Preproinsulin mRNA in the Rat Eye

G. Colin Budd,* Ben Pansky,† and Lou Glatzer‡

Purpose. The goal of this study was to extend the results of previous immunoassay, immunocytochemistry, and in situ hybridization studies showing the presence of insulin-related peptide in the rat retina by confirming the expression of insulin genes in the rat eye.

Methods. Total and poly(A)+ RNA were isolated from whole rat eyes, and separately from the retina, choroid, iris, lens, and vitreous. The poly(A)+ RNA was used for preparation of insulin-specific cDNA according to a coupled reverse transcription polymerase chain reaction (RT-PCR) protocol under high stringency conditions. Southern transfers, restriction fragment analyses, and nucleotide sequencing were used to characterize and identify the amplified cDNA products.

Results. Amplified cDNA fragments of 329 ± 6 base pairs (bp) were derived from whole rat eye and rat retina poly(A)+ RNA, but not from other regions of the eye. Southern blots probed with preproinsulin-specific primer demonstrated homology with similar-sized cDNA from rat pancreas. Restriction digests with 10 restriction enzymes and direct nucleotide sequencing confirmed that the 329-bp cDNA was identical to the previously known coding sequence for rat pancreatic preproinsulin, DNA.

Conclusions. The identification of retinal preproinsulin, mRNA was confirmed. This correlates with previous studies showing insulin immunoreactivity in rat eyes and in cultured retina, and verifies in situ hybridization evidence for the presence of insulin-related mRNA in retinal glial cells. Invest Ophthalmol Vis Sci 1993; 34:463-469.

Although other peptide products of the pancreatic islets are found in the brain and regions of the gastrointestinal tract, insulin traditionally has been considered to be a unique product of the vertebrate pancreatic islets of Langerhans. In previous studies using immunoassay,1 immunocytochemistry,2,3 and in situ hybridization,4,5 we have demonstrated insulin immunoreactivity in the brain, pituitary, retina, and retinoblastoma cells, and the presence of cell-specific expression of cytoplasmic insulin-related mRNA in cultured retinal glial cells, anterior pituitary cells, and in Y-79 human retinoblastoma cells. In the current studies, total RNA from whole rat eyes and poly(A)+ RNA from specific parts of the rat eye were investigated for the presence of preproinsulin mRNA using the combined techniques of reverse transcription and polymerase chain reaction amplification (RT-PCR) of specific cDNA using preproinsulin-specific oligonucleotide primers under conditions of high stringency. A 329-base pair (bp) DNA was amplified from whole eye total RNA and retinal mRNA that comigrated on electrophoresis with a cDNA amplified by the same RT-PCR methods from rat pancreas total RNA. Restriction enzyme digestion and nucleotide sequencing demonstrated that the DNA fragments derived from whole eyes and retina corresponded precisely to the coding sequence of rat pancreatic preproinsulin, DNA.
MATERIALS AND METHODS

Total RNA was isolated, free of DNA and protein, from 16 pairs of 2-year-old Sprague-Dawley rat eyes and separately from rat pancreas by the quanidinium isothiocyanate–CsCl method. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Poly(A) RNA was then purified directly from the total RNA on oligo (dt) cellulose according to the Polyattract and Ribosep mRNA isolation procedures. Alternatively, poly(A) RNA was purified individually from whole eyes, retina, choroid, iris, lens, and vitreous using a micro method (Micro Fastrack, Invitrogen, Inc., San Diego, CA). Aliquots (1–10 μg) of the poly(A)+ RNA were reverse-transcribed and then insulin-specific cDNA was generated according to the coupled RT-PCR procedure of Rappolee et al. The oligonucleotide primers (5'-GTT GAC CTC TTG ATG ACG TTG and 5'-ATG GCC CTG TGG ATG CGC TTC) corresponding to 21 bp at each end of the coding region equivalent to bases 4182 to 4202 and 4491 to 4511 in the rat preproinsulin, sequence (RATINSI, Genebank database tape release version 73.1; NCBI, Bethesda, MD), were specific for the known, highly conserved, rat preproinsulin, DNA sequence. PCR amplification was carried out with Ampli-Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) for 25 to 40 cycles in an Eppendorf programmable thermocycler under conditions of high stringency to eliminate nonspecific annealing of primer to template. PCR products were PCR’d in the absence of AmpliTaq DNA polymerase. Restriction analysis was performed using standard protocols, the appropriate unique buffer (Promega, Inc., Madison, WI) or with React buffers (BRL, Inc.) for 2 hr at 37°C. Enzymes used were: BamHI, EcoRI, HindII, HindIII, PstI, XhoI, HinfI, HpaII, HaeIII, and AluI. Four percent analytical agarose gels (3% NuSieve/1% agarose; FMC, Inc.) were used and typically run for 4400 volt-minutes in a minigel apparatus. They were prestained with ethidium bromide at 0.5 μg/ml agarose or poststained at 1 μg/ml in water for 20 to 30 min. Estimations of fragment sizes were carried out with a linear regression program and distances were measured relative to the size standards. Estimates were made with three points above and below the experimental point and no standards were used with a regression coefficient less than 0.995.

In all procedures, special care was taken to eliminate the possibility of cross-contamination between preparations, including isolation of tissues and poly(A)+ RNA in separate areas, sterile gloved handling of all samples, and meticulous care in the use of instruments and reagents.

RESULTS

The application of RT-PCR to poly(A)+ RNA derived separately from whole rat eyes and from rat retina produced amplified cDNA fragments with an estimated size of 329 ± 6 bp (Fig. 1A). No comparable fragments were obtained from our sampling of other regions of the eye (ie, choroid, iris, lens, and vitreous). Negative control studies, eliminating either the template or the Ampli-Taq DNA polymerase, showed no
329 ± 6 bp bands after PCR amplification. Amplified cDNA fragments with an estimated size of 329 ± 6 bp also were visualized when the pancreas was used as the source of poly(A)+ RNA (Fig. 1A). Southern blots probed with 32P-end-labeled 3'-primer are shown in Figure 1B. Both pancreas and eye products demonstrate rat preproinsulin homologous bands at the same position, just above the 310-bp marker. These results indicate that the products obtained from the eye are similar, if not identical to the pancreas-derived rat preproinsulin cDNA.

The identities of the rat eye and rat pancreas PCR products were investigated further by comparing the restriction patterns of the cDNAs amplified from both tissues. Restriction enzymes were used that should cut rat preproinsulin cDNA once, twice, or not at all (Fig. 2). Although a total of 10 restriction enzymes was used, the results of restriction endonuclease cleavage with 6 of the enzymes are shown in Figure 2. Lanes 1–3 and 8–10 are digests of the rat eye RT-PCR product. Lanes 5–7 and 12–14 are digests of the rat pancreas RT-PCR product. Lane 4 is a 1:1 mixture of uncut (no enzyme), purified 329-bp PCR product derived from both rat eye and rat pancreas RNA extracts used as a marker. Lane 11 is the φX174/Hae III standard marker. Lanes 1–7 represent the uncut products with nonrestricting enzymes (excluding lane 4, which has no enzymes present). Lanes 8–10 and 12–14 are cleaving restriction endonucleases. It should be noted that two of the enzymes, HinfI and AluI, are able to distinguish between preproinsulin 1 cDNA and preproinsulin2 cDNA. The close correspondence of the observed fragment sizes to the expected values for rat preproinsulin 1 cDNA underlines the identity of the product.

As expected for rat preproinsulin 1 (or preproinsulin 2) cDNA, the noncutting enzymes BamHI, EcoRI, HincII, HindIII, PstI, XhoI, and HinfI did not cleave the 329-bp linear band from either the pancreatic or rat eye products. HaeIII was predicted to generate four fragments, two of which (113 and 178 bp) would be expected to be visible in our gels. Lanes 9 and 13 (Fig. 2) show the observed fragments, estimated to be 115 and 182 bp. HpaII also produced four fragments whose visible bands should be 78 and 213 bp, respectively. The observed fragment (lanes 8, 14) was 218 bp. AluI was predicted to cleave rat preproinsulin 1 cDNA into two fragments of 130 and 200 bp. The actual
FIGURE 2. Agarose gel demonstrating the results of restriction enzyme digestions of rat eye and rat pancreas PCR products. Lanes 1–3 and 8–10 demonstrate digests of rat eye amplified product. Lanes 5–7 and 12–14 show digests of rat pancreas amplified product. Nocutting enzymes are shown in lanes 1 and 7 (EcoRI), lanes 2 and 6 (XhoI), and lanes 3 and 5 (HinfI). The enzymes that cut are shown in lanes 8 and 14 (HpaII, 218-bp fragment), lanes 9 and 13 (HaeIII, 182- and 115-bp fragments) and lanes 10 and 12 (AluI, 200- and 130-bp fragments). Lane 4 contains a 1:1 mixture of undigested pancreas and eye amplified products. Lane 11 contains phiX174/HaeIII standards.

observed fragments (lanes 10,12) were 130 and 200 bp. If the material were preproinsulin 2 cDNA, three fragments of 89, 110, and 130 bp would have been expected with AluI. The differentiation between preproinsulin1 cDNA and preproinsulin 2 cDNA is further confirmed by the failure of HinfI to cut the eye and pancreas products. HinfI normally cuts preproinsulin 1 cDNA into two fragments of 140 and 190 bp.

A Southern transfer from a gel containing the RT-PCR-amplified products from both rat eye and rat pancreas was probed with 32P-end-labeled 5'-primer and a strong annealed signal was seen at the 329-bp position of both the rat eye and rat pancreas products (Figs. 1A, B). The nucleotide sequences of purified cDNA obtained separately from whole rat eyes and rat retina were determined. The sequences were found to be identical to the previously known coding sequence of rat pancreatic preproinsulin1 DNA15 and to the sequence of the 329-bp amplified DNA that we generated after application of RT-PCR to poly(A)+ RNA isolated from rat pancreatic tissue. The sequences were identical in all respects, including the sequence encoding the insulin1 signal peptide, B-chain, C-peptide, and A-chain (Fig. 3).

DISCUSSION

The purified 329-bp cDNAs separately obtained from the whole eye and the rat retina were found by gel electrophoresis, Southern blotting, and restriction enzyme digestion to resemble the rat preproinsulin1 coding sequence obtained from rat pancreas RT-PCR products.

The identification of retinal preproinsulin, mRNA was confirmed by the direct sequencing of rat preproinsulin, cDNA in the reverse transcription products from total rat eye RNA and rat retina poly(A)+ RNA. This appears to be the first evidence for the expression of an insulin mRNA in normal mammalian eye tissue. When nonreverse transcribed total RNA was subjected to PCR amplification with the preproinsulin-specific primers, no detectable contamination with genomic preproinsulin DNA was observed. The sequenced rat eye cDNA product was identical to the preproinsulin1 coding sequence obtained by RT-PCR of rat pancreas poly(A)+ RNA and also identical to the published sequence.15

These findings correlate with our previous studies demonstrating immunoreactivity to insulin antisera in
FIGURE 3. Nucleotide sequence of the 329-bp amplified DNA produced by combined reverse transcription and PCR from poly(A)+ RNA purified from whole rat eye total RNA.

tissue slices of rat eye and retina and immunoreactivity to insulin, and the glial cell markers S-100 protein and glial fibrillary acidic protein, in retinal Müller cells in primary culture.\textsuperscript{2,3,16} Correlations can also be made with our previous in situ hybridization experiments using labeled rat preproinsulin cDNA probes to locate insulin-related mRNA in the cytoplasm of retinal glial cells cultured in serum- and insulin-free media.\textsuperscript{3,4}

The evidence that cells in the eye can synthesize preproinsulin, mRNA raises the possibility that insulin may have a local physiologic role in extrapancreatic tissues, being involved in intracellular (autocrine) and intercellular (paracrine) signaling. Also, locally produced insulin could serve as a growth factor, a neurotrophic factor, in organelle organization, in protein storage, in modulation of specific metabolic functions, including monoamine metabolism, or as a neuromodulator.

It is known that insulin stimulates the synaptic uptake of neurotransmitter amino acids in the adult rat brain and thus may be important as a neuromodulator\textsuperscript{17} or neurotransmitter,\textsuperscript{18} and that insulin has growth factor-like properties in the brain that are mediated through phosphorylation.\textsuperscript{19} The importance of insulin in glucose or amino acid metabolism in neural tissue, however, remains uncertain.\textsuperscript{20-22} The known neurotrophic effects of insulin (as well as nerve growth factor and insulin-like growth factor-I)\textsuperscript{23-24} suggests the possibility that insulin may interact with the cholinergic system and other systems in the brain and other tissues. Insulin has been shown to affect brain monoamine metabolism,\textsuperscript{25} dopamine release,\textsuperscript{26} and cerebroside synthesis,\textsuperscript{27} and can regulate membrane transport and influence enzyme activities as well as DNA and RNA synthesis.\textsuperscript{28} Treatment of neurons in culture with a depolarizing solution has been shown to induce a threefold stimulation of insulin release into the culture medium.\textsuperscript{29} Thus, locally produced insulin may be important for the growth and maintenance of neural tissue\textsuperscript{16,30} or in the control of neural and periph-
eral carbohydrate\footnote{31,32} and catecholamine metabolism.\footnote{33} Because Müller cells contain glycolytic enzymes and can synthesize and store glycogen,\footnote{34} it has been suggested that locally produced insulin could play a role in glucose or amino acid metabolism in the retina. Furthermore, the appearance of insulin in the developing retina and other areas of the developing eye before the initiation of pancreatic insulin synthesis\footnote{35,36} strongly suggests a significant role for insulin as a growth or trophic factor in the developing eye. These findings are strengthened by evidence showing that insulin-related molecules arose early in evolution and that such molecules can stimulate sugar uptake, lipid mobilization, and growth stimulation in nervous tissue and other tissues of invertebrates and vertebrates.\footnote{37}

Overall, much remains to be known about the control and importance of insulin synthesis occurring in the eye and other nonpancreatic tissues. Further work is in progress to determine the quantity of mRNA being produced in the eye, the identification of the specific localization and specific cells that are involved in its production, and the developmental time course leading to the appearance of insulin mRNA and insulin immunoreactivity in the fetal eye.

\textbf{Key Words}

cDNA, mRNA, polymerase chain reaction, preproinsulin synthesis, retina.

\textbf{Acknowledgments}

The authors thank Angela Lineen and Donald Zhou for expert technical assistance in the preparation of RNA samples and the amplification of some of the specific cDNA fragments.

\textbf{References}


Preproinsulin mRNA in the Rat Eye


