H and ^{14}C as described in Methods. (A) ^{3}H-labeled cell-free product with fish islet mRNA (●—●) compared with ^{14}C-labeled catfish proinsulin (○—○). (B) Tryptic peptides of [^{3}H]globin (●—●) compared with those of ^{14}C-labeled catfish proinsulin (○—○)

produced a peptide migrating ahead of bovine insulin, similar to catfish insulin. The trypsinized peptide was oxidized, chromatographed on Bio-Gel P-6 with oxidized bovine insulin carrier, and peaks of ^{3}H-labeled protein comigrating with bovine B chain, and slightly larger than A chain were observed.

B. Analysis by NaDodSO$_4$–Urea Polyacrylamide Gel Electrophoresis. To improve resolution and observe the proteins under denaturing conditions, proteins synthesized by the wheat germ extracts in the absence and presence of islet mRNA were electrophoresed on NaDodSO$_4$–urea slab gels. [^{35}S]cysteine-labeled messenger-dependent proteins of approximately 27 000 and 11 500 mol wt were observed (Figure 2A). Comparison of [^{14}C]leucine-labeled cell-free products with those synthesized by fish islets shows the in vivo preparation contains a protein comigrating with porcine proinsulin (Albert and Permutt, submitted for publication), whereas the cell-free product contained nothing smaller than the protein of estimated mol wt 11 000–12 000. The cell-free translations with [^{35}S]methionine gave better resolved radioautographs than with [^{35}S]cysteine. At least three islet mRNA dependent cell-free products of 11 000–12 000 daltons were observed (Figure 2B, lane 2).

C. Tryptic Peptide Analysis. The 11 000–12 000 dalton cell-free product (labeled with a mixture of ^{3}H-labeled amino acids) was mixed with catfish proinsulin (labeled with a mixture of ^{14}C-labeled amino acids) and trypsinized, and the peptides were adsorbed to a Beckman AA-15 cation exchange column. The peptides were eluted and fractions counted for ^{3}H and ^{14}C (Figure 3A). Since the cysteines in proinsulin were aminoethylated, we would expect eight tryptic peptides. At least four and perhaps as many as seven tryptic peptides of the cell-free product comigrate with tryptic peptides of catfish proinsulin (indicated by arrows). Only one peptide (fractions 34–36) was observed in the cell-free product which was not present in catfish proinsulin. When the tryptic peptides of [^{3}H]globin (Figure 3B) synthesized by wheat germ extracts were compared with those of ^{14}C-labeled catfish proinsulin, no overlap was observed. The elution profile of the tryptic peptides of [^{14}C]proinsulin differed for each experiment since the resin was removed from the column each time, washed, and repacked. The [^{14}C]proinsulin peptides served as an internal standard, however. It was therefore concluded that the 11 000–12 000 dalton cell-free product, larger than proinsulin, contained tryptic peptides of proinsulin, and was therefore tentatively identified as catfish preproinsulin.

III. *Characteristics of Cell-Free Translation of Fish Proinsulin mRNA.* A. Effects of mRNA, [K$^+$], and Mg^{2+}. Lomedico and Saunders (1976) found that bovine proinsulin mRNA had a very low affinity for initiation complexes relative to other bovine pancreatic mRNA, and others have reported widely different translation efficiencies of various mRNAs (Palmiter, 1974). Since translational efficiency may play a role in control of hormone biosynthesis, the effects of concentration of fish islet mRNA on total cell-free islet protein synthesis were compared with that of preproinsulin. Increasing the concentration of mRNA stimulated preproinsulin synthesis to the same extent as total protein, indicating that proinsulin mRNA does not have an affinity for initiation complexes appreciably different than other islet messengers. Total protein synthesis was optimal at 64 mM [K$^+$]. While total protein synthesis diminished with increasing [K$^+$] in the medium, preproinsulin synthesis decreased less, and comprised 23% of total synthesis at 85 mM [K$^+$]. Magnesium above 1.7 mM produced a small decrease in total protein synthesis, as well as a more pronounced decrease in preproinsulin.

B. Effects of m^{7}pG. Blocked methylated 5′-terminal structures of the type m′G5′pppNm have been identified at the 5′ end of a wide variety of eukaryotic and viral mRNA (Shatkin, 1976). Cap analogues such as m^{7}pG have been reported to be specific inhibitors of capped mRNA translation by wheat germ extracts (Groner et al., 1976). To determine whether m^{7}G caps exist on islet mRNA in general, and proinsulin mRNA in particular, increasing amounts of m^{7}pG were added to the cell-free system. At the highest concentration tested (0.5 mM), 80% inhibition of total protein synthesis was observed. In contrast, only a 12% inhibition was produced by m^{7}G (0.05 mM). [^{35}S]Methionine-labeled cell-free proteins

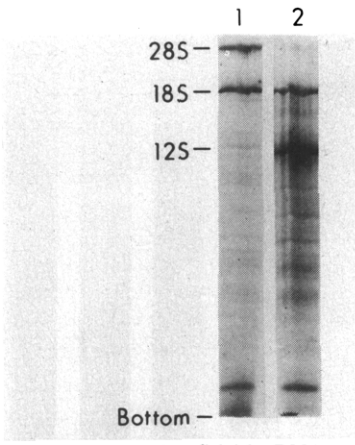

FIGURE 4: Electrophoresis of catfish islet RNA on 3% agarose-6 M urea slab gel. Poly(A)-minus RNA (50 μg) (lane 1) is compared with an equal amount of poly(A)-rich mRNA (lane 2). Electrophoresis and staining were as described in Methods.

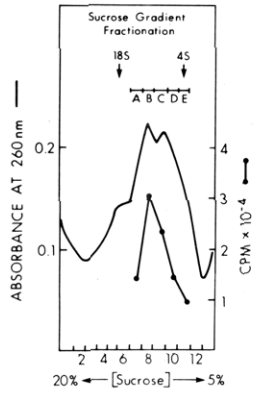

FIGURE 5: Sucrose gradient fractionation of islet poly(A)-rich mRNA. Poly(A)-rich mRNA (15 A_{260} units) was centrifuged on a 5–20% sucrose gradient, 0.1 M Tris, 0.1 mM EDTA, pH 7.8, in a SW-41 Beckman rotor at 36 000 rpm for 16 h at 4 °C. Fractions were collected from the bottom and those indicated (A–E) were ethanol precipitated and translated with wheat germ extracts; [^{35}S]methionine incorporation into trichloroacetic acid precipitable protein, cpm/μg RNA added (●—●); the solid line represents the absorbance at 260 nm. The migration of 4S and 18S RNA was determined with total cell RNA on a parallel gradient.

synthesized in the presence of m^{7}pG were electrophoresed on NaDodSO$_4$–urea slab gels and radioautographs quantitated by densitometric tracings. It was determined that initiation of all of the islet mRNA was inhibited, suggesting the presence of a m^{7}G cap on catfish proinsulin mRNA.

IV. *Partial Purification of Proinsulin mRNA.* Electrophoresis of non-oligo(dT)-cellulose bound [poly(A)-minus] islet RNA (Figure 4, lane 1) is compared with the poly(A)-rich mRNA fraction (lane 2). The gels were purposely loaded with 50 μg of RNA in each slot to visualize the minor RNA bands. Prominent 28S and 18S ribosomal bands were observed in the poly(A)-minus RNA as well as minor bands in the 4–18S region. The poly(A)-rich mRNA was greatly enriched in an RNA band which sedimented on sucrose gradients at 12 S (see below). In addition, some contaminating 18S ribosomal RNA was still present, as well as multiple smaller RNAs.

To determine whether prominent 12S mRNA was coding for preproinsulin or a larger islet protein, about 15 A_{260} units of poly(A)-rich mRNA was centrifuged on a sucrose gradient (Figure 5). The 28S rRNA was pelleted to optimally separate RNA in the 4–18S region. The A_{260} profile indicated 18S ribosomal RNA contamination as well as two mRNA peaks at

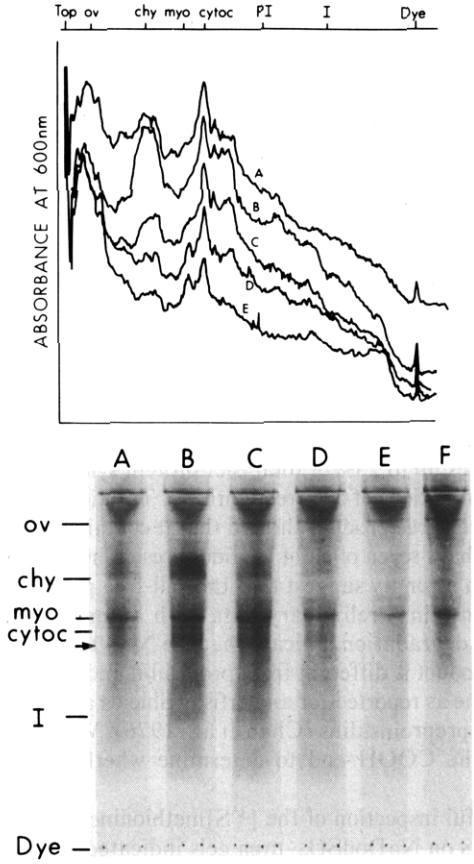

FIGURE 6: NaDodSO$_4$-polyacrylamide slab gel electrophoresis of the translation products of sucrose gradient poly(A)-rich mRNA fractions. The mRNA in each sucrose gradient fraction (A–E) of Figure 5 was translated by wheat germ extracts and approximately 50 000 cpm of each electrophoresed and autoradiographed as in Methods. The markers are as described in legend of Figure 2. (F) minus mRNA control.

12 S and 8 S. Five fractions were pooled across these peaks, and the RNA was concentrated by ethanol precipitation and translated in wheat germ extracts. RNA from each fraction was translatable, with maximum template activity in fraction B (Figure 5). Next the translation products synthesized in the presence of RNA from each fraction were electrophoresed on NaDodSO$_4$–urea polyacrylamide gels (Figure 6). Endogenous wheat germ activity was higher in these translations since a protein band about 17 000 mol wt was observed in the absence of added islet mRNA (Figure 6E). Translation of the sucrose gradient fractionated mRNA revealed that fraction B was enriched in mRNA coding for the 27 000 mol wt protein, and fraction C enriched in mRNA coding for preproinsulin (arrow). Densitometric tracings of the radioautographs showed that the 27 000 mol wt protein(s) comprised 63% of islet mRNA dependent proteins in fraction B, whereas preproinsulin accounted for 61% of islet mRNA dependent protein in fraction C. Next mRNA from each sucrose gradient fraction was electrophoresed on agarose–urea gels (Figure 7). Fraction A contains predominantly 18S ribosomal RNA, fraction B the 12S mRNA, fraction C several bands which migrated in the 8S region on sucrose gradients, and fractions D and E predominantly RNA smaller than 8 S. Since the major translation product of the mRNA in fraction B was in the 27 000 dalton region, the 12S mRNA most likely codes for this protein(s).

Discussion

Translation of poly(A)-rich mRNA from catfish islets by wheat germ extracts yields a protein of approximately 11 500

TABLE II: Partial Purification of Proinsulin mRNA.

Sample	RNA (mg)	Proinsulin mRNA act.[b]	Fold purification[c]
Total RNA	50[a]	516	1
Poly(A)-rich mRNA	0.668	16 400	32
Sucrose gradient-fx C	0.030	81 166	157

[a] Isolated from 4.6 g of pooled catfish pancreatic islets. [b] Activity in cpm of [^{35}S]methionine incorporated into total protein × the fraction in proinsulin determined by densitometric tracings of the $NaDodSO_4$ gel radioautographs, cpm/μg × 10^{-3}. [c] Relative to total RNA.

daltons on $NaDodSO_4$–urea gel electrophoresis. This protein comprises up to 23% of total islet protein synthesis depending on the conditions of cell-free synthesis. Tryptic peptide analysis of this cell-free product showed that it contained at least four, and perhaps seven of eight peptides present in proinsulin. Thus the data strongly suggest that the cell-free product is catfish preproinsulin. Preliminary data with automated sequential Edman degradation indicate that the NH_2 terminus of the in vitro product is different from proinsulin and is relatively rich in leucine as reported for anglerfish (Shields and Blogel, 1977) and rat preproinsulins (Chan et al., 1976). We have not evaluated the COOH end to determine whether it is also altered.

Careful inspection of the [^{35}S]methionine-labeled cell-free products on $NaDodSO_4$–urea gels indicated three bands between 11 000 and 12 000 daltons (Figure 2C). It appears that a similar result was obtained when sea raven islet mRNA was translated (Shields and Blobel, 1977). The $NaDodSO_4$ gel electrophoresis products of rat islet mRNA (Chan et al., 1976; Duguid et al., 1976) were sliced and counted, and no autoradiographs were published. The resolution of sliced gels is not as good as autoradiography. Autoradiography of translation products of rat preproinsulin mRNA have been reported to yield three bands between 11 500 and 13 500 (Forest Fuller, personal communication). The tryptic peptide analysis of catfish cell-free product (11 000–12 000 daltons) indicates that it is not homogeneous, but since overlapping peptides with respect to proinsulin were observed, this suggests that preproinsulin is the major cell-free peptide of this size. Some of the heterogeneity is probably due to endogenous wheat germ protein (Figure 2A, lane 1).

Fish islets have been demonstrated to synthesize glucagon precursors with mol wt of 11 400 and 9000 (Noe and Bauer, 1971, 1975). These precursors were detected when islet proteins were labeled with [^{3}H]tryptophan, an amino acid present in glucagon but not in proinsulin. In contrast, when islets were labeled with [^{3}H]leucine, no glucagon precursors were detected relative to incorporation into proinsulin. This would be predicted since the number of α cells is probably less than one-tenth the number of β cells in catfish islets (Bencosme et al., 1965). Similarly while somatostatin has been detected in pancreatic islets (Weir et al., 1976), the content of somatostatin was 0.18 ng/mg pancreas compared with 104 ng/mg of insulin (Ganda et al., 1977). While fish islets are a potential source of mRNA for other polypeptide hormones, these calculations suggest that proinsulin mRNA will be on the order of 10–1000 times more abundant.

Failure of the catfish preproinsulin to bind to any of the anti-insulin serum tested to any extent greater than nonimmune serum is unexplained. Guinea pigs immunized with purified catfish insulin developed potent antibodies which

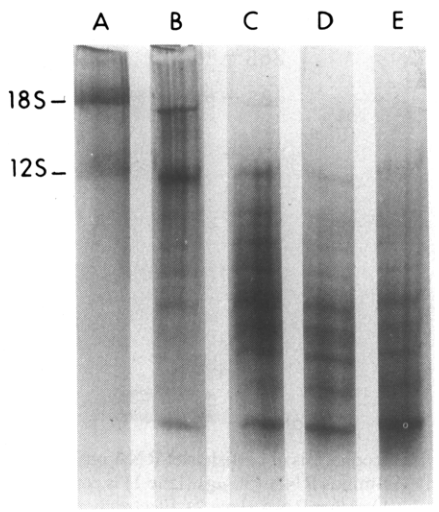

FIGURE 7: Agarose–urea slab gel electrophoresis of mRNA from the sucrose gradient fractions. Approximately 10–20 μg of mRNA from sucrose gradient fractions A–E (Figure 5) were electrophoresed on 3% agarose–6 M urea gels as described in Methods.

bound catfish insulin and proinsulin. Duguid et al. (1976) reported that only 20–30% of rat preproinsulin was bound to anti-insulin serum. Shield and Blobel (1977) showed that anti-insulin serum bound only 2–3% of the total acid precipitable radioactivity when sea raven mRNA was translated. This was equivalent to 10–20% of the radioactivity in the preproinsulin band; sequence data suggested that the peptide was homogeneous and the antibody binding incomplete.

Fish islets should prove to be a useful source of proinsulin mRNA. In this report we obtained 4.6 g of pooled islets from about 400 lb of catfish. About 50 mg of total nucleic acid was extracted (Table II). The yield of poly(A)-rich mRNA was about 1.3%. The translational activity, defined as the incorporation of [^{35}S]methionine into preproinsulin/μg of RNA, increased 32-fold by the oligo(dT) step. This is consistent with the gel electrophoretic pattern which clearly indicates ribosomal contamination of the oligo(dT)-bound mRNA, similar to that observed by others (Rosen, 1976). Sucrose gradient centrifugation of catfish poly(A)-rich mRNA yielded an 8S fraction of mRNA with a fivefold increase in translational activity. The overall purification was 157-fold by the two steps. While the 8S region of the sucrose gradient contained the peak preproinsulin translational activity, there were a large number of RNA bands observed when this RNA was electrophoresed (Figure 7). The heterogeneous nature of the RNA in the 8S region is unexplained, since preproinsulin comprised almost two-thirds of the islet mRNA dependent products of translation of fraction C. This suggests that these bands are either partially degraded ribosomal RNA, small nuclear RNA, or perhaps a heterogeneous population of proinsulin mRNAs with various amounts of poly(A)s on the 3′ end as has been described for other mRNAs (Estratiadis et al., 1975; Monahan et al., 1976; Rosen, 1976). Proinsulin mRNA partially degraded at the 5′-initiator site may account for some of the untranslated mRNA.

The size of catfish proinsulin mRNA, based on peak translational activity of sucrose gradient fractionated mRNA, was estimated to be approximately 8 S. Rat preproinsulin mRNA was similarly estimated by peak translational activity from a sucrose gradient (Duguid et al., 1976). This RNA was labeled and determined to have sedimentation coefficients of 6.1–9.3 on various sucrose gradients and approximately 600 nucleotides when electrophoresed on polyacrylamide gels in

98% formamide. Ullrich et al. (1977) succeeded in cloning 90% of the structural gene for rat preproinsulin and from the sequence determined that the mRNA contained 354 nucleotides terminated at the 3′ end by varying lengths of poly(A). The 5′ end of the mRNA was missing, which must contain at least 30 nucleotides to complete the structural portion of the mRNA. The number of nucleotides existing at the 5′-initiator region of the rat preproinsulin mRNA has not been determined.

The isolation and preliminary characterization of fish proinsulin mRNA have been reported. Analysis of the cDNA generated from this preparation by hybridization kinetics and restriction endonuclease mapping should give further information on the complexity of this fraction.

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