

Overexpression of Parathyroid Hormone-related Protein in the Pancreatic Islets of Transgenic Mice Causes Islet Hyperplasia, Hyperinsulinemia, and Hypoglycemia*

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Parathyroid hormone-related protein (PTHrP) is produced by the pancreatic islet. It also has receptors on islet cells, suggesting that it may serve a paracrine or autocrine role within the islet. We have developed transgenic mice, which overexpress PTHrP in the islet through the use of the rat insulin II promoter (RIP). Glucose homeostasis in these mice is markedly abnormal; RIP-PTHrP mice are hypoglycemic in the postprandial and fasting states and display inappropriate hyperinsulinemia. At the end of a 24-hour fast, blood glucose values are 49 mg/dl in RIP-PTHrP mice, as compared to 77 mg/dl in normal littermates; insulin concentrations at this time are 6.3 and 3.9 ng/ml, respectively. Islet perfusion studies failed to demonstrate abnormalities in insulin secretion. In contrast, quantitative islet histomorphometry demonstrates that the total islet number and total islet mass are 2-fold higher in RIP-PTHrP mice than in their normal littermates.

PTHrP very likely plays a normal physiologic role within the pancreatic islet. This role is most likely paracrine or autocrine. PTHrP appears to regulate insulin secretion either directly or indirectly, through developmental or growth effects on islet mass. PTHrP may have a role as an agent that enhances islet mass and/or enhances insulin secretion.

Parathyroid hormone-related protein (PTHrP)¹ was initially discovered in 1987 through its causative role in the most common of the hypercalcemic paraneoplastic syndromes, humoral hypercalcemia of malignancy (1–3). It is now widely appreciated that PTHrP is the product of a gene, which is expressed not only in a broad spectrum of human and animal cancers, but in almost every normal human and rodent tissue in which its expression has been sought (3, 4, 6). Oversecretion of PTHrP into the systemic circulation by cancers (including carcinomas of the pancreatic islet; Ref. 7–9) leads to hypercalcemia in a

classical endocrine fashion through binding of PTHrP to parathyroid hormone receptors in bone and kidney (1–6, 10). In contrast, there is growing consensus that the normal physiologic roles of PTHrP are most often paracrine or autocrine (1–6), or perhaps even “intracrine” as suggested by Kaiser, Karaplis, and colleagues (11, 12). The study of the normal physiologic roles of PTHrP is in its infancy. These roles have been reviewed recently (3–6, 13) and appear to include: 1) regulation of transepithelial calcium fluxes in the nephron, in the breast, in the placenta, and in the distal oviduct or shell gland of the hen; 2) regulation of uterine, vascular, gastrointestinal, and urinary bladder smooth muscle tone; 3) regulation of growth and differentiation in a broad variety of cell types and organs including the keratinocyte, the chondrocyte, the osteoblast, the cells of the proximal nephron, the breast, and many others. Importantly in the current context, PTHrP is a key developmental factor. This is apparent from the fact that in each of the transgenic mouse models of PTHrP overexpression or of disruption of the PTHrP gene or its receptor, dramatic developmental abnormalities occur: targeted overexpression of PTHrP in the breast leads to striking mammary ductular hypoplasia (14), and overexpression of PTHrP in the epidermal keratinocyte leads to failure of hair follicle development (15). Disruption of the PTHrP gene or its receptor leads to severe and lethal skeletal developmental abnormalities (16, 17).

One of the tissues that produces PTHrP under normal circumstances is the pancreatic islet. Drucker, Goltzman, and colleagues have shown that PTHrP can be identified in normal rat and human pancreatic islets; that PTHrP colocalizes immunohistochemically with insulin, glucagon, somatostatin, and pancreatic polypeptide in β , α , δ , and PP cells within the islet; and that mRNA encoding PTHrP is present in isolated rat pancreatic islets (7). PTHrP is also produced by islet cell adenomas and carcinomas (7–9). We have confirmed that PTHrP is present immunohistochemically in the rat islet (18) and, as shown below, the mouse islet. We have shown that cultured rat insulinoma cells of the line m5F display cytosolic calcium responses to doses of PTHrP within the physiologic range, and that these responses are mediated by receptors distinct from the classical PTH receptor (18). The observations: (a) that PTHrP is normally produced in the pancreatic β cell, (b) that pancreatic β cells contain receptors for PTHrP, and (c) that under all normal circumstances studied to date, PTHrP plays local paracrine or autocrine roles, together suggested that PTHrP could play a normal regulatory or developmental role within the pancreatic islet.

In order to begin to define a possible normal physiologic role

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¹ The abbreviations used are: PTHrP, parathyroid hormone-related protein; RIP, rat insulin II promoter; bp, base pair(s); RIA, radioimmunoassay.

for PTHrP in the pancreatic islet, we have prepared two lines of transgenic mice in which PTHrP has been targeted to, and overexpressed in, the pancreatic islet using the rat insulin II promoter (RIP). These RIP-PTHrP mice display a syndrome that includes islet cell hyperplasia, hyperinsulinemia, and hypoglycemia.

MATERIALS AND METHODS

Construction of Transgene and Generation of Transgenic Mice—The RIP-PTHrP transgene was constructed by placing a 650-bp segment of the promoter region of the rat insulin II gene truncated 30 bp upstream of the initiation codon on exon 1 (19, 20) (generously provided by Dr. Richard Flavell, Yale University) upstream of a 568-bp *EcoRI*-*StyI* cDNA fragment of the human PTHrP(1–141) cDNA. This sequence contains the entire pre-pro and coding region sequences of the PTHrP(1–141) isoform but only 20 bp of 3' untranslated sequence (15). Thus, the 3' AU-rich instability sequences normally present were deleted from this construct in an effort to enhance mRNA stability and thereby enhance the level of expression of the transgene. Instead, at the 3' end of the transgene, human growth hormone sequences containing transcription termination, polyadenylation, and splicing signals were added as we have described previously (15). The resulting 3.4-kilobase pair transgene was isolated, purified, and microinjected into the pronuclei of (C57Bl6 × SJL)F2 mouse ova in the Yale University Transgenic Mouse Core Facility to generate founder transgenic mice. Transgenic mice were identified by Southern blot analysis of *BglI*-digested genomic DNA made from the tails of mice. The 568-bp hPTHrP cDNA fragment was used as probe.

Using these procedures, 26 founder generation mice were obtained from seven mothers. Of these 26 animals, seven were transgenic. Three markedly dwarfed founders containing the highest copy number of the transgene died shortly after birth, and two others were determined to be mosaics in that they failed to transmit the transgene to their progeny. Two true-breeding lines were generated from the two remaining founders, animals 1799 and 1807. These were outbred onto a Sencar background. The studies described herein were performed on animals derived from the 1799 and 1807 lines. The two lines were maintained separately, but because of their similar biochemical phenotypes, data from the two lines have been pooled except where indicated below. All of the studies described in this manuscript were performed on animals between the ages of 5 and 12 weeks, unless specifically indicated. All procedures were approved by the Yale University Animal Care and Use Committee and the West Haven VA Medical Center Animal Studies Committee.

RNA Analysis—Pancreas, heart, lung, liver, stomach, intestine, spleen, kidney, testis, ovary, skin, pituitary, and brain from control and transgenic animals as described under "Results" were harvested and immediately frozen in liquid nitrogen. Total RNA was prepared using a modification of the guanidinium thiocyanate-cesium chloride method (21) or using the Trizol (Molecular Research Center) method. RNase protection analysis was performed as described in detail previously (22) using four different cRNA probes, which protected the following sequences: (i) a 349-bp band corresponding to an *AvrII*-*PvuII* fragment of the mouse PTHrP gene, (ii) a 307-bp band corresponding to a *PvuII*-*SacI* cDNA fragment of human PTHrP gene, (iii) a 220-bp band corresponding to a *Sau3A*-*Sau3A* cDNA fragment of mouse cyclophilin gene, and (iv) a 230-bp band corresponding to mouse insulin sequences protected by a *PstI*-*AvaI* mouse insulin genomic fragment (mouse insulin genomic sequences courtesy of Dr. Shimon Efrat, Albert Einstein College of Medicine, New York, NY).

Northern blotting of pituitary RNA was performed as described in the legend to Fig. 11 using a mouse growth hormone cDNA probe, generously provided by Dr. Daniel Linzer at the University of Chicago, and a mouse proopiomelanocortin probe, generously provided by Dr. Richard Mains at Johns Hopkins University.

Pancreatic Protein Extracts—Freshly dissected pancreata were immediately frozen in liquid nitrogen, weighed, and extracts prepared by the acid-urea method (23). Total protein concentrations of the extracts were measured by the Bradford method (Bio-Rad). Insulin radioimmunoassays and PTHrP immunoradiometric assays were performed as described below.

Plasma Analysis—Mice were bled retroorbitally into heparinized capillary tubes and plasma separated by spinning in a microcentrifuge for 10 min at 4 °C. Glucose was measured on whole blood using an Accu-Chek III instrument (Boehringer Mannheim). Plasma calcium was measured by atomic absorption spectrophotometry. Plasma insulin levels were quantitated using two different RIAs as described in Fig. 5.

The first insulin RIA has been described previously (24) and was performed in the laboratory of Dr. W. Zawulich. Briefly, this assay uses a polyclonal guinea pig insulin antiserum (ICN Biomedicals, Costa Mesa, CA) and a rat insulin standard (Eli Lilly, Indianapolis, IN). The detection limit of the assay is 195 pg/ml. The second insulin RIA was performed using a kit purchased from Linco Research Inc., St. Louis, MO, as described by the manufacturer. This assay has a detection limit of 2.5 microunits/ml. PTHrP was measured using a rat modification (25) of a human PTHrP(1–74) immunoradiometric assay, which has been described previously in detail (26). The detection limit of this assay in mouse plasma is 4 pM.

Plasma insulin-like growth factor 1 was measured by radioimmunoassay as described previously (27).

Immunohistochemistry and Quantitative Islet Histomorphometry—Pancreas was obtained from representative animals as described in Results below, fixed overnight in 10% buffered formalin or Bouin's solution, and embedded in paraffin using standard techniques. Sections were placed onto glass slides, deparaffinized with xylene, and either stained with hematoxylin and eosin or incubated with affinity-purified primary antisera recognizing human and mouse PTHrP (prepared in our laboratory as described previously; Refs. 26 and 28) or with insulin, glucagon, and somatostatin antisera purchased from Biogenix (San Ramon, CA). Visualization of staining was accomplished using species-appropriate avidin-biotin complex second antisera as described previously (28). Importantly, in all studies, sections of normal and transgenic mouse pancreas were placed on the same slide to ensure identical staining conditions. Sections from five normal and six transgenic mice were stained with insulin and used for histomorphometric analysis. Quantitative islet histomorphometry was performed using a Nikon (Nikon Inc., New York, NY) Labophot microscope coupled to an Osteotablet (Osteometrics, Atlanta, GA) planimeter and analyzed using the Osteomeasure software package (Osteometrics) as we have reported previously for use in quantitative bone histomorphometry (29).

Islet Perfusion—Pancreata were digested and islets isolated as described in detail previously (30). Briefly, mice were anesthetized using nembutal (50 mg/kg), the abdomen opened, and the pancreas removed and incubated for 5–7 min in Collagenase P (5 mg/pancreas). The digest was washed four times in Hank's balanced salt solution, and individual islets were manually harvested under a dissecting microscope and placed onto a nylon membrane. Extreme care was taken to select islets of equal size. The nylon membranes were then placed into perfusion chambers and perfused at a rate of 1 ml/min with glucose added in the concentrations indicated in the legend to Fig. 8. Fractions were collected at 1-min intervals and assayed for insulin using the first insulin RIA described above (24). Matched groups of 20–40 islets were used for each perfusion assay. Four normal, four 1799 transgenic mice, and four 1807 transgenic mice were used for the experiments shown in Fig. 8.

RESULTS

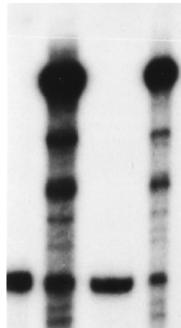
RIP-PTHrP Mice Display a Dwarfed Phenotype—Each of the seven founder RIP-PTHrP mice and every member of the F₁ and subsequent generations of each of the two true-breeding lines of RIP-PTHrP mice displayed a dwarfed phenotype (Fig. 1). The mice appeared normal in size at birth but were smaller than their littermates within 1 week of life. The animals were normally proportioned and appeared vigorous and healthy. The size discrepancy was such that by 8 weeks of life, the RIP-PTHrP mice were approximately 2/3 the weight (19 g *versus* 28 g for RIP-PTHrP *versus* normals) of their normal littermates. The size discrepancy remained for the duration of their life span. The pathophysiology responsible for the dwarfed phenotype is discussed below. For the current study, animals between the ages of 5 and 12 weeks of age were selected. This age window was selected for study because by this age the animals were old enough to permit easy access to blood samples for multiple biochemical studies, but young enough that an ample pool of animals could be maintained for the studies described below. While animals outside the 5–12-week window have not been studied to date in the detail described below, all information available indicates that the hormonal abnormalities described below are present throughout the life span of the animals.

Overexpression of the RIP-PTHrP Transgene Is Targeted to the Pancreatic Islet—In order to determine the level of expres-



FIG. 1. Appearance of the RIP-PTHrP mouse at 8 weeks of life (left) as compared to an age-matched normal littermate (right). Note that the RIP-PTHrP mouse is normally proportioned and healthy appearing. The size discrepancy first becomes apparent at 2–3 weeks of age and persists throughout life.

24 HR Exposure



72 HR Exposure

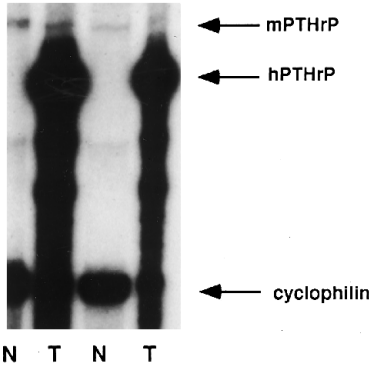


FIG. 2. RNase protection analysis of total RNA prepared from pancreas from normal mice (N) and transgenic (T) RIP-PTHrP mice. The panel on the left shows a 24-h exposure, and the panel on the right shows the same gel exposed for 72 h. Note that the level of pancreatic expression of the human transgene (hPTHrP) is dramatic at the 24-h time point when the endogenous murine mRNA (mPTHrP) is invisible, and that the endogenous mRNA only becomes visible after 72 h of exposure. A mouse cyclophilin probe serves as an internal control.

sion of the RIP-PTHrP transgene in the pancreas, RNase protection analysis of total RNA prepared from the pancreas of normal and transgenic animals was performed (Fig. 2). As can be seen in the figure, marked overexpression of the human PTHrP was evident at the mRNA level in the pancreas of the transgenic animals. As compared to the level of expression of the endogenous murine PTHrP gene, the level of expression of the transgene is roughly estimated to be 10–30-fold greater. Human PTHrP mRNA was also expressed in other tissues, including liver, stomach, heart, brain, kidney, and skin, but the level of expression in tissues other than the pancreas was low, comparable to the level of expression of the endogenous murine PTHrP mRNA shown in Fig. 3, and considerably below the level of expression observed in the pancreatic islet.

In order to confirm that the transgenic animals overexpress the PTHrP at the peptide level, immunohistochemistry using two region-specific anti-PTHrP antisera was performed. As can be seen in Fig. 4, overexpression at the protein level is easily apparent. PTHrP appears to be expressed in essentially all of the cells of the islet using both PTHrP antisera. By rough estimate, PTHrP expression would appear to be 3–10 times higher in the RIP-PTHrP islet than in the normal islet.

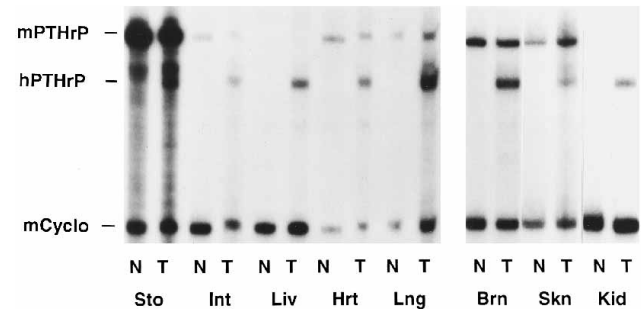
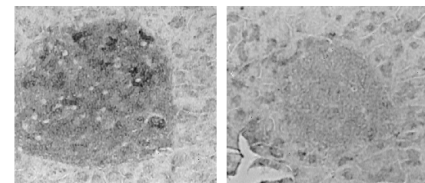


FIG. 3. Extra-islet expression of the RIP-PTHrP transgene. Total RNA (100 μ g) prepared from the tissues shown in the figure, both from normal littermates (N) and transgenic animals (T), was analyzed using RNase protection analysis. As can be seen, low levels of transgene expression, as detected using the hPTHrP probe, were observed in the stomach (Sto), intestine (Int), liver (Liv), heart (Hrt), lung (Lng), whole brain (Brn), skin (Skn), and kidney (Kid). In general, mRNA expression was comparable to endogenous mRNA as detected using the mPTHrP probe. mCyclo indicates mouse cyclophilin. Gastric expression of the endogenous PTHrP gene is higher than in any other organ. These samples were obtained from animals with full stomachs; gastric distention has been reported to increase PTHrP expression (reviewed in Ref. 13).

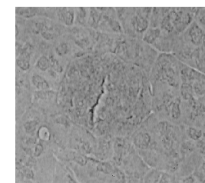
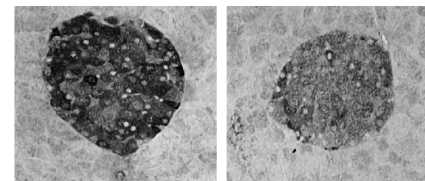
anti-PTHrP(1–36)

anti-PTHrP(37–74)

Normal Mouse



Transgenic Mouse



Competition

FIG. 4. Immunohistochemical staining for PTHrP. Immunohistochemistry for amino-terminal PTHrP (left two panels) and mid-region PTHrP (right two panels) in normal mouse pancreas (upper two panels) and RIP-PTHrP transgenic mouse pancreas (middle two panels). Normal and transgenic pancreas sections were processed identically on adjacent portions of the same slide as described under "Materials and Methods." The bottom panel shows staining of a RIP-PTHrP section with the mid-region antibody following preincubation of the antibody with 10^{-6} M PTHrP(37–74). Identical loss of staining was obtained when the amino-terminal antibody was preincubated with excess PTHrP(1–36) (not shown). The results indicate that PTHrP, both amino-terminal and mid-region epitopes, is present in the islets of the RIP-PTHrP mouse in substantially greater than normal amounts. Although colocalization studies using antibodies directed against insulin, somatostatin, glucagon, and pancreatic polypeptide were not performed, the pattern of staining is consistent with expression of the transgene in β -cells and perhaps α -, δ -, and PP cells as well.

Since the level of expression of the transgene was high, and since the pancreatic islet is a secretory cell with direct access to the circulation, it was important to determine whether systemic oversecretion of PTHrP occurred. Serum calcium concentrations were normal in both lines of RIP-PTHrP mice (mean \pm S.E. = 9.4 ± 0.1 versus 9.3 ± 0.2 mg/dl, normal versus RIP-

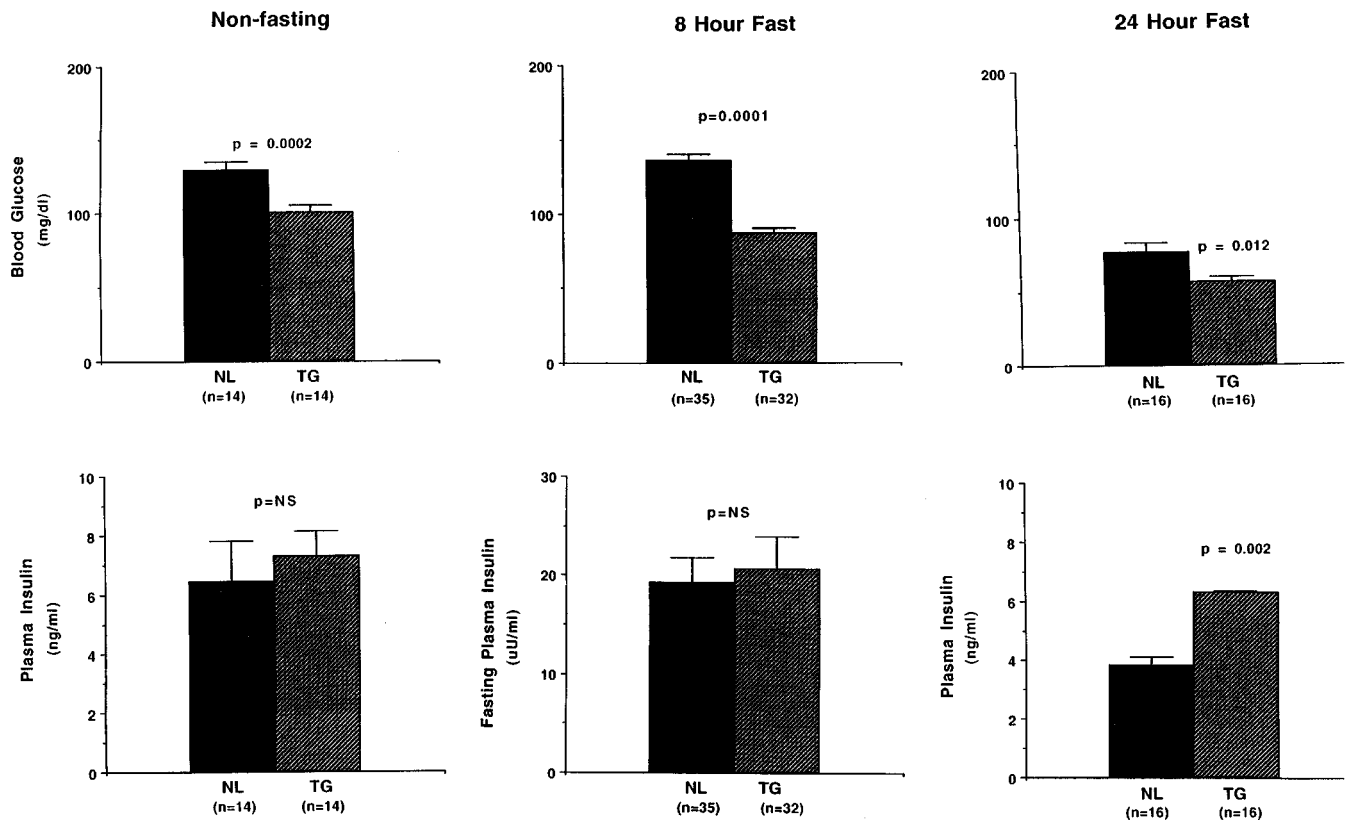


FIG. 5. Whole blood glucose (upper three panels) and plasma insulin (lower three panels) concentrations in non-fasting mice, after an 8-h fast, and after a 24-h fast. NL indicates normal littermates, and TG indicates RIP-PTHrP mice. Note that the insulin RIA employed for the non-fasting and the 24-h time points was the Zawalich insulin RIA (24), while that used for the 8 h time point was the Linco insulin RIA as discussed under "Materials and Methods" and "Results." The results indicate that glucose and insulin concentrations progressively and appropriately decline with fasting in the normal mice, whereas the RIP-PTHrP mice are hypoglycemic relative to their littermates at all time points studied, are inappropriately hyperinsulinemic at all time points, and have non-suppressible plasma insulin concentrations even in the face of marked hypoglycemia. The hyperinsulinemia is documented using two different insulin RIAs.

PTHrP, respectively, $n = 10$ animals in each group, $p = N.S.$), and circulating PTHrP concentrations as determined using an immunoradiometric assay for PTHrP(1-74) with a sensitivity of 4 pM in the mouse (25, 26) were undetectable in both lines of RIP-PTHrP mice. While appropriate samples of portal plasma could not be obtained from these miniature animals, it is worth noting that an elevation in the portal concentration of PTHrP has not been excluded.

RIP-PTHrP Mice Are Hypoglycemic and Hyperinsulinemic—The results of plasma glucose and insulin determinations performed on RIP-PTHrP and control mice between the ages of 5–12 weeks of age are shown in Fig. 5. As can be seen in the three upper panels, blood glucose concentrations in normal littermates are normal post-prandially in the non-fasting state (*i.e.* at an 8–9 a.m. bleed after unrestricted access to food over the preceding night), and after 8 h of fasting, and fall appropriately with progressively longer fasting. In contrast, the glucose values are lower in the RIP-PTHrP mice at every time point, both post-prandially and with progressively longer fasting, and this difference was statistically different at every time point.

As can be seen in the three lower panels of Fig. 5, plasma insulin values were measured on the same samples from the RIP-PTHrP mice and their normal littermates. Surprisingly, in the non-fasting state, and after 8 h of fasting, plasma insulin values were slightly (but not significantly) higher in the RIP-PTHrP mice than in their normal littermates. Given the relative hypoglycemia in the RIP-PTHrP mice in the post-prandial and 8-h fasted states, one would have expected the plasma insulin values to have been lower in the RIP-PTHrP mice than

in controls at these two time points. Further, the plasma insulin/glucose ratios were higher in the RIP-PTHrP mice than in their normal littermates at both of these time points; in the non-fasting state, the plasma insulin/glucose ratios (\pm S.E.) were 0.08 ± 0.01 versus 0.05 ± 0.01 ($p = 0.08$) in the RIP-PTHrP animals versus the normal littermates; corresponding values after 8 h of fasting were 0.23 ± 0.03 versus 0.14 ± 0.02 ($p = 0.001$).

After 24 h of fasting, insulin values were markedly higher in the RIP-PTHrP animals than in their normal littermates, and at this time point, the difference was highly significant in statistical terms ($p = 0.002$). Interestingly, the plasma insulin values after 24 h of fasting were no different than the corresponding values post-prandially (Fig. 5), indicating that plasma insulin is not suppressible by fasting hypoglycemia in the RIP-PTHrP mouse. Finally, in order to be certain that inappropriate hyperinsulinemia was present, plasma insulin concentrations were determined in two different laboratories using two different plasma insulin immunoassays as described under "Materials and Methods;" the values shown for the non-fasting and 24-h time points were performed in one assay (the Zawalich assay) and the 8-h fasting values in another (the Linco assay).

Levels of steady-state insulin mRNA were determined using RNase protection analysis of pancreatic total RNA. As shown in Fig. 6a, steady state insulin mRNA levels were 2–3-fold higher in the RIP-PTHrP mice as compared to their normal littermates. This overexpression of insulin was confirmed at the protein level by measuring insulin in pancreatic extracts by radioimmunoassay. As can be seen in Fig. 6b, RIP-PTHrP mice

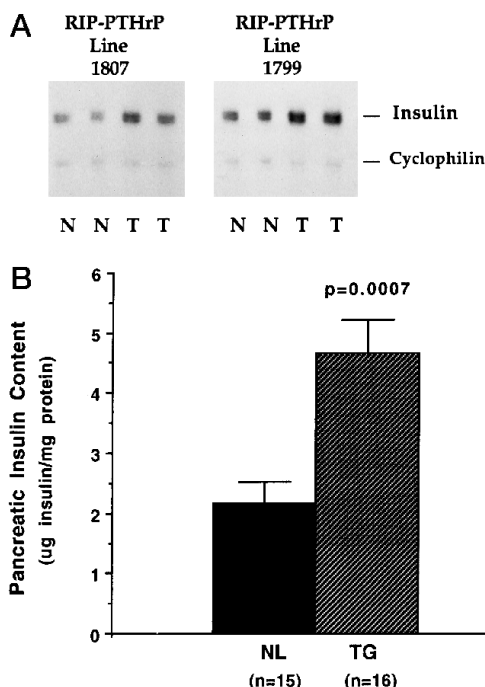


FIG. 6. **Pancreatic insulin mRNA and insulin content.** Panel A shows steady-state insulin mRNA levels as assessed using RNase protection analysis of total pancreatic RNA from normal littermates (N) and from RIP-PTHrP transgenic (T) animals from lines 1799 and 1807. The probes used were a mouse insulin and a mouse cyclophilin cRNA as indicated in the Fig. and described in detail under "Materials and Methods." Panel B shows the insulin content of normal and transgenic pancreas acid-urea extracts prepared and assayed as described under "Materials and Methods." Note that both pancreatic insulin mRNA and insulin protein are 2–3-fold higher in the RIP-PTHrP pancreata than in those of their normal littermates.

contained more than twice as much insulin as those of their normal littermates.

Immunohistochemistry using insulin, glucagon, and somatostatin antisera is shown in Fig. 7. The distribution of insulin-, glucagon-, and somatostatin-containing cells in the RIP-PTHrP islet appears to be normal. No distributional or quantitative differences were observed for any of these three islet peptides between the RIP-PTHrP animals and their littermates.

Isolated RIP-PTHrP Islets Respond Normally to Glucose—Hypoglycemia in the RIP-PTHrP mice could in theory arise from a defect in glucose sensing, in insulin biosynthetic rates, in the regulation of insulin release, or in a combination of the above. In order to study these possibilities, isolated islets were prepared from RIP-PTHrP mice and their littermates and perfused with varying concentrations of glucose. As shown in Fig. 8, neither perfusion with a low glucose concentration (2.75 mM or 50 mg/dl) nor perfusion using a high glucose concentration (20 mM or 360 mg/dl) revealed differences in insulin secretion between RIP-PTHrP and normal islets. Indistinguishable results also were observed when normal and RIP-PTHrP islets were perfused using 0 mM and 10 mM glucose perfusates (data not shown). These results suggested that the hyperinsulinemia and hypoglycemia observed *in vivo* and the elevated pancreatic insulin peptide and mRNA values may not result from abnormalities in individual islets or islet cells, but may reflect an increase in islet mass.

Islet Number and Islet Cell Mass Are Increased in the RIP-PTHrP Mouse—Initial unblinded histologic examination of the pancreata from RIP-PTHrP mice suggested that these animals had more than a normal allotment of pancreatic islets (Fig. 9). In order to determine whether this was in fact the case, a blinded systematic quantitative histomorphometric analysis of

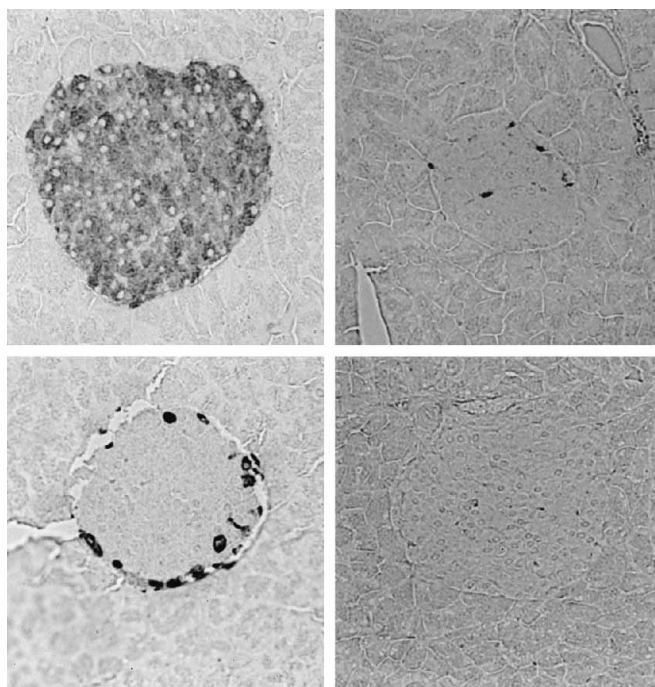


FIG. 7. **Immunohistochemical staining for insulin, glucagon and somatostatin in paraffin sections of RIP-PTHrP pancreas.** Staining for insulin (upper left), glucagon (lower left), and somatostatin (upper right) appear normal in the RIP-PTHrP islet, and are indistinguishable from staining patterns and intensities for the same three peptides in sections of pancreas of normal littermates (data not shown). The bottom right panel is a section for which the primary antibody was omitted, demonstrating the specificity of the staining.

the pancreata from normal littermates and RIP-PTHrP transgenic animals was performed. Five control animals and five age- and sex-matched transgenic animals were sacrificed, the pancreata completely removed, and sections from each pancreas (representing the pancreatic head, body and tail) obtained. Each section was stained for insulin so that all islets of all sizes were included. These sections were quantitated by a blinded histomorphometrist with respect to islet number per unit of exocrine pancreatic area and total islet volume per unit of exocrine pancreas volume. The results are shown in Fig. 10. Both islet number and the islet volume were found to be between 2- and 3-fold increased in the RIP-PTHrP animals as compared to their littermates. Extrapolating from these data, the mean volume of individual islets can be predicted to be normal or near normal in the RIP-PTHrP animals.

Dwarfism in the RIP-PTHrP Mouse Results from Growth Hormone Deficiency—The dwarfed phenotype was unexpected since hyperinsulinemia would be predicted, if anything, to result in *hypersomatism*. The results of pituitary growth hormone mRNA levels and of circulating IGF-1 are shown in Fig. 11. Messenger RNA for growth hormone is dramatically reduced in the pituitary of both strains of RIP-PTHrP mouse, while levels of proopiomelanocortin mRNA, the precursor for ACTH, are comparable in normal and transgenic animals. The reduction in growth hormone mRNA is accompanied by an equally striking reduction in plasma IGF-1 concentrations, also observed in both strains of RIP-PTHrP mouse.

DISCUSSION

These studies demonstrate that PTHrP overexpression in the pancreatic β cells of transgenic mice leads to a syndrome of hypoglycemia resulting from hyperinsulinemia. Taken together with the observations that PTHrP is normally produced in pancreatic β cells (7, 8, 18), and is capable in doses that are

well within the physiologic range (10^{-12} to 10^{-8} M) of eliciting cytosolic calcium responses in a cultured β cell line (18), these findings suggest that PTHrP may play a normal paracrine, autocrine or perhaps "intracrine" (11, 12) physiologic role within the pancreatic islet, and that this role directly or indirectly may involve the regulation, biosynthesis or secretion of insulin.

A primary question regards the mechanisms responsible for hyperinsulinemia in the RIP-PTHrP mouse. It is in theory possible that hyperinsulinemia and hypoglycemia result from

islet-specific effects of the promoter or to random insertional events relating to the location of the transgene in the murine genome. The RIP promoter used in these studies has been used extensively in the creation of other transgenic mouse models. In two models of RIP promoter-targeted transgenic mice, one involving yeast hexokinase (31) and the other involving vasoactive intestinal polypeptide (32), hypoglycemia and hyperinsulinemia did occur. In these cases, there were sound physiologic reasons for the occurrence of hypoglycemia. The other RIP-transgenic models, *e.g.* the RIP-Tag mouse (19, 20), the RIP- G_{α} mouse (33), the RIP-TNF- β mouse (34), and the RIP-MHC-II mouse (35), do not develop hypoglycemia, but more typically develop glucose intolerance or frank diabetes. Since most RIP transgenic models do not develop hypoglycemia, and since the hypoglycemia and hyperinsulinemia were observed in two independent lines of RIP-PTHrP mice, it is unlikely that random insertional mutagenesis or islet-specific effects of the RIP promoter can explain the findings. Rather, the results would appear to be a specific consequence of PTHrP overexpression in the pancreatic islet.

Hyperinsulinemia was accompanied by increases in pancreatic insulin peptide and mRNA content. No qualitative or quantitative abnormalities in glucagon or somatostatin immunohistochemistry could be detected.

Hyperinsulinemia could result in the RIP-PTHrP mouse from abnormalities in individual islet cells and/or within individual islets. These defects could include abnormalities in β cell glucose sensing, inappropriate rates of insulin biosynthesis, a failure of the normal regulation of insulin secretory mechanisms within the β cell or a combination of the above. In order to test these possibilities, perfusion experiments were performed using islets isolated from RIP-PTHrP transgenic animals and their normal littermates. When compared in this way, individual transgenic islets appeared to sense glucose and secrete insulin appropriately. Insulin secretion was normal in response to 0, 2.75, 10, and 20 mM glucose perfusion. These findings support the interpretation that there are no intrinsic abnormalities in the RIP-PTHrP islet or in individual RIP-PTHrP β cells, but rather that the hyperinsulinemia and hypoglycemia might result instead from the observed increase in islet cell mass.

Two observations made during the preliminary characterization of the animals suggested that an increase in islet cell mass was present in the RIP-PTHrP mice. First, in the course of islet

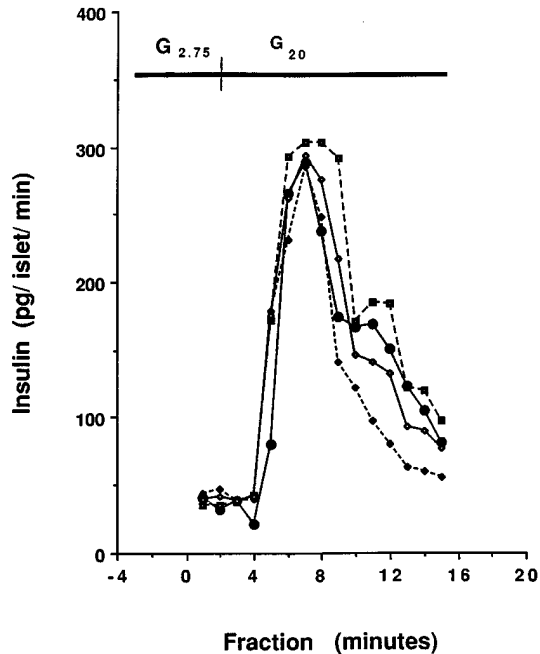


FIG. 8. Perfusion of isolated islets derived from RIP-PTHrP mice and their normal littermates. Perfusion was performed as described under "Materials and Methods" on isolated islets with either low glucose ($G_{2.75} = 2.75$ mM or 50 mg/dl glucose) or high glucose ($G_{20} = 20$ mM or 360 mg/dl glucose) for the times indicated. As indicated in the key on the right, large closed circles represent the results of four perfusions of islet from four normal animals, small closed circles the results of four perfusions of islets from four RIP-PTHrP mice of the 1799 line, and squares, four perfusions of the islets from four RIP-PTHrP mice of the 1807 line. The diamonds are the mean of all the transgenic data, and thus represent the results of eight perfusions of islets isolated from eight mice.

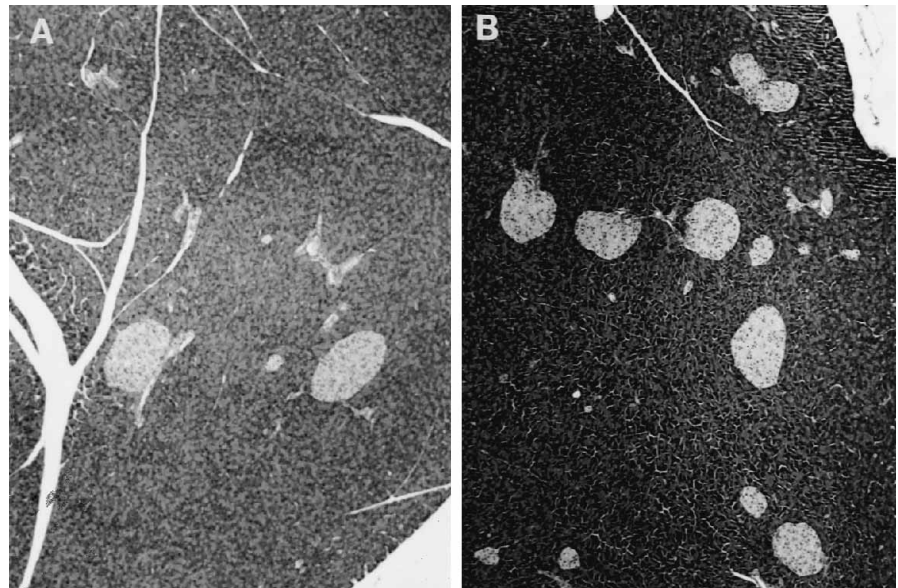


FIG. 9. Hematoxylin and eosin staining of representative sections of pancreas from normal mice (panel A) and RIP-PTHrP mice (panel B). Note that the RIP-PTHrP appear to have more than the normal number of islets, and that the islets appear to display a normal size distribution.

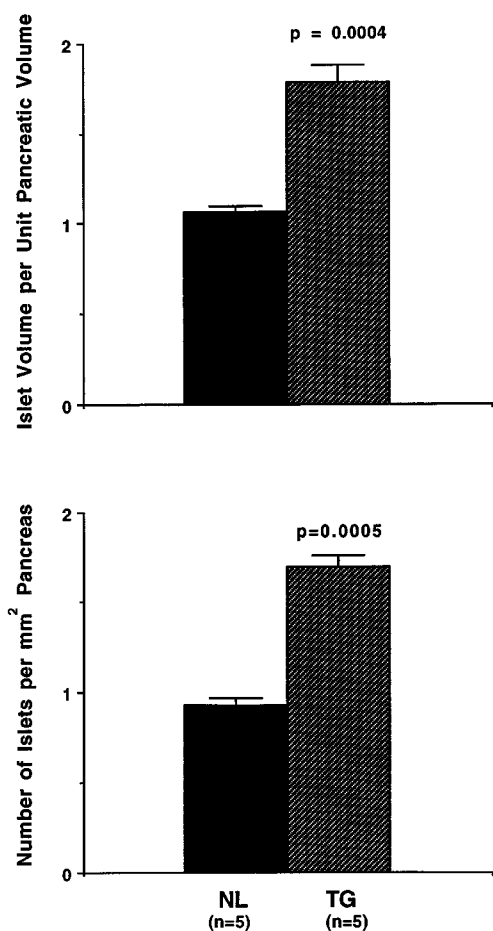


FIG. 10. Quantitative islet histomorphometry of normal (NL) and RIP-PTHrP transgenic (TG) pancreas. The upper panel shows the volume of pancreatic islets as a function of arbitrary total pancreatic volume units. The lower panel displays the number of islets per square millimeter of total pancreatic area. Although not directly measured, it can be extrapolated from these findings that the mean volume or area of individual islets in normal and transgenic animals is comparable.

isolation for the perfusion studies, islets appeared to be of normal size but were more abundant and therefore easier to harvest from the RIP-PTHrP mice than from those of their normal littermates. Second, in initial histologic study of the pancreata from the RIP-PTHrP mice, it appeared that islets were more abundant than in their littermates. In order to examine this possibility in a formal manner, multiple histologic sections were prepared from pancreata from multiple RIP-PTHrP and control animals and these were subjected in a blinded fashion to quantitative islet histomorphometry. The quantitative histomorphometric findings confirmed our initial subjective impression; the RIP-PTHrP mice had approximately twice as many pancreatic islets per unit area of exocrine pancreas as did their littermates; and the aggregate islet area or volume in the RIP-PTHrP mouse was approximately 2-fold higher in the RIP-PTHrP mice than their littermates. By extrapolation, the average islet size would be normal in the RIP-PTHrP mouse.

These observations raise two questions. The first question is, "Given that the RIP-PTHrP mice are smaller than their littermates, do the islet histomorphometric findings represent an appropriately normal islet mass in otherwise miniature mice or should the islet mass be reduced in miniature mice?" This question is difficult to answer unequivocally from available information, but several points bear mention. First, in the Snell

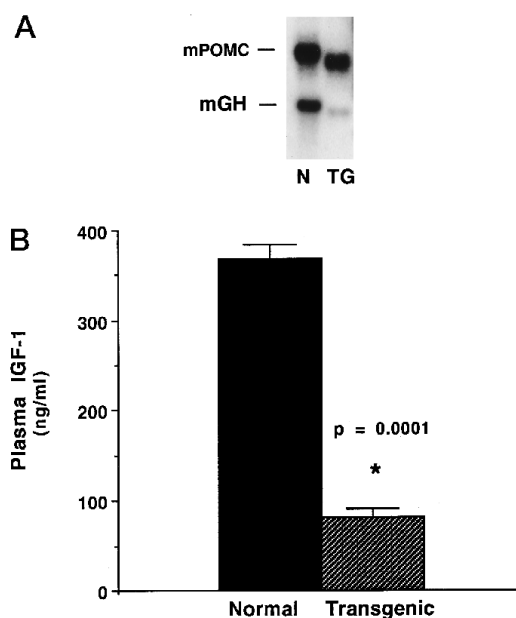


FIG. 11. Panel A, expression of murine growth hormone (*mGH*) and murine proopiomelanocortin (*mPOMC*) mRNA in the pituitary of normal littermates (N) and transgenic animals (T). Five μ g of pituitary total RNA prepared from pituitaries pooled from five animals was loaded in each lane. This blot was prepared from animals of the 1807 line, but indistinguishable results were found in the 1799 line as well. Panel B, circulating insulin-like growth factor 1 concentrations in normal and transgenic animals. See text for details.

and the Ames dwarf growth hormone-deficient mouse models, islet mass is reduced in concert with body size (36). Second, the hypoglycemia observed in the RIP-PTHrP mouse *per se* would be expected to reduce islet proliferation rates and thereby reduce, not increase, islet mass. Third, in preliminary studies, RIP-PTHrP mice of all ages and sizes are hypoglycemic and hyperinsulinemic.² Thus, it is difficult to avoid the conclusion that islet mass is inappropriately and absolutely increased in the RIP-PTHrP mouse.

The second question is, "Is a 2-fold increase in islet mass sufficient to cause hyperinsulinemia and hypoglycemia in otherwise normal mice?" Again, this question is difficult to answer from available data. Islet transplant experiments in which pancreatic islets have been harvested from syngeneic animals and transplanted into normal rats and mice in order to produce models of hyperinsulinemia and hypoglycemia have generally employed more than double the normal allotment of islets, but the viability of the transplanted islets is difficult to know in such studies and could conceivably be such that a 2-fold increase in islet mass was achieved. In patients with insulinomas, islet mass is probably not more than double, but insulinomas have intrinsic glucose sensing and insulin secretory abnormalities that account for insulin oversecretion (37). Our bias would be that a 2-fold increase in islet mass should be insufficient for the induction of hypoglycemia, and that despite the perfusion study results, suggesting that glucose sensing and the regulation of insulin secretion are normal, the RIP-PTHrP islets or individual β cells have intrinsic glucose-sensing or insulin regulatory abnormalities that lead to inappropriate insulin secretion, and that were not detected using our perfusion method. In preliminary studies, we have found that administration of synthetic PTHrP(1-36) or PTHrP(1-74) by perfusion to normal islets does not influence insulin secretion. Clarification of these possibilities must await further study.

PTHrP is produced in a broad range of normal tissues. It has

² R. C. Vasavada and A. F. Stewart, unpublished observations.

been shown to have important effects on growth, development, and differentiation in many tissues (reviewed in Refs. 1–6 and 13), including the epidermal keratinocyte, the osteoblast, the chondrocyte, the fibroblast, the mammary myoepithelial cell, the renal proximal tubular cell, embryonic teratoma cells, and others. No studies have been reported examining the possible role of PTHrP as a mitogen, or developmental or differentiating factor in the pancreatic islet. However, Drucker and collaborators have shown that butyrate, which induces differentiation in cultured pancreatic β cell lines, co-induced the expression of PTHrP in these cells (38). The increased pancreatic islet cell mass in the RIP-PTHrP mouse is consistent with a role for PTHrP as a factor that regulates islet cell mass. It is worth noting that disruption of the PTHrP gene has been accomplished in a mouse model by Karaplis and colleagues (16). The “PTHrP knockout” mouse has severe skeletal abnormalities. Unfortunately, however, these animals die immediately following parturition, prior to the complete development of pancreatic islets. This early lethality together with the absence of information regarding insulin and glucose homeostasis in these animals means that the consequences of PTHrP gene disruption on islet formation and function remain unknown in these animals. Further studies will be required to determine whether PTHrP is in fact a growth regulatory peptide in the islet, and if so, whether these effects occur primarily developmentally *in utero* or continue into adult life. The potential role for PTHrP as an islet growth factor is significant given the failure of the β cell in Type II diabetes mellitus, given the long term failure of islet cell transplantation despite the development of potent and effective immunosuppressive agents, and given the current paucity of well defined islet growth factors.

PTHrP is a prohormone that is posttranslationally endoproteolytically cleaved to yield a family of mature secretory peptides (3–5). These include an amino-terminal secretory form, which binds to and activates the recently cloned parathyroid hormone receptor (10), as well as several other mid-region, and carboxyl-terminal secretory forms of the peptide. In the current experiment, since the full-length PTHrP(1–141) cDNA was used to construct the transgene, these experiments do not provide information regarding which of the several secretory forms is (or are) responsible for the hyperinsulinemia and islet hyperplasia observed.

The dwarfed phenotype was particularly surprising given the presence of hyperinsulinemia. In preliminary studies, RIP-PTHrP mice are dwarfed as compared to their littermates throughout their normal lifespan.² The animals are healthy and vigorous-appearing throughout life, are not hypercalcemic, and have no organ or tissue abnormalities at post-mortem examination. Furthermore, none of the several other RIP transgenic models described to date have displayed dwarfism as a part of the phenotype (19, 20, 30–34). Thus, it is likely that dwarfism occurs not as an artifactual result of the transgene but as a specific consequence of PTHrP overexpression. Plasma IGF-1 concentrations were strikingly reduced, and these in turn would appear to have resulted from a similarly striking reduction in growth hormone. It seems likely that dwarfism results from low level “leaky” expression of the transgene in the hypothalamus or pituitary (low levels of transgene expression were observed in tissues outside the islet in the RIP-PTHrP mouse as they have in other RIP- transgenic mice; see Refs. 19, 20, and 31–35). Northern analysis of pituitary RNA failed to detect PTHrP mRNA in either normal or transgenic pituitary (not shown). PTHrP is slightly overexpressed in RNA prepared from whole brain of the transgenic animals (Fig. 3), but it is not yet clear whether there is overexpression of PTHrP in the hypothalamus. If overexpression were to occur in the hypothal-

amus, it would suggest that PTHrP may inhibit growth hormone or GHRH production or secretion, and would imply that PTHrP might play a normal physiologic role in their regulation. Studies designed to clarify these issues are under way. In the context of the current study, however, growth hormone deficiency or consequent insulin-like growth factor I deficiency would not be expected to lead to hyperinsulinemia, hypoglycemia and islet hyperplasia. Thus, the growth abnormality is almost certainly independent from the glucose homeostatic abnormalities observed.

In summary, these studies indicate that PTHrP may play a normal physiologic role as a regulator of islet cell mass, and through this mechanism or perhaps others, may play a normal role in the physiologic regulation of insulin secretion. Further studies are required to define the cellular mechanisms underlying the islet mass-regulating properties of PTHrP, to define the developmental pathobiology of the pancreas and glucose homeostasis in the RIP-PTHrP mouse, and to fully define the mechanisms responsible for dwarfing.

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