

# Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide

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## SUMMARY

The early progenitor cells to the pancreatic islets in the mouse have been characterized so as to re-examine their possible lineage relationships to the four islet cell types found in mature islets. Insulin and glucagon were both first expressed at embryonic day 9.5, and many cells co-expressed these two markers, as shown by light and electron microscopic analysis using double-label immunohistochemistry. Incubation of embryonic pancreas with 1% glutaraldehyde, a fixative commonly used by electron microscopists, abolished this reactivity, thereby explaining reported difficulties in detecting these precursor cells. Using antisera specific for neuropeptide Y (NPY) a peptide with considerable homology to pancreatic polypeptide (PP), we show that NPY first appears with insulin and glucagon immunoreactivity at E9.5, and is co-expressed with glucagon in a majority of adult  $\alpha$  cells. As we have previously reported, PP itself is first detectable immunocytochemically at

postnatal day 1 with PP-specific antibodies. However, antibodies raised against bovine PP are shown by dot blotting to recognize NPY with comparable avidity, indicating that a recent report of islet progenitor cells containing PP at E9.5 (Herrera, P. L., Huarte, J., Sanvito, F., Meda, P., Orci, L. and Vassalli, J. D. (1991) *Development* 113, 1257-1265), actually represents cross-reactivity to NPY. The data support a model in which early precursor cells to the endocrine pancreas co-activate and co-express a set of islet cell hormone and neural genes, whose expression is both selectively increased and extinguished as development proceeds, concomitant with a restriction to the patterns of expression characteristic of mature islet cell types.

Key words: insulin, pancreatic islet cell lineage, multi-positive progenitors, NPY, mouse pancreas, tyrosine hydroxylase, neuropeptide Y

## INTRODUCTION

Endocrine cells of the adult pancreas are organized into the islets of Langerhans which are scattered throughout the exocrine tissue. There are four major islet cell types:  $\beta$ ,  $\delta$ ,  $\epsilon$  and PP cells that synthesize glucagon (GLU), insulin (IN), somatostatin (SOM) and pancreatic polypeptide (PP), respectively. In addition to the four hormones, islet cells synthesize several neurone-specific markers such as tyrosine hydroxylase (TH) and neurone-specific enolase (Teitelman et al., 1981; Polak et al., 1984; Teitelman and Lee, 1987). The mouse pancreas develops by fusion of dorsal and ventral primordia that appear as evaginations of the gut. This occurs in embryos of about 20 somites, corresponding to day

9.5 of development (E9.5) in mouse and E10.5 in the rat (Wessels and Evans, 1968; Pictet and Rutter, 1972; Kaufman, 1992). The two primitive glands grow independently, forming both endocrine and exocrine tissues, and finally merge at E10.5 in mouse and E11.5 in rat (Pictet and Rutter, 1972). It is generally believed that, in embryos, the pancreatic duct contains undifferentiated precursor cells that migrate away from the duct to generate both islet and acinar cells. Endocrine cell differentiation begins while the pancreatic cell precursors are still within the pancreatic duct (Pictet and Rutter, 1972).

Previous studies suggested that all four islet cell types arise from common precursors and that, in these precursors, the hormone-specific genes become activated sequentially

during development (Alpert et al., 1988). Cells expressing GLU were first seen at E9.5. These primitive islet cells appeared in the epithelium of the foregut prior to morphogenesis of the pancreas. Cells containing IN immunoreactivity appeared two days later, at E11.5. Double-label immunohistochemical studies indicated that, at this stage, all the IN cells also contained GLU immunoreactivity. The number of IN<sup>+</sup> GLU<sup>+</sup> cells decreased during gestation and, by E14.5, a significant number of cells expressed only one antigen. Those studies also revealed that cells containing SOM first appeared at E15.5 while cells expressing PP arise at postnatal day 1 (P-1). Each of these cell types also co-expressed the other islet cell hormones when they first appeared (Alpert et al., 1988).

In that study, the assumption was made that, if islet cells co-expressed two or more hormones during development, these mixed cells probably represented precursors that would divide and ultimately differentiate into mature cells expressing a single specific product (Alpert et al., 1988). Identical approaches have been used to follow cell lineage relationships in the nervous and immune systems *in vivo* and *in vitro* (Raff, 1989; Guidos et al., 1989; Adkins et al., 1987). Similar to those developmental processes, we proposed that the four islet cell types arise from common multipotential precursors and that, during maturation, each phenotype is segregated to a different cell type.

Recently, utilizing RNA-PCR techniques, two groups reported that endocrine cell precursors present in murine embryos at E10.5 expressed mRNAs coding for all four pancreatic hormones (Herrera et al., 1991; Gittes and Rutter, 1992). These findings suggested that the genes encoding the synthesis of pancreas-specific hormones were simultaneously, rather than sequentially, activated during development. Immunohistochemical studies performed by Herrera et al. (1991) also raised questions regarding the derivation of the four islet cell types from common precursors, since they failed to detect cells co-expressing IN and GLU in embryonic pancreas. In addition, Herrera et al. (1991) reported the early expression of PP in islet cells and the presence of a large number of cells containing both GLU and PP. These findings suggested an alternate pathway of differentiation where at least two groups of islet precursor cells are present, one unique group that gives rise to insulin-containing cells and a second group which generates the other three islet cell types.

In light of this new model of the islet cell lineages, we re-evaluated our previous findings using improved immunological reagents. The present study had three objectives: first, we sought to determine the sequence of appearance of insulin and glucagon protein during development. In our previous studies of fetal mouse pancreas, we used antisera against human or bovine insulin. These two insulins are, however, only partially homologous to murine insulin (Wentworth et al., 1986; Steiner et al., 1989). The recent availability of antisera against rat C-peptide (CP), a by-product of the conversion of proinsulin into insulin (Steiner et al., 1989), provided a potentially more sensitive reagent to ascertain the time of initiation of insulin synthesis. Rat CP is highly homologous to mouse CP (for refs see Steiner et al., 1989). Our second objective was to determine, using immunohistochemical techniques both at the light and

electron microscope level, whether islet cell precursors indeed co-express IN with glucagon and other neuroendocrine markers. Finally, we used antisera of distinct specificities to determine the temporal appearance of cells expressing PP immunoreactivity.

## MATERIALS AND METHODS

### (1) Animals and tissue processing

Pregnant CD-1 mice were purchased from Charles River. The appearance of the vaginal plug was considered as day 0.5 of gestation (E0.5). This staging differs from that used in all our previous studies (Alpert et al., 1988 and references therein) in which the day of appearance of the vaginal plug was considered day 1 of development. We have adopted this method in view of its widespread acceptance in current studies on murine embryogenesis. Pregnant females were killed by cervical dislocation, the uterus was removed and placed in 4% paraformaldehyde buffered to pH 7.4 with 0.1 M sodium phosphate buffer (PBS). The embryos were dissected in the fixative solution and were postfixed for 1 hour in the same solution. Embryos were examined at E9.5, 11.5, 13.5 and 15.5. Postnatal and adult CD-1 mice were perfused through the heart with the fixative solution; the pancreas was then removed and postfixed for 1 hour. The fixed tissues were infiltrated overnight in 30% sucrose, mounted in embedding matrix (Lipshaw Co., Pittsburg, Pa) and 15-20 µm cryostat sections were collected onto gelatin-coated slides.

For semithin and thin sections, pancreata were removed from E13.5 embryos and fixed for 4 hours in Zamboni's fixative (85 ml of 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 plus 15 ml saturated picric acid), then stored overnight in 7% sucrose in phosphate buffer and dehydrated through a graded series of ethanols to propylene oxide. After dehydration, the tissues were transferred to a mixture of propylene oxide:Epon (1:1) for 2 hours, then placed in Epon 812-filled capsules at 37°C overnight, followed by a 24 hour incubation at 45°C and an overnight incubation at 60°C.

### (2) Source of antibodies and purified peptides

The following antisera were used to stain cryostat sections and dot blots: guinea pig antibodies to bovine insulin and rat C-peptide were purchased from Linco Research Inc (Eureka, MO); rabbit antiserum to human glucagon was purchased from Calbiochem (San Diego, CA); rabbit antiserum to bovine TH was a generous gift from Dr T. H. Joh (Cornell Univ. Medical College, NY); rabbit anti-bovine pancreatic polypeptide (batch #615-R110-146-14) was generously provided by Dr R. E. Chance (Lillie Research Lab, Indiana). Rabbit antisera to human PP, NPY and somatostatin were supplied by Peninsula Labs (Belmont CA). Biotinylated goat anti-rabbit IgG, goat anti-guinea pig IgG and avidin-labelled peroxidase were purchased from Vector Laboratories (Burlingame, CA).

The following antisera were used to immunostain Epon-embedded tissue: guinea pig anti-insulin and rabbit anti-glucagon were supplied by Milab (Malmö, Sweden). The secondary antibodies were peroxidase-conjugated rabbit anti-guinea pig (DAKO patts, Glostrup, Denmark) and biotinylated swine anti-rabbit (DAKO patts, Glostrup, Denmark).

Source of peptides: purified bovine pancreatic polypeptide, human NPY, bovine insulin and bovine/porcine glucagon were purchased from Sigma (St Louis, MO).

### (3) Immunolabeling of cryostat sections using peroxidase techniques

Sections on slides were transferred to Tris-saline solution (TS; 0.9% NaCl in 0.1 M Tris, pH 7.4) and were immunostained using the avidin-biotin-HRP method. In brief, the sections were

incubated sequentially in: (a) 0.3% Triton X-100 in 1% solution of goat serum in TS for 15 minutes; (b) a 1:30 dilution of goat serum (Gibco) in TS for 30 minutes; (c) an empirically derived optimal dilution of control serum or primary antibody raised in species 'X' containing 1% goat serum in TS for 18 hours; (d) a 1:50 dilution of anti-(species x) biotinylated IgG solution in 1% goat serum in TS for 30 minutes and (e) a 1:100 dilution of peroxidase-avidin complex for 30 minutes. Following these incubations, the bound peroxidase was visualized by reaction for 6 minutes in a solution containing 22 mg of 3, 3'-diaminobenzidine (DAB) and 10  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub> in 100 ml of 0.1 M TS. All incubations were carried out at room temperature. After the DAB step, sections were dehydrated and mounted with Permount. Antibodies were used at the following dilutions: guinea pig anti-bovine insulin, 1:400; guinea pig anti-rat insulin C-peptide, 1:300; rabbit anti-human glucagon, 1:12,000; rabbit anti-human somatostatin, 1:8,000; rabbit anti-human pancreatic polypeptide, 1:20,000 and rabbit anti-bovine pancreatic polypeptide (batch #615-R110-146-14), 1:300,000.

The specificity of the anti-bPP, anti-hPP and anti-human NPY sera used for immunostaining was tested by incubating cryostat sections of embryonic and adult mouse pancreas with antisera that had first been absorbed overnight at 4°C with homologous or heterologous peptides. The peptides (human NPY or bovine PP) were dissolved and adjusted to a concentration of 50  $\mu$ g/ml in anti-bovine PP sera (diluted at 1:10,000), in anti-human PP sera (1:5,000 dilution) or in anti-human NPY sera (diluted 1:5,000). The following day the solutions were centrifuged at 12,000 *g* for 30 minutes and the supernatant used for immunostaining.

#### (4) Electron microscopy

Semithin serial sections (1  $\mu$ m thick) were treated with immunocytochemical techniques in order to locate areas of interest for examination at the light and electron microscope level. The sections were incubated with guinea pig anti-insulin diluted 1:1000 in Tris HCl-buffered saline, pH 7.4 (0.05 M NaCl, TBS) and rabbit anti-glucagon

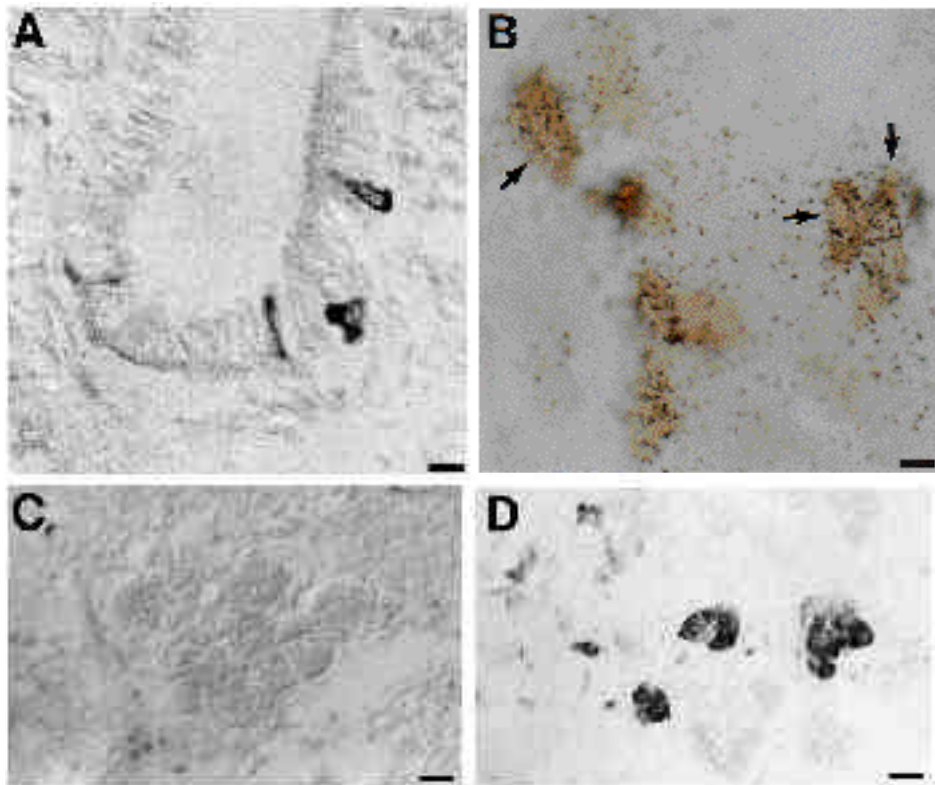
diluted 1:1000 in TBS. Sections were then incubated with peroxidase-conjugated rabbit anti-guinea pig diluted 1:200 in TBS and biotinylated swine anti-rabbit diluted 1:20 in TBS. The biotinylated antiserum was visualized with the avidin-biotin complex.

After analysis of the semithin sections revealed regions of IN- and GLU- positive cells, adjacent segments of the blocks were trimmed for electron microscopy, and ultra thin sections taken and mounted on nickel grids. The grids were incubated in normal goat serum 1:50 for 30 minutes and overnight at 4°C in a mixture of guinea pig anti-insulin and rabbit anti-glucagon, both diluted 1:1000 in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, pH 7.4; 0.15 M NaCl) containing 0.1% bovine serum albumin (BSA, Sigma). Following thorough rinses, the grids were placed in a drop containing a mixture of the secondary antibodies: goat anti-guinea pig IgG conjugated to 10 nm gold particles (diluted 1:50 in PBS + 1% BSA), and goat anti-rabbit IgG conjugated to 15 nm gold particles (diluted 1:50 in PBS + 1% BSA), for 1 hour at room temperature (Varndell et al., 1982). After several washes, the grids were counter stained with uranyl acetate and lead hydroxide and observed with a Zeiss EM 10 CR electron microscope.

The specificity of the immunostaining procedure was confirmed in semithin sections. Each antibody was incubated with 10 nmol/ml of the homologous or a heterologous antigen overnight at 4°C prior to the immunostaining. Preabsorption with homologous antigens precluded the staining while the heterologous antigen did not interfere with the immunolabelling.

#### (5) Immunoperoxidase combined with immunoradiographic labeling

The immunoradiographic method has been described in detail previously (Pickel et al., 1985; Teitelman and Lee, 1987; Alpert et al., 1988). In brief, cryostat sections were prepared as described above and processed at room temperature through the following incubations: (a) an empirically derived dilution of a first primary antibody (i.e. GLU at 1:100,000) in 1% goat serum in TS for 18



**Fig. 1.** (A) Cells containing insulin C-peptide (IN-CP) appear at E9.5. Immunocytochemical localization of IN-CP in epithelial cells of the foregut of mouse embryos at E9.5. Note that few cells contain immunoreactive product. Bar, 10  $\mu$ m. (B) Immunocytochemical autoradiographic co-localization of IN-CP and glucagon (GLU) at E9.5 of development. Localization of IN-CP and GLU antibodies are visualized by the brown reaction product of the DAB and by silver grains, respectively. Arrows indicate double-label cells. Bar, 5  $\mu$ m (C) Incubation with 1% glutaraldehyde abolished IN-CP immunoreactivity. Photomicrograph shows that a pancreas from an E13.5 mouse embryo, which has been fixed with a solution containing 1% glutaraldehyde, sectioned and incubated with IN-CP antisera sera, lacks immunopositive cells. Small dark cells in lower half of section are red blood cells. Bar, 20  $\mu$ m. (D) Photomicrograph shows, for comparison, IN-CP<sup>+</sup> cells in E13.5 pancreas fixed with 4% paraformaldehyde. Bar, 20  $\mu$ m.

hours; (b) three 15 minute washes in TS; (c) a 1:50 dilution of  $^{125}\text{I}$  anti-rabbit IgG (Amersham) with a radioactive concentration of 100  $\mu\text{Ci}/\text{ml}$  in 1% goat serum in TS for 2 hours, (d) six 10 minute washes in TS; and (e) labeling of a second antigen (i.e. IN) with immunoperoxidase by the ABC method. Sections were dehydrated with ascending alcohols and xylene, and then rehydrated, air dried and dipped in Ilford L-4 emulsion (at 50°C) diluted 1:1 with distilled water. The autoradiographic preparations were air dried and exposed in light-proof boxes with desiccant for 4°C for periods of 8-30 days. At the end of the exposure periods, the slides were developed for 2 minutes at 16-17°C using Kodak D-19 developer, rinsed in water and then placed in Kodak Ektaflo fixer for 8 minutes, washed in running water for 1 hour, dehydrated, cleared and mounted with a coverslip using Histoclad or Permount.

This double-label procedure was used to compare the localization of two antisera that were produced in the same species (i.e., rabbit). It takes advantage of the fact that the autoradiographic procedure is far more sensitive than the avidin-biotin technique. To avoid cross-reactivity of the second secondary antibody (i.e., goat anti-rabbit IgG) with the first primary antibody (i.e. GLU), the first primary antibody was diluted below the sensitivity of the ABC technique. If, for instance, sections were stained with antisera to GLU (visualized with  $^{125}\text{I}$ IgG) and to IN (visualized with DAB), we first determined the appropriate dilution of the GLU antisera. Histological sections of pancreas were incubated with increasing dilutions of GLU antibodies and processed with the ABC technique. The dilution of GLU antisera selected was the one that gave no immunohistochemical signal (no DAB precipitate). Next, we tested whether the chosen concentration of GLU antisera gave a clear autoradiographic signal. Sections were incubated with the preferred dilution of GLU antisera and donkey anti-rabbit  $^{125}\text{I}$ -IgG, and processed for autoradiography; these sections displayed silver grains over the cytoplasm of cells. This indicated that the selected dilution of the GLU antibody was below the sensitivity of the ABC technique but it was detected by the immunautoradiographic procedure. Substitution of the first (i.e. GLU) primary antisera in the double-label procedure by preimmune serum eliminated specific immunolabelling visualized by silver grains; substitution of the second primary antisera (i.e. IN) by preimmune serum eliminated the presence of DAB precipitate.

### (6) Dot blots

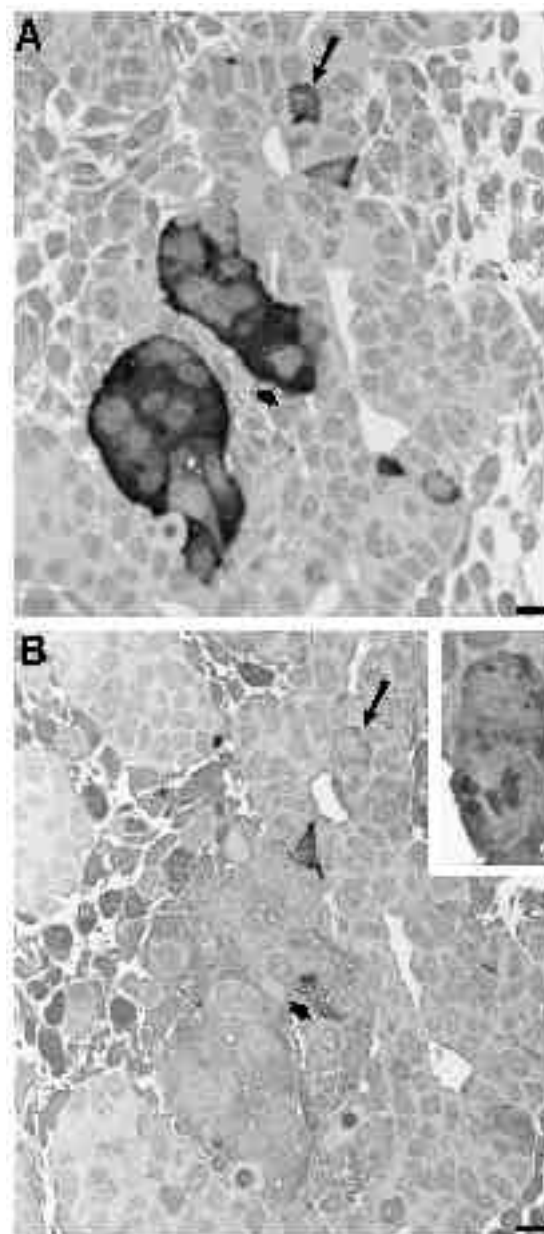
The peptide antibodies were tested for specificity using a dot blot technique (Larsson, 1981). Briefly, human neuropeptide Y and bovine pancreatic polypeptide were dissolved in water to yield an initial concentration of 1 mg/ml which was then serially diluted. Decreasing concentrations of peptides were spotted in 10  $\mu\text{l}$  drops on strips of Whatman No. 1 filter held in place by a filtration manifold (Schleicher and Schuell, Keene, NH). The peptides were air dried on the filter paper and exposed to paraformaldehyde vapors at 80°C for 1 hour to fix them to the paper. The dot blots were stained immunocytochemically using the ABC method with the working dilution of the desired antibody and visualized with a DAB reaction.

## RESULTS

### (1) Concurrent appearance of insulin and glucagon in islet cell precursors

Embryonic pancreas rudiments were analysed for islet cell hormone expression beginning at embryonic day E8.5 and continuing through development to postnatal day 1. The more sensitive antibody to insulin C-peptide was used instead of antisera to insulin itself.

Cells containing insulin C-peptide (IN-CP<sup>+</sup>) were first seen in the epithelium of the foregut at E9.5 (Fig. 1A) by single label immunocytochemistry. At this stage, the epithelial layer also contained cells that expressed immunoreactive glucagon (GLU<sup>+</sup>) and tyrosine hydroxylase (TH<sup>+</sup>) (not shown). The number of IN-CP<sup>+</sup> cells ( $3 \pm 1.41$ ,  $n=5$ ) per pancreas was significantly lower than the number of GLU<sup>+</sup>



**Fig. 2.** Immunocytochemical localization of IN and GLU in semithin (1  $\mu\text{m}$ ) serial sections. Photomicrographs illustrate a section of E13.5 mouse pancreas immunostained with antisera to glucagon (A) and a consecutive section immunostained with antisera to insulin (B). Two cells within the cluster of GLU<sup>+</sup> cells (indicated with short-tail arrow) also contain insulin. A and B show another cell that contains both GLU and IN (indicated with long-tail arrow) which is located next to a duct and adjacent to a cell in mitosis. Insert is a high magnification photomicrograph of the cell to illustrate the presence of insulin immunoreactivity. Bar, 7  $\mu\text{m}$ .

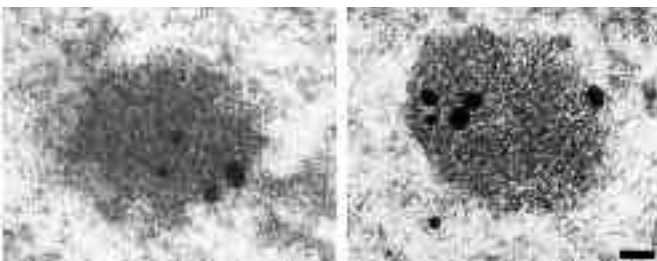
or TH<sup>+</sup> cells (GLU<sup>+</sup> = 29±3, n=5). At E10.5, immunoreactive cells were found both within the epithelium and in the surrounding parenchymal tissue. The number of IN-CP<sup>+</sup>-containing cells increased gradually during development and became the predominant cell type of the mature islets.

We previously reported that IN<sup>+</sup> cells present in E11.5 embryos co-expressed glucagon (Alpert et al., 1988). To determine whether the antigenic phenotype of IN<sup>+</sup> cells at E9.5 is similar to those at E11.5, sections were processed for double immunocytochemical labeling. We found that all the IN-CP<sup>+</sup> cells present at E9.5 co-expressed glucagon (Fig. 1B). Many GLU<sup>+</sup> cells, however, lacked IN-CP immunoreactivity. In confirmation of our previous findings (Alpert et al., 1988), GLU<sup>+</sup> IN-CP<sup>+</sup> cells were also present at E11.5 and E13.5, and the percentage of double-label cells decreased after E14.5 of development. In adults, only a small fraction of GLU<sup>+</sup> cells expressed IN-CP.

In a recent study, Herrera et al. (1991) failed to detect cells co-expressing insulin and glucagon during development. These authors used a fixation protocol different from the one that we follow. Their method exposed cytospin and tissue sections to high concentrations (1%) of glutaraldehyde, which is a fixative routinely used in electron microscopy. To ascertain whether the fixative used by Herrera and colleagues could account for their inability to find cells co-expressing IN and GLU in embryonic pancreas, we tested the effect of increasing concentrations of glutaraldehyde on immunoreactivity. Pancreata from E13.5 embryos were fixed for 1 hour with either 0.125%, 0.25%, 0.5% or 1% glutaraldehyde dissolved either in 0.1 M PBS or in a solution of 0.1 M PBS containing 2% paraformaldehyde. After fixation, the tissues were sectioned and processed for immunohistochemistry. We found that the number of IN-CP<sup>+</sup>, IN<sup>+</sup> and GLU<sup>+</sup> cells decreased dramatically with increasing concentrations of glutaraldehyde (Fig. 1C). Double-label immunocytochemistry was performed on tissues exposed to fixative solutions containing 1% glutaraldehyde. No cells immunostained for both IN and GLU or IN-CP and GLU could be detected (not shown), in clear contrast to the situation where glutaraldehyde was omitted.

## (2) E.M. colocalization of insulin and glucagon in the same cell

To confirm that the embryonic pancreas contained cells co-



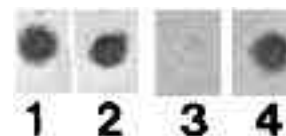
**Fig. 3.** E.M. immunocytochemistry of endocrine cells in an E13.5 mouse pancreas. Photomicrographs show two storage vesicles, both with colocalization of IN (visualized with 10 nm gold particles) and GLU (visualized with 15 nm gold particles) in the same vesicle. Bar, 30 nm.

expressing IN and GLU, we processed tissues for visualization of immunostained cells in semithin serial sections (1 µm thick) at the light microscopic level. We chose E13.5 pancreas for this study because a significant population of GLU<sup>+</sup> IN<sup>+</sup> cells was previously shown to exist at that stage (Alpert et al., 1988). The analysis of semithin serial sections revealed the existence of three cell populations in the 13.5-day-old mouse pancreas: GLU<sup>+</sup> cells, IN<sup>+</sup> cells and a small population of GLU<sup>+</sup> IN<sup>+</sup> cells (Fig. 2A,B). GLU<sup>+</sup> cells were much more abundant and were grouped into clusters, IN<sup>+</sup> cells were found scattered in the ducts and among the pancreatic parenchyma, while the double-label cells predominantly appeared next to ducts. In some cases, a very faint staining for insulin could be observed in GLU<sup>+</sup> clusters.

In order to investigate the co-localization of GLU and IN and to study if the two hormones were stored in the same or in distinct vesicles in the double-labeled cells, sections from E13.5 embryos were processed for immunocytochemical visualization of the two antigens using the electron microscope. This examination demonstrated the presence of cells that contained both GLU and IN immunoreactivity. At higher magnification, it was evident that some vesicles contained both immunoreactive products (Fig. 3). The number of vesicles varied greatly in the developing endocrine cells: some double-label cells contained only a few vesicles (two to three per section) while others contained many (80-100) secretory granules (not shown). In general, the number of secretory granules in IN<sup>+</sup> cells was lower than in the GLU<sup>+</sup> cells. Thus, the analysis of semithin (1 µm) sections by light microscopy and of thin sections by electron microscopy confirmed our previous observation, using 15-20 µm cryostat sections, that IN and GLU are co-expressed in developing islet cells.

## (3) Early islet cell precursors express neuropeptide Y and not pancreatic polypeptide

We and others (Sundler et al., 1977; Yoshinari and Daikoku, 1982) have reported the initial appearance of cells containing pancreatic polypeptide (PP) during the perinatal period. In contrast, Herrera et al. (1991) found abundant PP expression in pancreas from E10.5 of development; these authors also reported that most PP cells of embryos co-expressed GLU. One possible explanation for this discrepancy is a previous observation that apparent PP labeling can result from cross-reactivity of PP antisera to neuropeptide Y (NPY) (DiMaggio et al., 1985), a neuropeptide with considerable sequence homology to PP (Tatemoto et al., 1982). Therefore, we tested the specificity of the PP antisera used by Herrera et al. (1991), anti-bovine PP serum batch # 615-



**Fig. 4.** Western dot blot. Dot 1, bovine PP incubated with anti-bPP sera; dot 2, human NPY incubated with anti-bPP sera; dot 3, bovine PP incubated with anti-human NPY sera; dot 4, human NPY incubated with anti-human NPY sera.

**Table 1. Specificity of anti-bovine PP and anti-human NPY determined by dot blot analysis**

Antigen	Antisera	
	anti-bPP	anti-human NPY
bovine PP	+	-
human NPY	+	+

**Table 2. Specificity of anti-bovine and anti-human PP sera determined by immunohistochemistry**

	Antisera			
	bPP(-NPY <sup>1</sup> )	bPP(-PP <sup>2</sup> )	hPP(-NPY <sup>3</sup> )	hPP(-PP <sup>4</sup> )
E13.5	-	+	-	-
neonates	+	+	+	-
adults	+	+	+	-

<sup>1</sup>bPP sera immunoabsorbed with NPY. It labels PP cells.

<sup>2</sup>bPP sera immunoabsorbed with PP. It labels NPY cells.

<sup>3</sup>hPP sera immunoabsorbed with NPY.

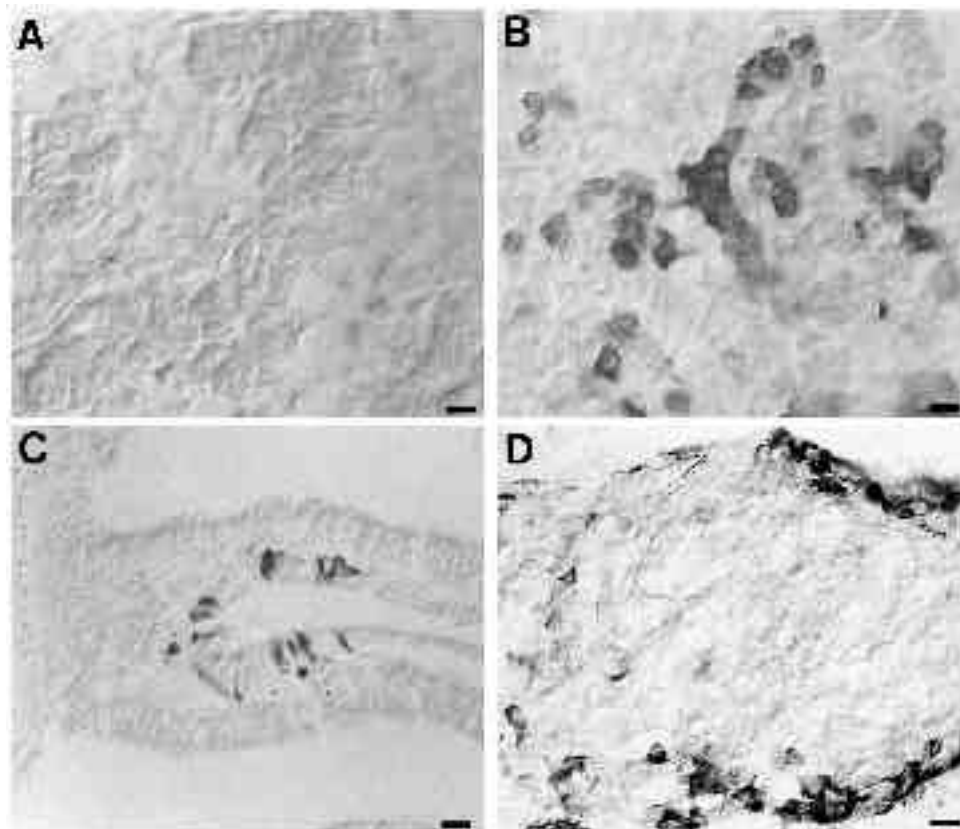
<sup>4</sup>hPP sera immunoabsorbed with PP.

R110-146-14 (bPP), by performing dot blot analysis. As shown in Fig. 4 and summarized in Table 1, this bPP antisera binds to both bovine PP and human NPY. In contrast, antisera raised against human NPY did not cross-react with bovine PP.

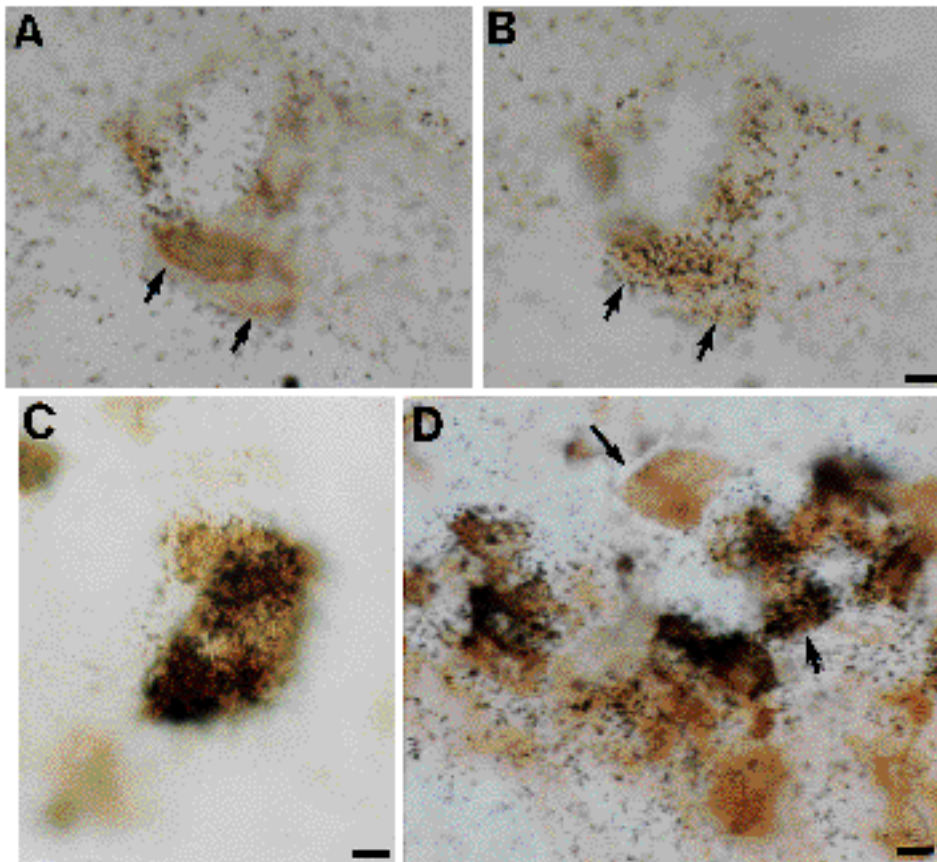
To determine whether the presence of cells that immunolabel with anti-bovine PP serum in the pancreas during embryogenesis reflected the presence of PP or NPY, we

incubated sections of pancreas from E13.5 embryos, neonates and adults with anti-bovine PP serum that was previously absorbed overnight with human NPY. This serum, therefore, labeled only PP<sup>+</sup> cells. We found that the E13.5 pancreas completely lacked immunostained cells (Fig. 5A). Neonate and adult pancreas contained, however, immunoreactive cells in the periphery of the islets where PP<sup>+</sup> cells have been shown to be localized (Solcia et al., 1985). In contrast, both embryonic and adult pancreas contained immunoreactive cells when the sections were incubated with anti-bovine PP serum that was previously absorbed with PP itself (not shown). These labeled cells presumably contained NPY, which reacted with bPP sera. Preabsorption of the antisera to PP previously used by us (rabbit anti-human PP sera, anti-hPP) with human NPY did not affect the previously determined time table of appearance of PP<sup>+</sup> cells at postnatal day 1 or their distribution in islets of neonates and adults. In contrast, absorption of rabbit anti-human PP sera with bovine PP eliminated the staining. The results of these experiments, summarized in Table 2, demonstrate a clear difference in reactivity between the antisera previously used by us (anti-hPP) and that used by Herrera et al. (anti-bPP) in that the anti-bovine PP serum has considerable reactivity towards the related peptide NPY, as previously reported (DiMaggio et al., 1985).

To ascertain whether the embryonic cells labeled with anti-bovine PP were indeed expressing NPY rather than PP, we determined the time table of appearance of NPY<sup>+</sup> cells in islet cell development. Using the previously characterized NPY antisera (which does not cross-react with PP), we found that cells containing NPY immunoreactivity first



**Fig. 5.** (A) Pancreas of E13.5 mouse embryos lack cells expressing PP immunoreactivity. The photomicrograph shows embryonic pancreas incubated with rabbit anti-bovine pancreatic polypeptide (bPP) sera previously absorbed with human NPY. Note the absence of immunostained cells. (B) Section of an E13.5 pancreas incubated with non-absorbed bPP antisera. The immunostained cells are actually NPY<sup>+</sup> cells (see text). Bar, 20  $\mu$ m. (C) Immunolocalization of NPY in mouse pancreas. Cross-section of an E9.5 mouse pancreas illustrates the presence of several NPY<sup>+</sup> cells in gut epithelium. In this photograph, the dorsal region is located to the left and ventral region to the right. (D) Immunocytochemical staining of adult mouse pancreas with specific NPY antisera. Bar, 20  $\mu$ m.



**Fig. 6.** (A,B) Immunocytochemical autoradiographic localization of IN-CP (visualized with DAB) and NPY (visualized with  $^{125}\text{I}$ gG). (A,B) Photomicrographs of the same field taken at different planes of focus: (A) the focus is on the cells, while (B) the focus is on the overlying silver grains. The photographs illustrate the presence of double-labelled (IN-CP<sup>+</sup> NPY<sup>+</sup>) cells (arrows). (C,D) Double immunocytochemical visualization of Glucagon (DAB) and NPY (silver grains). (C) At E9.5, all cells are double labelled. (D) In adults, some islet cells contain both GLU and NPY (short arrows) while other Glu<sup>+</sup> cells lack NPY (long arrows). Bar, 5  $\mu\text{m}$ .

appear at E9.5, within the epithelium of the gut, and persisted in the pancreas throughout life (Fig. 5C,D). Double-label immunocytochemical analysis revealed that, at E9.5, a subset of NPY<sup>+</sup> cells expressed insulin C-Peptide (Fig. 6A,B) and that all NPY<sup>+</sup> cells contained glucagon (Fig.

6C). Sections of E11.5 and E13.5 pancreas incubated with IN and NPY also revealed the presence of IN<sup>+</sup> NPY<sup>+</sup> cells. NPY was also expressed by SOM<sup>+</sup> cells and PP<sup>+</sup> cells from the time that they first appeared at E15.5 and P-1, respectively (not shown). In adults, NPY<sup>+</sup> colocalizes predomi-



**Fig. 7.** Schematic diagram of the pancreatic islet cell lineage. Shown is a proposed pathway for the formation of pancreatic islet cells in mouse embryos. The model is consistent with the immunohistochemical co-localization of endocrine and neuronal markers in the same cells during development. Since Som and PP mRNA are detected at E9.5, it is possible that those two genes are expressed by precursor cells from the time they first differentiate. These precursor cells could give rise to cells expressing only one hormone as illustrated. For clarity, the figure illustrates only the time when each cell type first appears. It should be noted that many mixed cell types are present throughout prenatal development and that some persist in adults.

nantly with GLU (Fig. 6D) in  $\beta$  cells although it is expressed by occasional  $\delta$ ,  $\epsilon$  and PP cells (not shown).

## DISCUSSION

Elucidation of the mechanisms that specify differentiation of the four pancreatic islet cell types will be facilitated by characterization of the initial stages of their development, in particular possible lineage interrelationships. Two goals of this work were (1) to reassess the temporal onset of insulin and glucagon-immunoreactive cells during embryogenesis and (2) to confirm our previous observation that islet precursor cells co-express these two hormones. We have now detected cells synthesizing insulin (as revealed by C-peptide immunostaining) at E9.5, rather than beginning at E11.5, and confirmed, using E.M. and high resolution light microscopy, that IN-CP<sup>+</sup> cells co-express glucagon when they first appear. Immunoelectron microscopic studies from other laboratories have also revealed double-labelled IN<sup>+</sup> GLU<sup>+</sup> cells in pancreas of human and porcine embryos (De Krijger et al., 1992; Lukinius et al., 1992). In contrast, Herrera et al. (1991) failed to observe IN<sup>+</sup> GLU<sup>+</sup> precursor cells, presumably as a result of analyzing tissues that were fixed with high concentrations of glutaraldehyde (1%). We show that this fixation protocol dramatically decreases the sensitivity of the immunostaining reaction for IN and GLU, rendering these double-positive precursors virtually undetectable.

The third goal of this study was to assess the recent proposal that a distinct islet cell precursor expressed pancreatic polypeptide beginning at E9.5 (Herrera et al., 1991). Instead, we have determined that these cells express neuropeptide Y, a structurally similar peptide, but not PP itself. NPY, one of the most abundant neuropeptides in the central nervous system (Foster and Woodhams, 1992), belongs to a family of polypeptides that includes pancreatic polypeptide and peptide YY (PYY) (Ali-Rachedi et al., 1984). It has been reported that many antibodies prepared against bovine pancreatic polypeptide efficiently cross-react with NPY (DiMaggio et al., 1985), which we have confirmed. Our analysis revealed that embryonic islet cells contain NPY from the time that they first appear; however, those cells did not express authentic PP immunoreactivity until the perinatal period. Double-label immunocytochemical analysis demonstrated that, at E9.5, all the NPY<sup>+</sup> cells contained GLU and TH. During islet cell maturation, the expression of NPY segregated with GLU. In the adult, many  $\beta$  cells co-expressed both antigens. Adult islets, however, contained cells that expressed NPY but lacked GLU immunoreactivity, suggesting that NPY<sup>+</sup> cells may be a previously unrecognized pancreatic endocrine cell type.

We initially postulated that the genes encoding pancreatic hormones are activated sequentially during development (Alpert et al., 1988). However, using an improved immunological reagent (anti-IN-CP) we can now detect co-activation of insulin and glucagon (and TH and NPY) in precursors first appearing at E9.5, consistent with RNA-PCR analysis indicating transcriptional activation of both insulin and glucagon genes at this time (Gittes and Rutter, 1992). Yet, while all the E9.5 precursor cells expressed glucagon,

TH and NPY, only a fraction expressed IN-CP. We hypothesize that those precursor cells lacking insulin immunoreactivity activated their insulin genes but have not yet translated its message to detectable levels. Consistent with this proposition, our previous analysis of a transgenic mouse line that expressed the SV-40 large T-antigen (T-ag) under the regulation of the rat insulin II promoter revealed that 100% of the GLU<sup>+</sup> cells of the E9.5 pancreas also expressed the transgene (Alpert et al., 1988). This finding implied that the transcriptional machinery required for expression of the endogenous insulin gene was already active in all the cells that contained glucagon.

We further hypothesize that a similar dichotomy between transcription and translation exists for the SOM and PP genes. The somatostatin and pancreatic polypeptide genes are first transcribed at E9.5 in the pancreatic diverticulum, as revealed by RNA-PCR (Herrera et al., 1991; Gittes and Rutter, 1992) and yet the proteins are only detectable immunocytochemically beginning at E15.5 and P-1, respectively. As with insulin, it is possible that the protein products of the SOM and PP genes are not detected earlier either due to a delay in the initiation of translation or insufficient levels of protein synthesis for immunodetection. It is notable that a previous analysis of exocrine and endocrine genes revealed a pattern in which these pancreatic genes were initially expressed at low levels, and then substantially up-regulated as development proceeded (Han et al., 1986).

Those observations collectively suggest that establishment of islet cell hormone expression is a stepwise process in which islet precursor cells first activate low level transcription and then gradually increase synthesis of the specific hormones, concomitant with their restriction to assume mature cell type properties. The data are consistent with a model in which the islet progenitors co-express a characteristic set of neuroendocrine genes from the time that they are born (Fig. 7), and thus represent a common developmental lineage for the four islet cell types of the adult pancreas.

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