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Partial purification and characterization of the mRNA for rat preproinsulin

[oligo(dT)-cellulose chromatography/formamide–polyacrylamide gel electrophoresis/islet cell tumor]

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ABSTRACT **Electrophoretically homogeneous messenger RNA for rat preproinsulin has been prepared from an islet cell tumor by the use of oligo(dT)-cellulose chromatography and sucrose density gradient centrifugation. The molecular weight of the mRNA is about 210,000, as determined by polyacrylamide gel electrophoresis in formamide, and its sedimentation coefficient is 9.3 S in sucrose gradients containing 0.2 M NaCl. These results indicate that the synthesis of preproinsulin is directed by a monocistronic mRNA 600 nucleotides in length and requires approximately 55% of the molecule's coding capacity.**

Recent studies in this laboratory and others (1–3) have shown that the translation product of proinsulin mRNA in cell-free protein synthesizing systems is an 11,500-dalton polypeptide, preproinsulin, having an extension of 23 residues at the NH_2-terminus of the proinsulin sequence. Although preproinsulin mRNA can be obtained from rat islets of Langerhans in amounts sufficient for cell-free translation studies (1), a more abundant source of this mRNA is required for its isolation and characterization. For this purpose we have examined a variety of rat islet cell tumors which were induced either chemically (4) or by x-irradiation (5, 6). Both kinds of tumor contain amounts of proinsulin mRNA comparable to that of normal islet tissue as judged by the stimulation of incorporation of amino acids into preproinsulin in the wheat germ ribosomal system. For the studies reported here on the partial purification and characterization of preproinsulin mRNA we have used tissue from a transplantable x-ray-induced islet tumor recently characterized by Chick *et al.* (6). Because of the limited availability of this material we have used an iodinated preparation enriched in preproinsulin mRNA for characterization of its physical properties.

METHODS

RNA Sources. Ribosomal RNA and tRNA were isolated from post-nuclear supernatants of rat liver homogenates by standard techniques. X-ray-induced insulin-producing tumors were maintained in NEDH inbred rats (6). Nucleic acid extractions were performed as described (1), except that frozen tumors, stored at −70°, were first pulverized in a stainless steel mortar maintained at dry ice temperatures. After extraction, RNA was precipitated by the addition of 0.25 volume of 10 M LiCl. After 1 hr at 0° the precipitate was collected by centrifugation at 15,000 × *g* for 10 min. The recoveries in the pellet of absorbance units and translation activity were 50% and 80%, respectively.

Chemicals. Phenol was redistilled and sodium dodecyl sulfate ($NaDodSO_4$) was obtained from Serva, Heidelberg. All other chemicals were reagent grade, except where noted. Tris, urea, and sucrose, which were Ultra Pure grade, were from Schwarz/Mann, Orangeburg, N.Y. ^{125}I was obtained from Industrial Nuclear Corporation, St. Louis, Mo., and thallium chloride was from ICN Pharmaceuticals, Plainview, N.Y. Sodium decyl sulfate was from Pfalz and Bauer, Stamford, Conn. All buffers were made 0.2–0.5% diethyl pyrocarbonate (Aldrich) and autoclaved twice before use. All glassware was baked at 200° for 8 hr. Plastic surfaces were treated with 0.5% diethyl pyrocarbonate and washed exhaustively with autoclaved buffer.

Sucrose Density Gradient Centrifugation. Sedimentation behavior was studied on 15–30% linear sucrose gradients in the buffer system used by McKnight and Schimke (7), 10 mM Tris·HCl, 5 mM EDTA, 1% $NaDodSO_4$, pH 7.4. Samples were heated to 60° for 10 min and rapidly cooled in an ice bath before centrifugation. A Beckman SW 60Ti rotor was used, unless otherwise noted, operating at 60,000 rpm at 22°. Gradients were fractionated with an Isco apparatus equipped with a model UA-5 absorbance monitor. Translation assays using the wheat germ cell-free system and immunoprecipitations were as described previously (1).

$NaDodSO_4$–Urea–Polyacrylamide Gel Electrophoresis. Polyacrylamide gels described by Swank and Munkres (8) were prepared with acrylamide and bisacrylamide obtained from Bio-Rad Laboratories, Richmond, Calif. Translation assay aliquots were treated as described (1) except that after the 80° incubation in 10% trichloroacetic acid, the precipitates were lyophilized, washed with diethyl ether, and dried. The samples were denatured with the buffer described (8) and electrophoresed on 10 cm gels for 17 hr at 3 mA per gel. Fractionation of the gels was achieved with a Gilson gel-extruding device and radioactivity was determined as described (1).

Oligo(dT)-Cellulose Chromatography. Oligo(dT)-cellulose was obtained from Collaborative Research, Waltham, Mass. After washing with 0.1 M KOH in the cold, the column, containing approximately 0.1 g of oligo(dT)-cellulose, was equilibrated in the cold with 0.2 M NaCl, 10 mM Tris·HCl, 1 mM EDTA, 0.1% sodium decyl sulfate, pH 7.4. RNA samples were applied to the column in 1 volume of this buffer and the column was washed with 5 volumes of the same buffer. Poly(A)-containing RNA was eluted with buffer minus NaCl, at 60°. Alternatively, in later experiments (see Fig. 4) the chromatography was performed at room temperature in buffers containing 0.1% $NaDodSO_4$ instead of sodium decyl sulfate. Experiments with iodinated tumor nucleic acid indicated that approximately 1% of the RNA was retained in the poly(A)-containing fraction after two cycles of chromatography. The recovery of radioactivity was quantitative. The recovery of translation activity was low, generally 30–40%; 80% of this translation activity was recovered in the fraction eluted at low-salt concentration.

Iodination of RNA. RNA was iodinated after LiCl precipitation and ethanol precipitation by the method of Commerford (9) with the following modifications. It was found that iodina-

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

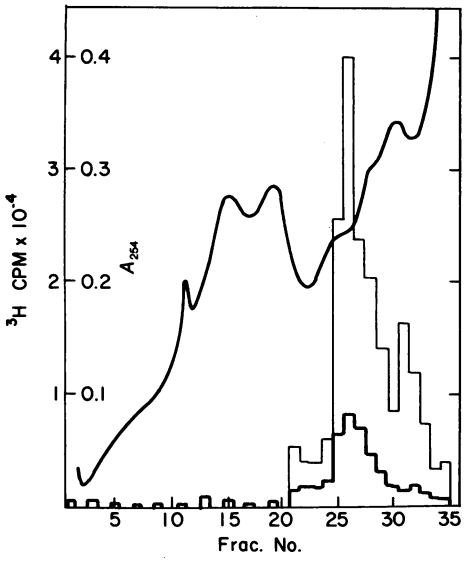

FIG. 1. Sedimentation of tumor RNA with translation activity. Tumor RNA (100 μg) was centrifuged in a low-salt gradient for 10.5 hr at 60,000 rpm, as described under *Methods*. RNA in indicated fractions was ethanol-precipitated with 12.5 μg *Escherichia coli* tRNA (Gibco) and assayed for translation activity (thin line histogram, 5000 cpm background incorporation subtracted). Recovery of translation activity was 66%. Results of immunoprecipitation with guinea pig antiserum (Miles-Yeda Ltd.) directed against porcine insulin are shown as the thick line histogram. Absorbance at 254 nm was measured and is shown as the smooth curve.

tion was much more reproducible after LiCl precipitation (10). RNA at a concentration of 1 mg/ml was incubated at 60° in 50 μl of 0.2 M NaOAc, pH 5.0, with 1–2 mCi of carrier-free Na^{125}I and 2 μl of 0.1 M $TlCl_3$. After 10 min, free iodide was removed by gel filtration on Sephadex G-25, generally in the same high-salt buffer used in oligo(dT)-cellulose chromatography. Specific activities of the order of 2×10^7 cpm/μg were obtained. ^{125}I radioactivity was determined using a Packard gamma counter.

Polyacrylamide Gel Electrophoresis in Formamide. The molecular weight of preproinsulin mRNA was determined by the method of Staynov *et al.* (11) with the following modifications. After deionization, the pH of the formamide was adjusted to 7.8 by titration with a saturated aqueous solution of Tris. The electrophoresis was performed in cylindrical gels (4% acrylamide).

RESULTS

Analysis of the Crude Tumor RNA. The sedimentation behavior of the translation activities present in the nucleic acid extract, after removal of DNA by LiCl precipitation, was investigated by means of sucrose density gradient centrifugation. After centrifugation for 10.5 hr, the material active in stimulating incorporation of [^{3}H]leucine into protein in the wheat germ cell-free system was resolved into a major (fractions 25–29) and a minor (fractions 30–33) component, as shown in Fig. 1. Shorter centrifugations revealed the presence of polydisperse translatable RNA components with *s* values as high as 18 S (see Fig. 3).

Translation products obtained in the cell-free synthesis assays shown in Fig. 1 were analyzed both by immunoprecipitation with insulin antiserum and by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and urea (8). The immunoprecipitation results, presented as the thick line histogram in Fig. 1, demonstrate the synthesis of an insulin-like component

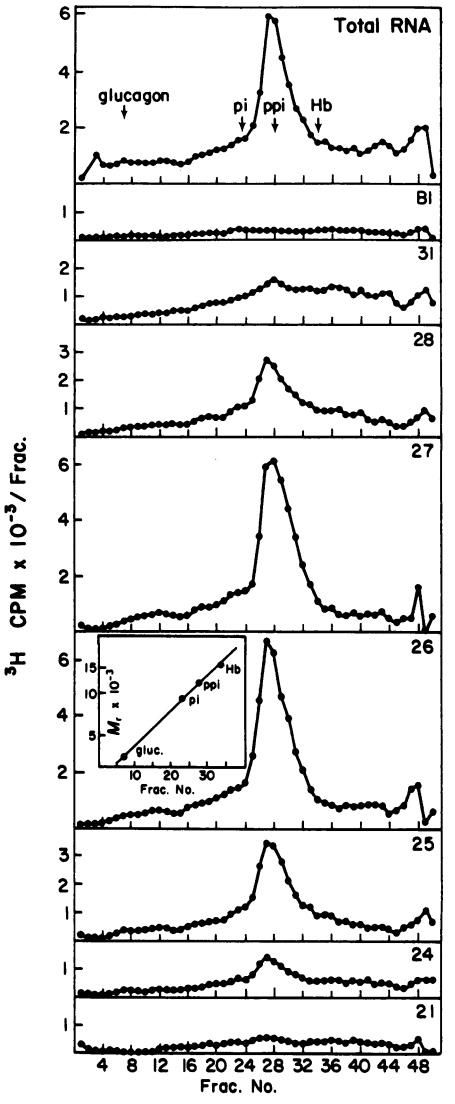

FIG. 2. $NaDodSO_4$-polyacrylamide gel electrophoresis of translation products. The electrophoretic behavior of the translation products directed by unfractionated tumor RNA and by fractions across the gradient shown in Fig. 1 are shown. Bl represents the background synthesis of the wheat germ system. Glucagon (gluc.) and proinsulin (pi) were labeled with ^{125}I. Preproinsulin (ppi) and globin (Hb) were synthesized in the wheat germ cell-free system and were labeled with [^{3}H]leucine.

directed by the mRNA in the main peak of translation activity, while the product coded for by the mRNA in the minor translatable peak contained a smaller proportion of immunoprecipitable material.

$NaDodSO_4$ gel electrophoresis of the labeled translation products directed by the total RNA extract, shown in the top panel of Fig. 2, demonstrated the presence of a large peak of material corresponding in size to preproinsulin (1), and heterogeneous material of higher and lower molecular weight. The electrophoretic analyses of translation products directed by fractions across the main peak of translatable RNA (fractions 25–29, Fig. 1) confirm the findings of the immunoprecipitation experiments of Fig. 1, that this activity is expressed mainly in the synthesis of protein with the properties of preproinsulin. The cell-free products directed by the minor peak of translation activity in the sucrose gradient of Fig. 1 were also analyzed by electrophoresis and shown to consist mainly of high-molecu-

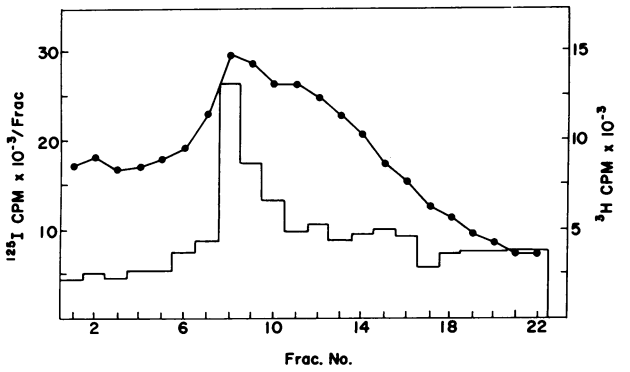

FIG. 3. Sedimentation of ^{125}I-labeled poly(A)-containing tumor RNA. ^{125}I-Labeled poly(A)-containing RNA, purified by oligo-(dT)-cellulose chromatography at 4°, was mixed with 100 μg of total nucleic acid and centrifuged for 6 hr at 50,000 rpm in a Beckman SW 50.1 rotor. Translation activity was determined as in Fig. 1, and is shown as the histogram. ^{125}I-Labeled RNA comigrating with the major peak of translation activity (fractions 8 and 9) represents 17% of the applied radioactivity, or 0.17% of that applied to the oligo(dT)-cellulose column. 18S rRNA derived from the tumor migrated to fraction 15 in this gradient.

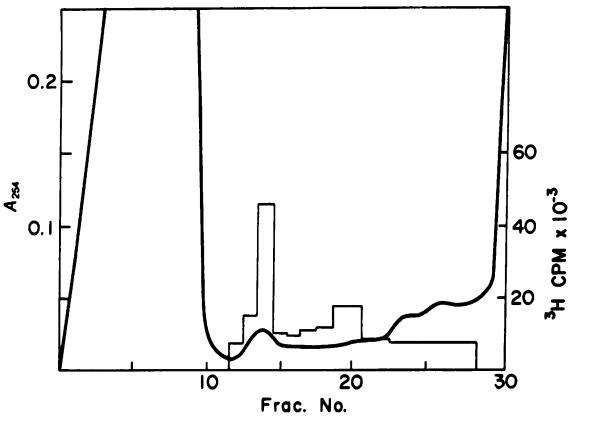

FIG. 4. Sedimentation of unlabeled poly(A)-containing tumor RNA. Poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography at room temperature, as detailed in *Methods*. This procedure decreased the polydisperse RNA sedimenting more rapidly than preproinsulin mRNA (see Fig. 3). Centrifugation was for 6 hr at 60,000 rpm. Absorbance measurements of 254 nm reveal preproinsulin mRNA as a discrete peak cosedimenting with the major component of translation activity. The baseline for the absorbance measurements was determined using a parallel gradient containing only tRNA. Translation assays were performed on 10 μl aliquots of the indicated gradient fractions, after coprecipitation with 12.5 μg of tRNA, shown as the histogram.

lar-weight heterogeneous material. In addition there was a small peak of material at the position of preproinsulin in the product directed by mRNA in this fraction, consistent with the small amount of immunoprecipitable material present in the translation products from this region.

Partial Purification of ^{125}I-Labeled Preproinsulin mRNA. After iodination, the poly(A)-containing fraction of the tumor RNA was purified by two cycles of oligo(dT)-cellulose chromatography. The sedimentation behavior of this RNA was compared with that of the translatable RNA in the crude nucleic acid extract as shown in Fig. 3. A peak of labeled RNA, which was more prominent in other experiments not shown, was found to cosediment with the main peak of translation activity. Material corresponding to this peak (i.e., equivalent to fractions 8 and 9 in Fig. 3) was collected from a preparative sucrose gradient, run in parallel with the analytical gradient shown in Fig. 3, but without added crude nucleic acid, and was used for the further characterization of preproinsulin mRNA.

In a separate experiment the sedimentation behavior of unlabeled preproinsulin mRNA was studied by both translation assays and by absorbance measurements at 254 nm, as shown in Fig. 4. Preliminary results indicate that the preproinsulin mRNA obtained in this experiment (peak at fraction 14) has a somewhat higher specific translation activity than purified globin mRNA (data not shown).

Determination of the Sedimentation Coefficient of Preproinsulin mRNA. The sedimentation velocities of rat liver 28S and 18S rRNA and of rat preproinsulin mRNA were measured in sucrose gradients both with and without 0.2 M NaCl. Fig. 5 shows the results of experiments with sucrose gradients not containing NaCl, conditions which have been reported to minimize aggregation (7). The slopes of these lines, and those generated using data from gradients 0.2 M in NaCl, are presented in Table 1. The sedimentation coefficients we have determined for preproinsulin mRNA are 9.3 S in 0.2 M NaCl, 7.1 S in the low-salt gradients, and 6.1 S after formaldehyde treatment.

Formamide–Polyacrylamide Gel Electrophoresis of Preproinsulin mRNA. The ^{125}I-labeled preproinsulin mRNA preparation obtained as shown in Fig. 3 yielded a symmetric peak on electrophoresis in formamide. The electrophoretic mobility of preproinsulin mRNA was found to be unaffected by treatment with formaldehyde, as shown in Fig. 6, confirming that complete denaturation was achieved in this system.

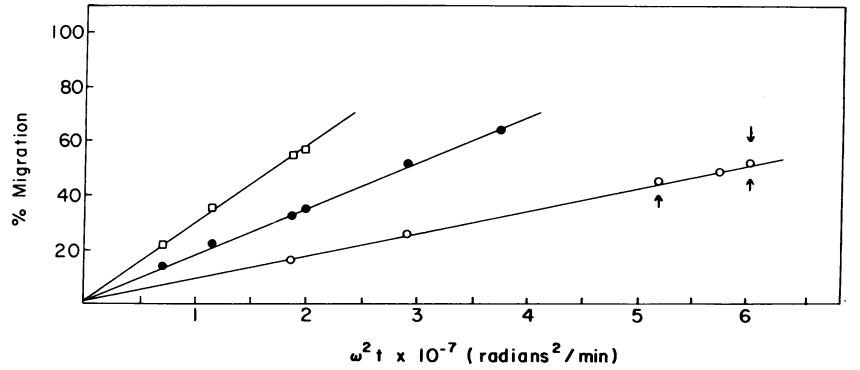

FIG. 5. Sedimentation behavior of preproinsulin mRNA. Aliquots of rRNA and purified preproinsulin mRNA were mixed, treated as described, and applied to low-salt gradients in approximately 80 μl of buffer. The migration of the rRNAs was determined with an Isco UV monitor and that of the mRNA by radioactivity determinations, except where noted (single arrow, by translation activity; double arrow, by both translation activity and absorbance) in a gradient without added tumor RNA. □, 28S rRNA; ●, 18S rRNA; ○, preproinsulin mRNA.

Table 1. Determination of sedimentation coefficients

	Formaldehyde treated		Low salt		High salt	
	Slope $\times 10^6$	*s* value (S)	Slope $\times 10^6$	*s* value (S)	Slope $\times 10^6$	*s* value (S)
28 S	2.23	19.1	2.82	24.2	3.63	31.1
18 S	1.38	11.8	1.68	14.4	2.02	17.3
Preproinsulin mRNA	0.716	6.14	0.824	7.06	1.08	9.26

Data were obtained as described in legend to Fig. 5. Samples were treated with formaldehyde as described in the legend to Fig. 6 before application to low salt gradients. Samples applied to high salt gradients were not heated. Sample volume was compensated for by including a data point of 1% migration at time 0. The contributions of acceleration and deceleration were determined graphically. Lines were generated from data by a least-squares fit. Coefficients of correlation were greater than 0.999 and the uncertainty in the slopes determined was less than ±3%, in all cases. Slopes were converted to *s* values by a conversion factor, 8.57×10^6, the mean of those required to convert the slopes of the two rRNAs in high salt to the *s* values for these RNAs determined by Noll (12), i.e., 30.2 S and 17.8 S.

The molecular weight determination (inset in Fig. 6) was performed in a separate experiment using iodinated 28S and 18S rRNA and tRNA from rat liver as standards. The average of three independent determinations of the molecular weight of preproinsulin mRNA was 212,000.

DISCUSSION

The evidence presented here indicates that preproinsulin mRNA is a major component of the mRNA population of the islet tumor cells, and that its physical properties correspond well with its function in directing the synthesis of preproinsulin. The preproinsulin mRNA is present in the major peak of translatable RNA as shown in Fig. 1. However, only 20–30% of the protein product whose synthesis is directed by aliquots of the fractions across the main peak of translation activity (Fig. 1) is precipitable with insulin antiserum, even though the nonprecipitated portion appears to migrate identically on $NaDodSO_4$ gel electrophoresis. We also have encountered this problem in the translation of nucleic acid preparations from normal islets (1). A plausible explanation for this phenomenon could be that the reaction with antiserum is incomplete due to aggregation or improper 3-dimensional folding of a considerable proportion of the product peptides under the artificial conditions of *in vitro* translation. It is likely that two nonallelic preproinsulins are synthesized in thissystem (1, 5). Although slight differences in the properties of these two proteins might produce some heterogeneity in the translation product, the skewing of the peaks observed in the experiment shown in Fig. 2 was not seen with standards and suggests systematic heterogeneity. The sequence homogeneity of the translation product directed by purified preproinsulin mRNA must be studied in greater detail to clarify these problems.

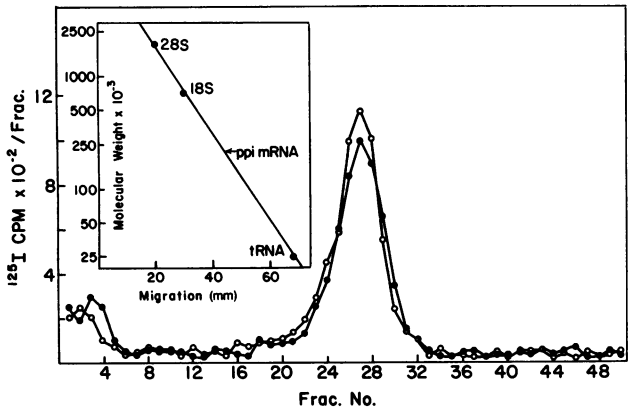

FIG. 6. Formamide–polyacrylamide gel electrophoresis of preproinsulin (ppi) mRNA. Aliquots (10 μl) of the purified ^{125}I-labeled mRNA were added to 89% (vol/vol) formamide, 2 mM NaCl, 7% sucrose, pH 7.8, both with (○) and without (●) prior treatment with 3.7% formaldehyde in 0.1 M phosphate, pH 7.5, for 10 min at 60°. Samples in formamide were heated to 60° for 1 min and rapidly cooled before electrophoresis (3 mA per gel for 7 hr). Iodinated standards were run in a parallel gel; molecular weight values were those used by Staynov *et al.* (11). The line shown in the inset was generated by a least-squares fit (coefficient of correlation, −0.999; uncertainty of slope, ±1%). The width of peaks for the rRNAs was between 6.5 and 7.5 mm in several experiments. In the experiment shown above, the peak-width at half-height was 8.4 and 9.6 mm for formaldehyde-treated and untreated preproinsulin mRNA, respectively.

The amount of secondary structure in preproinsulin mRNA, as revealed by the relative difference in sedimentation coefficients determined in low-salt and high-salt gradients, and after formaldehyde treatment, is similar to that in 28S and 18S rRNA. The sedimentation coefficient determined for preproinsulin mRNA in high-salt gradients, 9.3 S, is somewhat lower than the value of 10 S reported for globin mRNA (14), although the molecular weight these authors have determined is equal to that of preproinsulin mRNA, 210,000.

The molecular weight we have determined for preproinsulin mRNA corresponds to a length of approximately 600 nucleotides, as the unhydrated sodium salt. Only 55% (330 nucleotides) of the coding capacity of this mRNA is used for the direction of preproinsulin synthesis, a significantly smaller proportion than the 70% of globin mRNA which codes for the synthesis of globin (15), but comparable to the proportion that directs preimmunoglobulin light chain synthesis (16), approximately 55% of the coding capacity of the mRNA. Our preliminary results indicate the preproinsulin mRNA from this tumor as well as from normal rat islets bears a 7-methylguanosine residue at the 5′-terminus (17) which is required for its translation in the wheat germ system (G. Hortin and S. Chan, unpublished data).

A further point of interest is the presence of a small amount of preproinsulin in the translation product directed by RNA sedimenting more rapidly than the bulk of the preproinsulin mRNA (Figs. 1 and 2). While this finding might be due to aggregation of the mRNA, an alternative explanation might be the presence of a precursor RNA in this fraction.

The sequence complexity of the purified preproinsulin mRNA has not yet been determined; however, our preparation yields a symmetric peak on electrophoresis under denaturing conditions (see Fig. 6). The relative broadness of this peak relative to 28S and 18S rRNA (see legend to Fig. 6) might be explained by a slight difference in the length of the two rat preproinsulin mRNAs; however, a poly(A)-tract length heterogeneity similar to that found in globin mRNA (13) could fully account for the apparent dispersity.

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