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Evidence for Biosynthesis of Preproinsulin in Gut of Rat

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Glucagon and other pancreatic peptides are made in the gut, but there is little evidence for the formation of insulin. The demonstration of insulin receptors on the mucosa of gut epithelium suggests that there may be an autocrine or paracrine role for insulin made in the gut. Such insulin may control cell division, the secretion of other peptides from the same or neighboring cells, or motility and absorption. To search for the ability of the gut to make insulin, sections of freshly excised segments of rat gut were treated with an antiserum against porcine insulin. Intracellular immunoreactivity appeared in glandular cells in the stomach and colon but not in the small intestine. Preproinsulin mRNA was detected in similar cells in the stomach and colon by in situ hybridization, using specific oligonucleotide probes. Rat preproinsulin 1 and 2 mRNAs were transcribed by reverse transcriptase to the corresponding cDNAs, which were then amplified by polymerase chain reaction, utilizing specific oligonucleotide primers. Restriction analysis confirmed the identity of rat preproinsulin 1 and 2 mRNA in the colon and rat preproinsulin 1 mRNA in the stomach. Neither was found in the small intestine. Base sequences of the cDNAs were identical to the coding regions of pancreatic rat preproinsulin 1 and 2 messages. These observations are strong evidence for the synthesis of preproinsulin in the gut of the rat.

Key Words: Motility; rat gut preproinsulin; autocrine/paracrine preproinsulin; mRNA; cDNA; reverse transcriptase/polymerase chain reaction.

Introduction

Evidence for the existence of extrapancreatic insulin or insulin-like material has been documented in several tissues. Expression of rat preproinsulin 2 in fetal, postnatal, and adult brain in reverse transcription polymerase chain reaction (RT-PCR), and preproinsulin 1 and 2 expression in fetal rat liver and yolk sac has been reported *(1)*. Immunocytochemistry has demonstrated insulin/insulin-like reactivity in the fetal and neonate rat retina *(2)* and rat prostate *(3)*.

The occurrence in the gut of polypeptides of pancreatic islets, such as glucagon, somatostatin, peptide YY, etc., is also well documented *(4)*. However, there is virtually no mention of insulin in the gut *(5)*. Oral insulin, introduced directly into the gut of dogs made diabetic by total pancreatectomy, provoked a consistent, large reduction in the plasma level of glucagon-like immunoreactivity, even when increases in plasma insulin were minimal and there was little or no decrease in plasma glucose *(6)*. In these depancreatized dogs, the gut was the only source of plasma glucagon-like immunoreactivity. These observations suggest that glucagon secretion by gut cells may be suppressed by direct exposure to insulin.

Although insulin-sensitive glucose uptake in the gut may involve insulin receptors on the basolateral surfaces of the epithelial cells *(7)*, insulin receptors, apparently identical to those in the liver and other tissues, have been located on the mucosal (lumenal) side of the gut by Pillion et al. *(8)*. The receptors on the lumenal side are located to bind insulin descending from the upper gut, but it is also possible that these receptors are well placed to bind insulin produced in the same or neighboring cells for autocrine and paracrine control of gut functions.

Because of the possibility of an autocrine or paracrine effect of insulin in the gut, we instituted a search for the presence of preformed intracellular insulin or its precursors and for the mRNA for preproinsulin in the gut of the rat as evidence that the gut is capable of the biosynthesis of insulin.

Results

Immunocytochemical Detection of Insulin and Insulin Precursors (Fig. 1)

Immunoreactivity, against a porcine insulin antibody that reacts to both rat insulin and its precursors, was present in superficial cells of the colon (Fig. 1A) and in the crypts of Lieberkuhn. Positive control samples of pancreas from the

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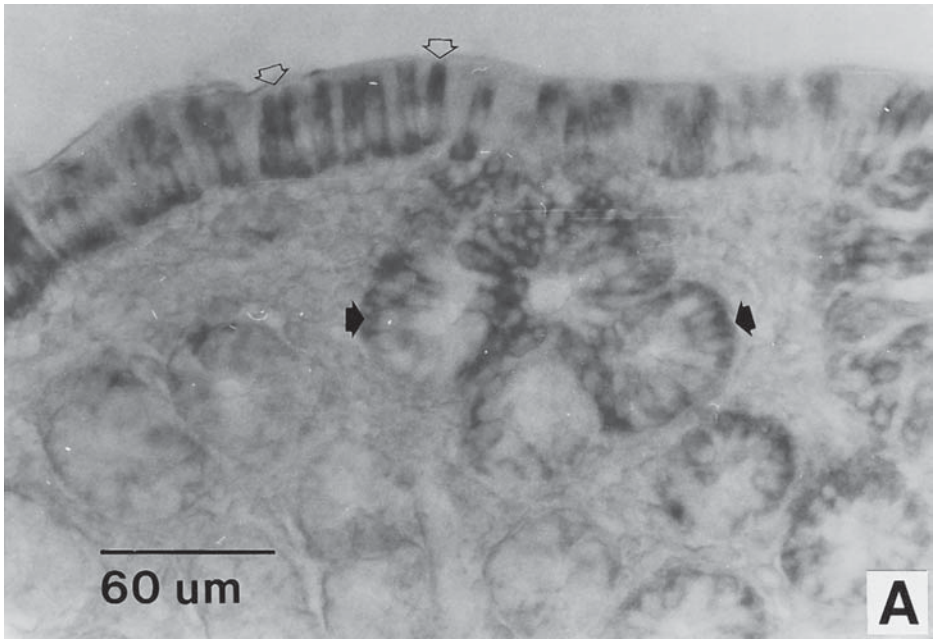

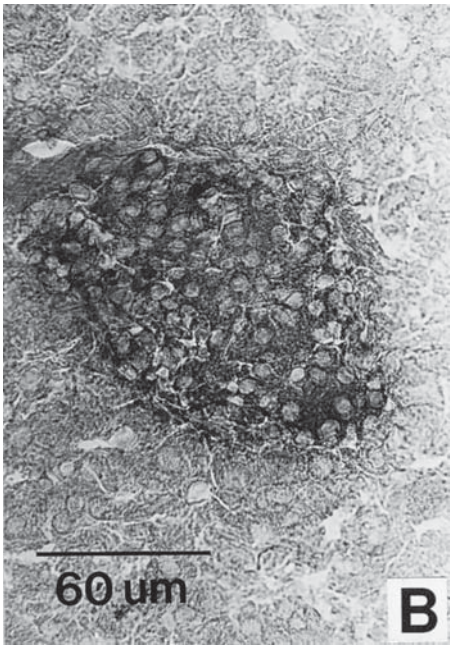

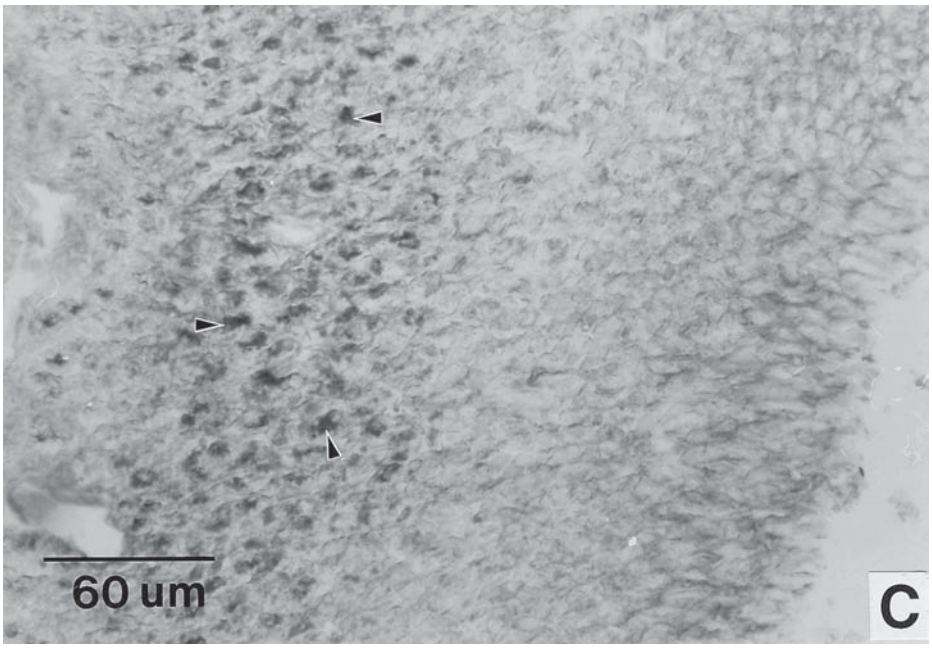

same animals exhibited specific staining of the β-cells of the islets of Langerhans (Fig. 1B). Immunoreactivity was also present in some, but not all, glandular cells of the stomach (Fig. 1C,D). There was no staining in the small intestine. The stain was confined to the cytoplasm in all cells. Negative controls, in which the primary antiserum was omitted or immunoabsorbed, showed no staining of the islets or gut tissue.

In situ Hybridization (ISH) of Preproinsulin mRNA (Fig. 2)

Hybridization with digoxigenin-labeled DNA probes directed at the mRNAs for rat preproinsulins was found in the colon in scattered epithelial cells in the crypts and in the superficial epithelium. Sections of rat pancreas, treated similarly, developed the blue-black stain product in the cytoplasm of β-cells in the islets of Langerhans (Fig. 2B). In the stomach (Fig. 2C), the distribution of stain products was similar to that in the colon. No mRNA was detected in the small intestine. The distribution of mRNA was very similar to that of immunoreactivity in the stomach and colon.

Reverse Transcription Polymerase Chain Reaction (Fig. 3)

Combined reverse transcription and PCR amplification produced cDNA products of about 223 bp from the pancreas, colon, and stomach. Electrophoresis patterns of the

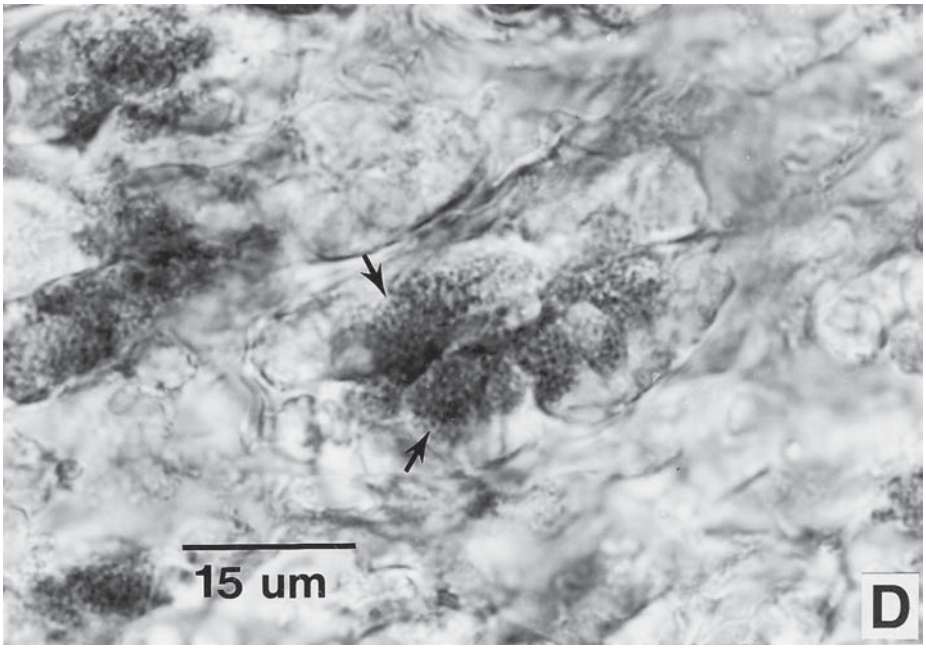

Fig. 1. Immunocytochemical staining of the colon, pancreas, and stomach of the rat. **(A)** Colon: immunoreactivity using antiporcine insulin antiserum in surface epithelial cells (open arrows) and in epithelial cells in crypts of Lieberkuhn (closed arrows) (X450); **(B)** pancreas: staining restricted to the islets (X450). **(C)** stomach: staining in groups of epithelial cells (arrowheads) in the basal region of gastric glands (original magnification X450). **(D)** stomach: higher magnification of stained cells (arrows) in the basal region of gastric glands (X2000).

products derived from the colon, stomach, and pancreas were identical (Fig. 3).

Digestion of the PCR products from rat colon, stomach, and pancreas with specific restriction enzymes resulted in identical restriction patterns for rat preproinsulin 1 and 2 in these tissues.

cDNA Sequences (Fig. 4)

The sequences of the amplified cDNAs from the colon (Fig. 4A,B) corresponded precisely with the established rat preproinsulin 1 and 2 DNA coding sequences derived from pancreatic β-cells *(9)*. The sequence of the cDNA from the stomach (not shown) corresponded with the known rat preproinsulin 1 DNA coding sequence derived from rat pancreatic β-cells.

Discussion

Our work describes evidence for the presence of immunoreactivity with an antiinsulin antibody in rat gut epithelial cells. Preproinsulin mRNA in similar cells hybridized in situ with specific synthetic oligonucleotide probes. Authentic preproinsulin mRNA was also demonstrated by the combined reverse transcription, PCR, restriction enzyme analysis, and nucleotide sequencing. The presence of insulin receptors on the mucosal surface in the gastrointestinal tract *(8)*, and the presence of the tools for making "insulin" by lumenal cells, suggests that there may be an important role for insulin in the gut that requires its local synthesis.

In view of the presence of insulin receptors on the mucosal surface of the gut, it is possible that insulin might be manufactured in gut cells facing the lumen to communicate with the insulin receptors as autocrine or paracrine couples. These may operate in the control of cell division, the development of local environments conducive to neoplastic transformation, the secretion of peptides from the same or neighboring cells, absorption, or gut motility. Our observation that insulin precursors and the mRNAs needed for their manufacture are indeed present in cells of the stomach and colon facing the lumen provides evidence for a possible role of insulin of enteral origin in the control of gut motility.

A common complication of diabetes mellitus is gastroparesis, which is widely considered to be a result of a defect in autonomic control of gastric emptying (10). Eliasson et al. *(11)* observed that high parenteral doses of insulin in healthy human volunteers reduced gut motility and slowed carbohydrate absorption. In these experiments neuropathy was not a factor. The insulin in these subjects reached the gut from the blood. Similar inhibition of gut motility was observed in the rat when an insulin solution was substituted for drinking water *(12)*. Some of these rats died. On autopsy, the stomach was engorged with undigested food. Gut motility was apparently inhibited. In this case, the insulin was delivered directly to the gastric mucosa.

A possible role for gastric insulin would be the detection of glucose from a meal, either preformed or resulting from salivary amylase, to control the process of peristaltic move-

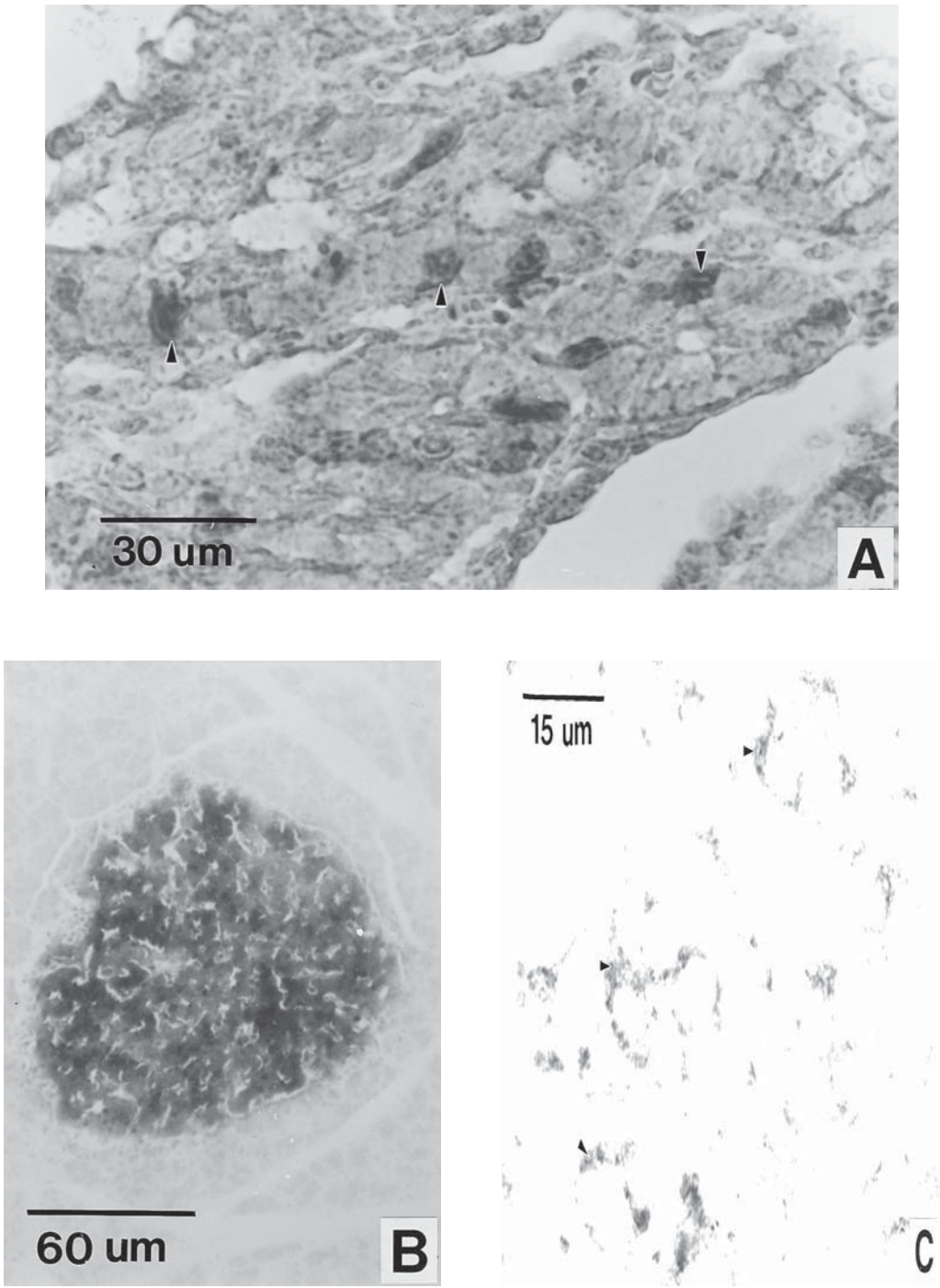

Fig. 2. *In situ* hybridization (ISH) with digoxigenin-labeled preproinsulin-specific probes. **(A)** Colon: stain product in epithelial cells in the crypts of Lieberkuhn (arrowheads) (X900); **(B)** pancreas: stain product is specifically localized in cells of the islet of Langerhans (X450); **(C)** stomach: stain product in basal epithelial cells (arrowheads) of the gastric glands (X2000).

ment of the oncoming food from the stomach to the small intestine. In the colon, insulin secretion may be triggered by unabsorbed glucose in the digestive residue, leading to a slowing of peristalsis to provide sufficient time for complete absorption of digesta. Insulin would join splanchnic somatostatin to regulate the absorption and distribution of nutrients *(13)*. A response of the pancreas to nutrients in the gut lumen involves the release of a number of peptide hormones from the gut, which in turn stimulate the release of pancreatic hormones, particularly insulin, in anticipation of an increase in the absorption of glucose and amino acids. Insulin introduced into the colon appears to regulate glucagon release from the gastrointestinal tract *(6)* and could, in turn, regulate pancreatic glucagon secretion. Insulin may therefore be considered to be one of the battery of gut peptides (e.g., glucagon, gastric inhibitory peptide, cholecys-

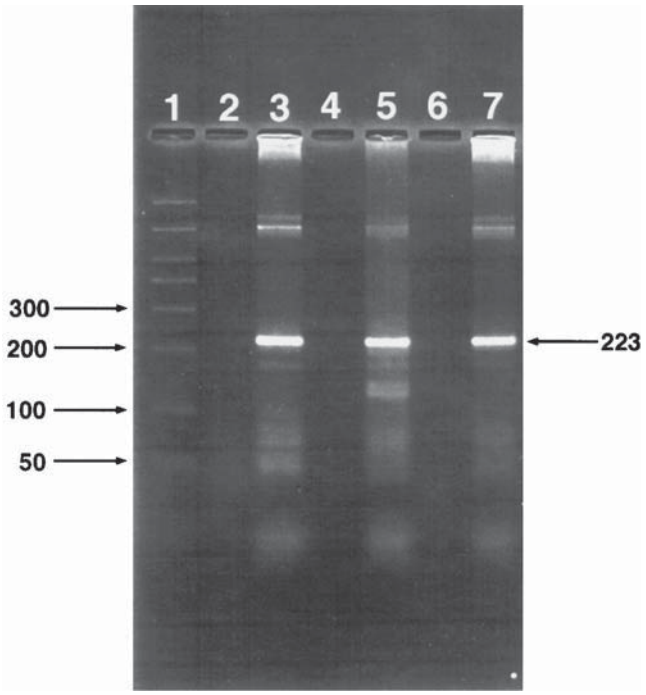

Fig. 3. Electrophoresis of approx 223-bp coding region of preproinsulin cDNA prepared by RT-PCR. Ethidium bromide-stained (3% NuSieve GTG/1% agarose) gels of PCR products are shown. Lane 1, standard DNA ladder; lane 2, blank; lane 3, pancreas; lane 4, blank; lane 5, colon; lane 6, blank; lane 7, stomach. Measured size of amplified cDNA is indicated.

tokinin, secretin, gastrin, and vasoactive intestinal peptide) that coordinate and regulate the function of the gut and secretion by pancreatic islet cells.

Materials and Methods

Rat Tissues

Male and female Sprague Dawley rats were anesthetized with pentobarbital and the viscera were exposed. The pancreas, stomach, small intestine, and colon were removed. The lumenal contents were gently expressed, the tissue was briefly rinsed in deionized water, and the tissue samples were divided into two sets. One set of samples of pancreas and gut was fixed in buffered formalin (pH 7.0) overnight, dehydrated in increasing concentrations of ethanol in water treated with diethylpyrocarbonate (DEPC) to eliminate RNase, further dehydrated in absolute ethanol, cleared in xylene, and embedded in Paraplast. Sections cut from the embedded tissues were used for immunocytochemistry or for ISH histochemistry (*see* below). The other tissue samples were quickly frozen individually on solid dry ice and either used immediately for the extraction of RNA or stored at –70°C.

Preparation of RNA

The frozen tissues were ground to a fine powder in a cooled mortar at –56°C and were immediately transferred

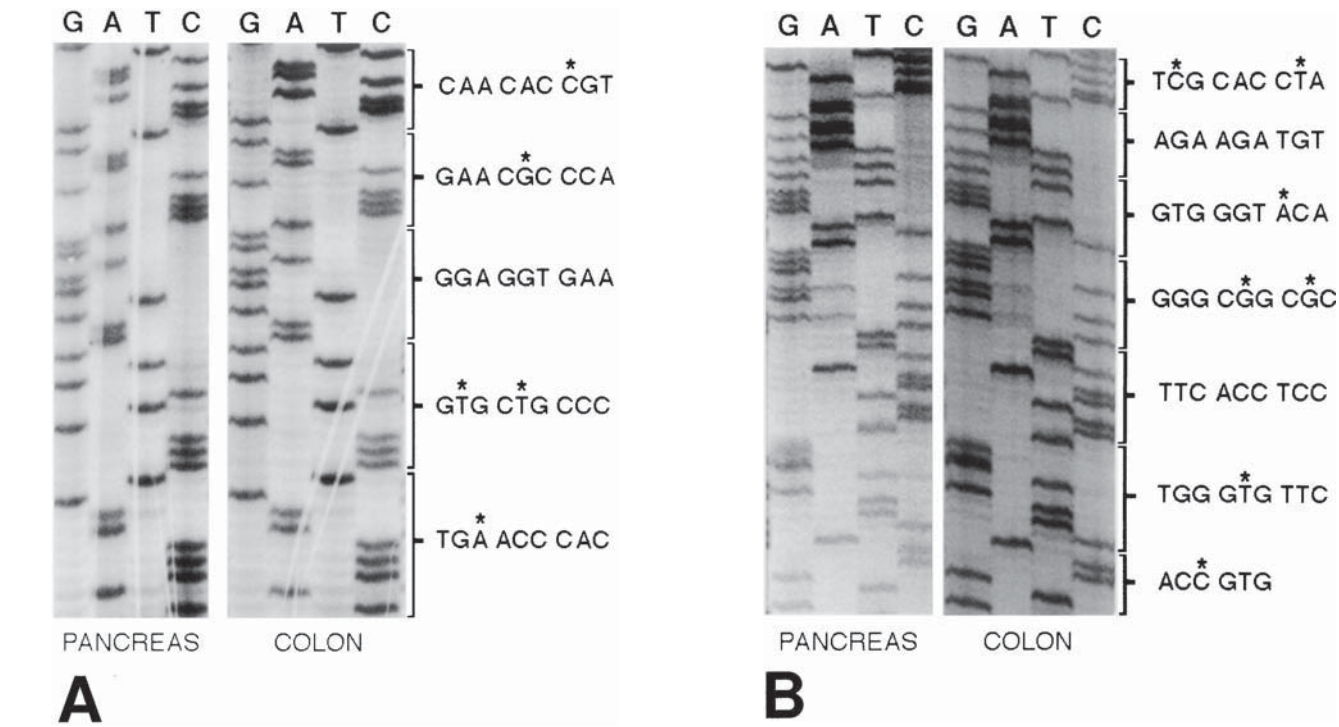

Fig. 4. Partial sequences of rat preproinsulins. (A) Preproinsulin 1 from pancreas and colon. The corresponding amino acid sequence, with the reading frame starting at the second pictured nucleotide, is QPVQPDEVERRSKP. (RATINS1, Genbank database, accession no. J00747). (B) Preproinsulin 2 from pancreas and colon. The corresponding amino acid sequence, with the reading frame starting at the third pictured nucleotide, is RGFFYTPMSRREVEDPQVA. (RATINS2, Genbank database, accession no. J00748). Asterisks indicate the nucleotides that are specific for each preproinsulin sequence.

to a tube containing lysis solution with ribonuclease inhibitors (TRIzol Reagent; Life Technologies, or Micro Fastrack Procedure, Invitrogen). For the extraction of total RNA, the pulverized tissue/TRIzol mixture was homogenized by drawing through a 21-gauge needle, incubated for 5 min, and the phases separated by the addition of chloroform. Total RNA was precipitated from the upper aqueous phase with isopropanol, centrifuged, washed with 75% ethanol, dried under vacuum, and redissolved in RNase-free water.

Reverse Transcription

The total RNA was reverse transcribed with Moloney Murine Leukemia Virus RT (GeneAmp RNA PCR Kit; Perkin-Elmer Cetus) using a specific 20-base oligonucleotide complementary to the 3'-downstream end of the rat preproinsulin 2 DNA coding sequence (5'-A GCA CTG ATC CAC GAT GCC G), amino acid sequence (CQDVIG).

Polymerase Chain Reaction

The cDNA products were amplified through 35 PCR cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) using recombinant *Taq* DNA polymerase (Perkin-Elmer Cetus) and a 25-base oligonucleotide primer specific for the 5'-upstream end of the rat preproinsulin 2 coding sequence (5'-GCC CAG GCT TTT GTC AAA CAG CAC C), amino acid sequence (AQAFVKQH), and the specific aforementioned downstream primer. The primers (prepared in the laboratory of Ming You M.D., Ph.D., Department of Pathology, Medical College of Ohio) were derived from the rat preproinsulin 2 sequence (RATINS2, Genbank database, accession no. J00748; National Center for Biotechnology Information, National Library of Medicine) using the Primer Detective software package (Clontech Labs). The specific upstream primer includes 25 nucleotides corresponding to bases 1178–1202, and the specific downstream primer includes 20 nucleotides corresponding to bases 1880–1899 in the coding region for the known, highly conserved rat preproinsulin 2 DNA sequence. This primer set traverses an intron (intron B in the rat preproinsulin 2 gene, corresponding to nucleotides 1302–1800, 498 bp in length) in the rat preproinsulin 2 gene; however, there is no comparable intron in the gene for rat preproinsulin 1.

Contamination of preproinsulin genomic DNA was eliminated by treatment of aliquots of total RNA with RNase-free DNase (Ambion) prior to reverse transcription and PCR. No amplification occurred when the treated aliquots of total RNA were subjected to 35 cycles of PCR without prior reverse transcription. Neither did amplification occur when reverse-transcribed products were subjected to 35 cycles of PCR in the absence of DNA polymerase.

Isolation of Individual PCR Products

The oligonucleotide primers were designed to amplify both the rat preproinsulin 1 and 2 mRNAs. Both the upstream and downstream primers are 100% homologous to the rat preproinsulin 2 sequence. The upstream primer is 100% homologous and the downstream primer is 90% homologous to the rat preproinsulin 1 sequence. In tissues expressing both preproinsulin 1 and 2, the initial amplification contained a mixture of rat 1 and 2 cDNA products. Aliquots were treated with specific restriction enzymes to remove either one or the other cDNA product from the mixture and then secondarily amplified through another 35 cycles to yield an enriched PCR product of only a single cDNA sequence.

Restriction Analysis

The PCR products were separated by electrophoresis in 3% Nusieve/1% agarose (FMC) and visualized under ultraviolet light after staining with ethidium bromide. The molecular size of the cDNA was estimated by comparison with known standards. The cDNA was excised from the gel and purified on a silica matrix (Prepagene; Bio-Rad). Care was taken to eliminate the possibility of cross-contamination between preparations by isolating tissues and RNA in separate laboratory areas, using sterile gloves to handle all samples, using sterile disposable plasticware, treating all water used with DEPC, and using meticulous care with instruments and reagents *(9)*. Restriction analysis was performed by digesting aliquots of the purified cDNA with the restriction enzymes *Rsa*I (specific for rat preproinsulin 1), *Bst*EII, and *Hin*fI (both specific for rat preproinsulin 2), incubated with appropriate React buffers (BRL) for 1 to 2 h at 37°C. The digests were separated by electrophoresis on 4% analytical agarose gels (3% NuSieve/1% agarose), that were poststained with ethidium bromide (1 μg/mL in water) for 20–50 min.

Base Sequences

Aliquots of the purified cDNA were directly sequenced according to a modification of the Sanger et al. method *(14)*, incorporating ^{35}S and using the Sequenase PCR Product Sequencing Kit (U.S. Biochemical). Both the 3'- and 5'-oligonucleotide primers used for PCR amplification were employed as separate primers for double-stranded sequencing in both directions. Electrophoresis was carried out in 8% polyacrylamide/8 *M* urea at approx 2000 V, 60 W. The sequencing gels were dried under vacuum at 65°C and were autoradiographed with Biomax film (Kodak) for 24–72 h. The sequences were read and compared with published sequences *(15,16)* derived from cDNA prepared from rat pancreatic tissue and sequences recorded in the Genbank database.

Immunocytochemistry and ISH

Sections, approx 5–7 μm thick, from the Paraplast-embedded tissues were mounted onto polylysine-coated, DEPC-washed slides. Some of these sections were stained immunocytochemically, using a polyclonal antiserum to porcine insulin (Dako), which crossreacts with

rat insulin and 30% of rat insulin precursors, as described elsewhere *(2)*.

Other sections were used to localize preproinsulin mRNA *in situ*, using a cocktail of six equimolar 30-base exon-specific, digoxigenin-labeled oligonucleotide probes (British Biotechnology, England, available through R & D Systems, Minneapolis, MN). The decerated sections were rinsed twice for 10 min each in absolute ethanol, air-dried, washed twice in DEPC-treated water, and incubated with 5 μg/mL of proteinase K (30 min at 37°C). After washing in DEPC-treated water for 5 min, the sections were postfixed in 0.4% paraformaldehyde for 10 min at room temperature and washed again twice in DEPC-treated water for 5 min. The sections were then dipped in 0.3% Triton X-100 in DEPC-treated water and finally washed twice in DEPC-treated water for 5 min.

The slides were prepared for hybridization by applying a prehybridization solution and incubated for 1 h in a humidified chamber at 37°C. (5 mL of deionized formamide, 2 mL of 20X saline sodium citrate [SSC], 0.2 mL of 50X Denhardt's solution, 0.5 mL of herring sperm DNA, heat denatured [10 mg/mL], 0.25 mL of yeast tRNA [10 mg/mL], and 2.0 mL of Dextran sulfate [50% in water])

The mRNA in the tissue was hybridized by the application of a hybridization solution (200 ng of digoxigenin-labeled probe/mL of prehybridization solution) at 37°C for 18 h. The slides were then washed sequentially with several changes of 4X SSC (0.6 *M* NaCl, 0.06 *M* sodium citrate, pH 7.0), 2X SSC, 0.2X SSC, and DEPC-treated water. The slides were washed for 1 min in a 100 m*M* Tris buffer (pH 7.5) containing 150 m*M* NaCl and then incubated in the same buffer solution containing 2% normal sheep serum in 0.3% Triton X-100 in Tris-HCl/NaCl buffer (30 min), followed by incubation in 1:200 to 1:500 sheep antidigoxigenin antibody tagged with alkaline phosphatase for 1 h. After washing with Tris buffer (pH 7.5) for 10 min and with Tris buffer (pH 9.5) for another 10 min, the slides were stained in a solution of nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) containing a phosphatase inhibitor for 18–72 h. The staining was stopped by washing in a solution of 10 m*M* Tris and 1 m*M* EDTA (pH 8.0), and the slides were dehydrated in increasing concentrations of ethanol in water, followed by two changes of absolute ethanol, rinsed in xylene, mounted in Permount, and examined microscopically.

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