

Evaluation of rat insulin messenger RNA in pancreatic and extrapancreatic tissues

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Summary. The purpose of these studies was to determine whether insulin detected immunochemically in extrapancreatic tissues of the adult rat is synthesized in situ by quantitating mRNA in these tissues. A blot hybridization assay was utilized with cloned ³²P-proinsulin cDNA. The lower limit of detection was estimated to be 3pg. Proinsulin mRNA concentration was found to be 1000–1500 µg in isolated pancreatic islets and was easily detected in total pancreatic RNA at 10–15 pg/µg. Proinsulin mRNA was quantitated in rat insulinoma cells adapted to culture at levels 1:50 those in normal islets. Samples of RNA (20–50 µg) enriched about 50-fold for mRNA sequences by repeated oligo-deoxythymidylate chromatography were assayed. No insulin mRNA was detected in 50 µg samples of RNA from brain or in 20 µg samples from subsec-

tions of brain or other extrapancreatic tissues. RNA samples were undegraded as assessed by ability to stimulate protein synthesis in a cell-free system. Proinsulin mRNA from pancreas was added to brain homogenates and recovered intact. Brain RNA samples with insulin mRNA levels 1:1000 that of pancreas would be predicted to have 50–75 pg proinsulin mRNA/50 µg sample assayed if present. Because none was found, brain must have a concentration <1:6,000 that of pancreas. These findings suggest that immunoassayable insulin detected in extrapancreatic tissues of the adult rat is synthesized by the pancreas.

Key words: RNA blot hybridization, rat, proinsulin mRNA, brain mRNA, pancreatic islets.

A number of peptides originally described as hormones from gut have been identified in brain [1, 2]. Some are present in approximately equal concentrations in hypothalamus and gut (e.g. somatostatin) [3]. Others, such as insulin, are present at levels 1000-fold less in brain than in pancreas [4, 5]. The identification of pg quantities of less abundant peptides per g of brain has been facilitated by radioimmunoassays and by localization with immunocytochemical techniques [1–5].

The immunochemical detection of hormones in brain leaves unanswered the important question of their origin. Demonstration of insulin synthesis in brain or elsewhere would strongly imply as yet undescribed functions for this peptide. There are no data to answer this question, except reports of increased levels of insulin in brain compared with plasma under various conditions [4–6]. The low abundance of insulin in brain precludes the use of radiolabelled amino-acid incorporation to document synthesis directly [7].

Using an RNA blot hybridization technique, we have assayed for proinsulin I and II mRNA's (which are 93% homologous) in brain and several other tissues of the adult rat, using a cloned ³²P-labelled probe complementary to rat insulin I mRNA [8]. Proinsulin I cDNA hybridizes readily to both genes under the conditions of hybridization employed [9]. The presence of proinsulin mRNA in extrapancreatic tissues would provide an independent line of evidence for synthesis. The

present limit of sensitivity of this assay is about 5 pg of proinsulin mRNA in 50 µg of RNA (0.00001%). Because amino-acid incorporation requires that a protein represent at least 0.1% of total protein synthesis, the mRNA assay is approximately 10,000-fold more sensitive. Using this approach, we have estimated the abundance of proinsulin mRNA in pancreas and in extrapancreatic tissue.

Methods

RNA extraction

Several organs were dissected from anaesthetised (pentobarbital sodium, 3 mg/kg) male Sprague-Dawley rats (weight 200–300 g), blotted, weighed and total and poly-adenylated (Poly A+) RNA prepared by the method of Chirgwin et al. [10] using sedimentation through CsCl. Poly A+ RNA was prepared from total cellular RNA by two cycles of oligo deoxythymidylate (dT)-cellulose (Collaborative Research, Lexington, MA) as described previously [11]. RNA recovered was 1.6–2.0% of input RNA. Islets of Langerhans were isolated by collagenase digestion [12] as described previously [13]. RNA pooled from 6–20 rats was used for analysis, excepting pancreas where RNA from 2–6 rats was used. Three preparation of RNA were analyzed on five occasions.

Quality of isolated RNA was checked by $A_{260\text{nm}}/A_{280\text{nm}}$ ratios > 2.0; electrophoresis under denaturing conditions of total RNA, which was visualized by staining with 1-ethyl-2-[3-(1-ethylnaphtho[1,2d]thiazolin-2-ylidene)-2-methylpropenyl] naphtho[1,2d]thiazolium bromide (Stainsall, Eastman-Kodak, Rochester, NY) (ratio of 28S to 18S

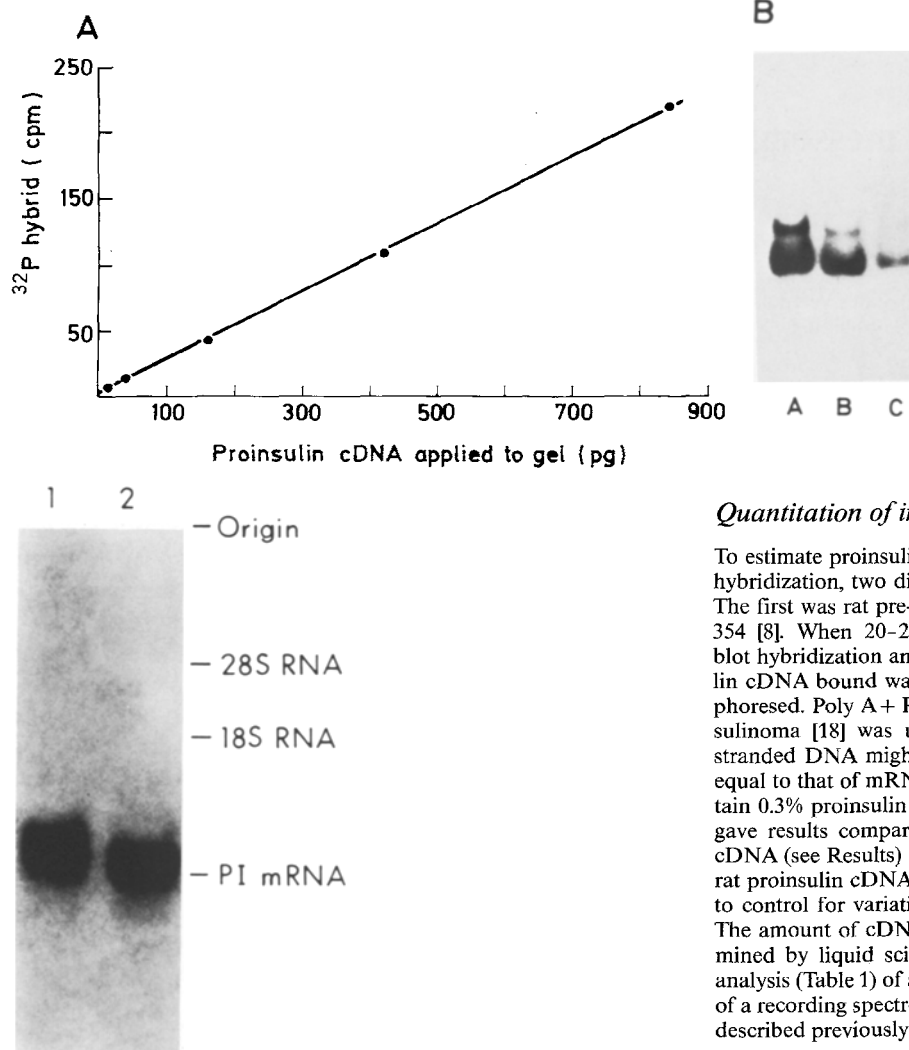


Fig. 1 A and B. Quantitation of proinsulin mRNA by blot hybridization assay using a cloned complementary DNA standard. Unlabelled plasmid pcRI 354 containing rat pre-proinsulin I cDNA [8] was digested with the restriction endonuclease Hind III, denatured with glyoxal, diluted, electrophoresed, transferred, hybridized with ³²P-proinsulin I cDNA and autoradiographed. **A** The ³²P-hybrid cpm in relation to pg proinsulin cDNA applied to the gel. **B** Autoradiographs of ³²P-proinsulin cDNA hybridized to 420 pg of proinsulin cDNA (lane 1), 160 pg (lane 2), and 42 pg (lane 3). Minor bands in lanes 1 and 2 represent partially digested plasmids (<5% of total). Exposure time, 60 h

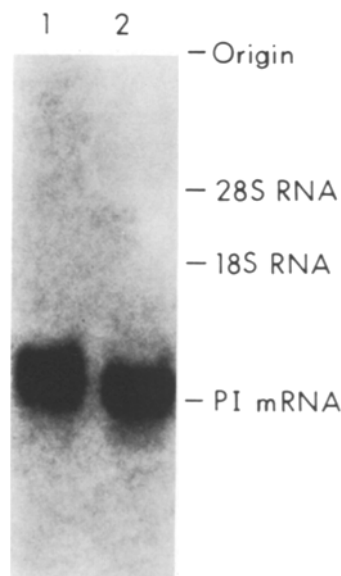


Fig. 2. Estimation of proinsulin mRNA in isolated rat pancreatic islet RNA and insulinoma cell RNA. RNA (20 µg) from insulinoma cells adapted to culture (lane 1) was compared to rat pancreatic islet RNA (1 µg, lane 2), by blot hybridization analysis with ³²P-proinsulin cDNA as described in the Methods. Size was determined from 18S and 28S RNA standards electrophoresed in adjacent lanes. Exposure time, 73 h

peak heights ~2), and cell-free translation. Brain poly A+ RNA was translated using a kit from rabbit reticulocyte lysate according to the direction of the manufacturer (Promega-Biotec, Madison, WI). Products were assayed by SDS-polyacrylamide (Eastman-Kodak) gel electrophoresis as described elsewhere [13].

RNA hybridization analysis

Poly A+ RNA (20–50 µg) was denatured with 1 mol/l glyoxal (Fisher Scientific, St. Louis, MO) in 50% dimethyl-sulfoxide (Sigma, St. Louis, Missouri, USA) [14] and electrophoresed on 2% agarose gels, pre-treated with 0.2% diethylpyrocarbonate (Sigma) to inactivate nucleases potentially present in agarose. Gels were run at 23 °C at 50 volts for 4 h. Proinsulin mRNA size was estimated from 18S and 28S ribosomal RNA. RNA was transferred from gels to diazophenylthio paper (Giddings, St. Louis, MO) [15] for at least 18 h using 100 mmol/l sodium acetate (pH 4) as the transfer buffer [16]. Filters were hybridized with pre-proinsulin I cDNA cleaved from the plasmid pcRI 354 [8] and labelled to 5×10^8 cpm/µg with ³²PO₄ by (New England Nuclear Corporation, Boston, MA) nick translation [17]. Hybridization was performed at 42 °C for 24–72 h, followed by washing and autoradiography as described previously [13].

Quantitation of insulin mRNA

To estimate proinsulin mRNA concentration in tissues by RNA blot hybridization, two different proinsulin mRNA standards were used. The first was rat pre-proinsulin I cDNA in the cloned plasmid pcRI 354 [8]. When 20–240 pg of proinsulin cDNA were subjected to blot hybridization analysis as in Figure 1, the amount of ³²P-proinsulin cDNA bound was proportional to the proinsulin cDNA electrophoresed. Poly A+ RNA from a subcutaneously transplanted rat insulinoma [18] was utilized as the second standard since double-stranded DNA might not hybridize to the probe with an efficiency equal to that of mRNA. The insulinoma RNA was estimated to contain 0.3% proinsulin mRNA [19] and in the RNA blot hybridization gave results comparable to those obtained with cloned proinsulin cDNA (see Results). In each RNA blot analysis a known amount of rat proinsulin cDNA was electrophoresed to provide a standard and to control for variations in efficiency of transfer and hybridization. The amount of cDNA bound to specific bands on filters was determined by liquid scintillation counting (Fig. 1) or by densitometric analysis (Table 1) of autoradiograms using the gel scanning accessory of a recording spectrophotometer (Beckman DU-8, Palo Alto, CA) as described previously [9, 26, 32].

Results

Proinsulin mRNA in pancreatic tissue

Isolated pancreatic islets. To determine the concentration of proinsulin mRNA in pancreatic β-cells, islets were isolated from pancreas by collagenase digestion. RNA blot hybridization with ³²P-rat proinsulin cDNA indicated that proinsulin mRNA was 600 ± 20 nucleotides in length (Fig. 2 lane 2). This is consistent with about 160 poly A+ residues attached to an mRNA of 330 coding and 110 non-coding nucleotides [8]. The concentration of proinsulin mRNA, determined using as standards either cloned rat proinsulin I cDNA (Fig. 1) or poly A+ RNA from a rat insulinoma, was estimated to be 1000–1500 pg/µg islet RNA.

Insulinoma cells adapted to culture. To demonstrate that the method may estimate proinsulin mRNA levels in tissues which synthesize less insulin, hybridization analysis was performed on RNA from rat insulinoma cells (Rin 5F) adapted to tissue culture [20]. RNA blot analysis (Fig. 2, lane 2) of 1 µg of islet RNA and 20 µg of insulinoma cell RNA (lane 1) indicated there was 20–30 pg proinsulin mRNA/µg insulinoma RNA or approxi-

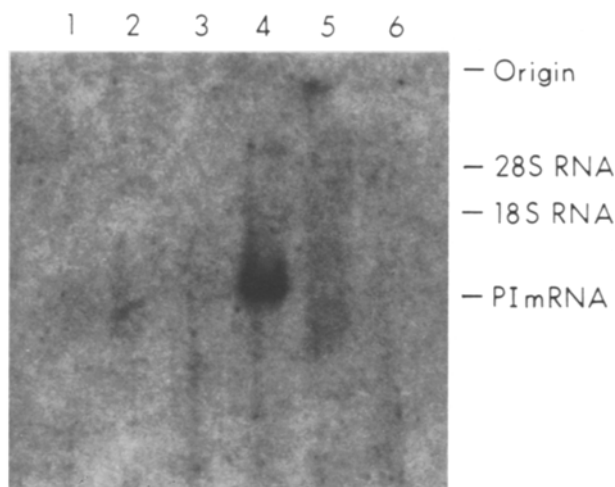


Fig. 3. Estimation of proinsulin RNA concentration of pancreatic and extrapancreatic tissues. This autoradiograph compares the amount of ^{32}P -proinsulin cDNA hybridizing to 20 μg of total RNA from rat pancreas (lane 4), or 20 μg of poly A+ RNA from testes (lane 1), kidney (lane 2), spleen (lane 3), liver (lane 5), and brain (lane 6). Hybridization time was increased 3-fold and the autoradiographs overexposed to facilitate detection of proinsulin mRNA in extrapancreatic tissues. The apparent band at the top of lane 5 corresponds to the origin of the gel. Exposure time, 9 days

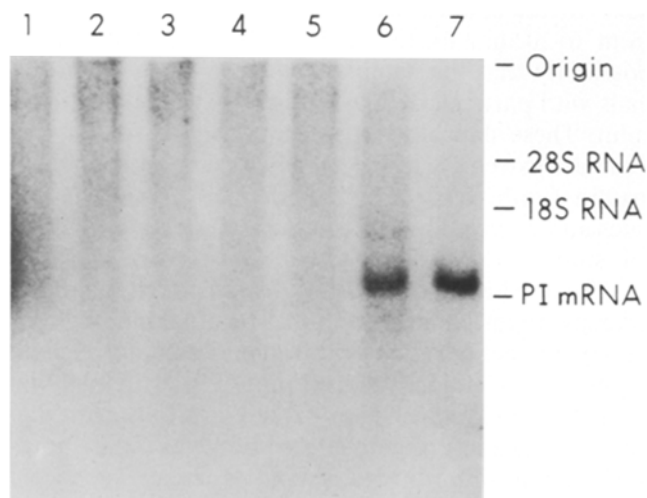


Fig. 4. Proinsulin mRNA content of poly A+ RNA from subsections of rat brain and guinea pig pancreas compared to that in total rat pancreatic RNA. Methods were as described in Figure 3 except that brains were dissected and poly A+ RNA (20 μg) from cerebrum (lane 1), olfactory lobes (lane 2), midbrain (lane 3), cerebellum (lane 4) and hindbrain (lane 5) were subjected to RNA blot hybridization. Guinea pig pancreas poly A+ RNA (20 μg , lane 6) was compared to total rat pancreatic RNA (20 μg , lane 7). Stringency was decreased by lowering the temperature of the posthybridization washes from 52° to 42° to facilitate detection of partially homologous sequences. Exposure time, 6 days

mately 1/50 that found in normal islet cells. Proinsulin mRNA from insulinoma cells appeared to be about 100 bases larger than that from normal islet cells, which is due to additional poly A+ residues on the 3' end (unpublished observations).

Total pancreatic RNA. The sensitivity of this assay allowed quantitation of proinsulin mRNA in RNA from whole pancreas without purifying poly A+ mRNA

(Fig. 3, lane 4, and Fig. 4, lane 7), even though islets represent only 1% of pancreas tissue, and mRNA only 1% of total RNA. When 20 μg of total RNA from pancreas was subjected to blot hybridization, it was estimated to contain 300 pg insulin mRNA (or 15 pg/ μg) which was readily detected.

Proinsulin mRNA in extrapancreatic tissues

RNA was isolated from several organs of rats which had been reported to contain levels of insulin greater than plasma including liver, spleen, kidney, testes, and brain [21]. To look for proinsulin mRNA in extrapancreatic tissues, polyadenylated mRNA was enriched approximately 50-fold by oligo dT-cellulose chromatography. The amount of proinsulin mRNA present in the poly A+ RNA fraction from these tissues (Fig. 3, lane 1-3, 5) was compared with that present in total pancreatic RNA (Fig. 3, lane 4). While proinsulin mRNA in total pancreatic RNA was readily detected (lane 4), repeated blot hybridization failed to reveal any proinsulin mRNA in any of the other tissues. The radiograph in Figure 3 was from an RNA filter subjected to increased hybridization time (from 24 to 72 h) with ^{32}P -proinsulin cDNA and deliberate overexposure which increased background. There was no detectable proinsulin mRNA in the extrapancreatic tissues examined.

To determine whether mRNA for insulin is present in brain at levels which might be detectable only in subsections, brains were quickly divided into olfactory lobes, cerebrum, midbrain, hindbrain, and cerebellum, and poly A+ RNA isolated. There was no measurable mRNA for insulin in poly A+ RNA from any subsection of brain (Fig. 4, lanes 1-5 compare with total pancreatic RNA, Fig. 4, lane 7). Background hybridization in Figure 4 is prominent because the washing temperature was lowered by 10°C in order to determine whether RNA with somewhat divergent sequences might be present in subsections of brain (see below).

It is possible that proinsulin mRNA in extrapancreatic tissues is different from that found in pancreatic islets. To determine whether rat proinsulin I cDNA could detect mRNAs for insulins which are as divergent in amino acid sequence as guinea pig [22] is from rat (about 65% homology), poly A+ RNA from guinea pig pancreas was examined. At the nucleic acid level, guinea pig mRNA differs from rat I and II proinsulin mRNA by about 20% at replacement sites, and up to 40% at silent sites [23]. A single hybridizing band of mRNA was readily detected in poly A+ guinea pig pancreatic RNA (Fig. 4, lane 6), which appeared identical in size to rat proinsulin mRNA (Fig. 4, lane 7). While there is no precedent for a messenger RNA being differentially polyadenylated in various tissues, non-polyadenylated RNA from brain was examined and no insulin mRNA was found.

Because no proinsulin mRNA was detected in extrapancreatic tissue, it is important to estimate the lower limits of detection of the RNA blot hybridization method. Blot hybridization assays have been shown by oth-

Table 1. Hybridization assay for proinsulin mRNA

RNA applied to gel		Area under curve (absorbance) optical density	
Total (μg)	Proinsulin mRNA (pg)	Experiment 1	Experiment 2
4	60	5.06	3.74
2	30	3.89	1.91
1	15	0.96	0.29
0.4	6	0.84	0.31
0.2	3	0.46	0.23
0.1	1.5	ND ^a	ND ^a

^a Not detected

ers to measure as little as 1–2 pg of mRNA [14, 24, 25]. The limits of the rat proinsulin mRNA assay is demonstrated by subjecting total pancreatic RNA containing 15 pg of proinsulin mRNA/ μg , to serial dilution and hybridization analysis. Autoradiographs were analyzed by densitometry (Table 1). The lower limits of detection of this assay was 3 pg of proinsulin mRNA.

To demonstrate that brain RNA did not interfere with detection of proinsulin mRNA, poly A+ RNA from pancreas was diluted 10,000-fold and added to a 20 μg sample of poly A+ RNA from rat brain, and analyzed. A faint but distinct band was recognized. To confirm the data in Figure 3 and 4 and to extend the sensitivity of the assay, 50 μg of poly A+ RNA from brain (the maximum capacity of the gel) was electrophoresed in an adjacent lane. No hybridizing band was detected in this or other similar experiments.

Failure to detect insulin mRNA in brain might be caused by degradation during the isolation process. To test this possibility, a small amount of pancreas was added to brain and RNA isolated. A strong signal for proinsulin mRNA was present in poly A+ RNA from the sample. Further evidence that brain mRNA was undegraded was provided by demonstrating that brain poly A+ RNA was active in stimulating synthesis of high molecular weight brain protein in a rabbit reticulocyte cell-free protein synthesizing system (data not shown).

Discussion

Blot hybridization provides a sensitive and specific method for identification and quantitation of messenger RNA's [9, 13, 26, 30]. Cloned cDNA probes can be labelled to specific activities similar to those obtained when iodinating peptides for use in radioimmunoassays. Thus, cDNA hybridization is theoretically as sensitive an assay for nuclei acids as are radioimmunoassays for proteins.

The nucleic acid hybridization assay for protein hormone mRNA's provides a distinct advantage over immunoassay for documentation of hormone biosynthesis in situ because presence of the mRNA is a prerequisite. Using this method we have quantified insulin mRNA in islets, insulinoma cells adapted to culture and total pancreas. Because β -cell number and RNA content are known for rat islets [13, 31], we can estimate the β -cell insulin mRNA copy number from the data presented

here to range from 50–150,000 molecules per cell. This estimate is similar to the copy numbers of other abundant mRNA's in differentiated tissues, (e.g. casein in mouse mammary gland [27], and ovalbumin in chick oviduct [28]).

These studies demonstrate that as little as 3 pg of insulin mRNA can be detected in a RNA sample. Because no signal was detected in a 50 μg sample of poly A+ RNA from brain, there is less than 0.06 pg of insulin mRNA per μg of brain messenger RNA. Similar results were obtained for all other extrapancreatic tissues examined. This compared to 1500 pg of insulin mRNA per μg of pancreatic mRNA. Havrankova et al. [4] and Rozensweig et al. [21] have presented data to show that levels of insulin in brain are about 20 ng/gm of tissue, as compared to 40 μg /gm of pancreas. Because the level of mRNA coding for a protein is roughly proportional to the amount of protein synthesized [29, 30, 32] one would estimate insulin mRNA in brain to be 2000-fold less abundant than in pancreas. The data here demonstrate that insulin mRNA is at maximum 6000-fold less abundant than in pancreas. In the studies by Havrankova et al. [4] and Rozensweig et al. [21] cited above, levels of insulin varied considerably from one animal to the next. In the studies presented here, RNA was pooled from 6–20 animals for each analysis, decreasing the possibility that the samples analyzed came from animals with particularly low levels of extrapancreatic insulin. These data do not rule out the possibility that there are extremely stable, slowly turning over pools of insulin synthesized in extrapancreatic tissues at low rates and encoded by levels of messenger undetected in this study.

The insulin in other tissues demonstrated by immunoassay might be an insulin-like peptide, although bioassay and gel filtration of this material do not seem to support this view [5, 21]. In support of such an hypothesis, Rozensweig et al. [37] presented data to suggest that insulin in extracts of guinea pig brain was more "pork-like" than insulin from guinea pig pancreas. The recent demonstration of only one gene for insulin in the guinea pig, encoding guinea pig pancreatic insulin [23], decreases support for distinct pork insulin-like molecules synthesized in brain. Nevertheless, we have screened for the presence of a partially homologous mRNA in brain by decreasing the stringency of hybridization. It is known that when RNA-DNA hybrids vary in sequence, they dissociate at lower temperatures than strictly homologous polynucleotides [33]. Decreasing the stringency of post-hybridization washes allowed detection of mRNA for guinea pig insulin, which is only 60–80% homologous to the rat proinsulin I mRNA [23]. These results do not exclude the possibility that a less homologous mRNA encodes the insulin reported in brain. Examples of distantly related members of the insulin gene family include IGF I and II [34, 35] and pre-prorelaxin [36]. These mRNA's which are partially homologous to proinsulin mRNA encode proteins with distinctly different immunoactivity and biological activity. Because insulin mRNA is not found in extrapancreatic tissues at

levels considerably lower than those predicted by their insulin content, this suggests that the insulin found in brain and other tissues is not synthesized there, but more likely originates in pancreatic β -cells.

Acknowledgements. We would like to thank Dr. W. Chick for generously supplying us with insulin-producing rat insulinoma cells adapted to tissue culture, and Dr. P. Lomedico for supplying poly A+ RNA from rat insulinoma. Dr. P. Rotwein provided valuable advice and reviews of this manuscript. We would like to thank C. Fleischmann for excellent technical assistance and P. Rader and P. Stewart for excellent assistance in preparing the manuscript. This work was supported by National Institute of Health grant AM-16746 and the United States Veterans Administration.

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Received: 26 October 1984
and in revised form: 26 March 1985

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