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Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas

(insulin)

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ABSTRACT **We observe Glut2 protein in day 11 (E11) rat embryos in an endodermal domain containing the pancreatic primordium. Glut2 expression continues as the endodermal epithelium evaginates into the surrounding mesenchyme to form the pancreatic buds. Cells of the dorsal and ventral pancreatic buds maintain Glut2 expression as the epithelium grows and branches to form ducts. As acini form at the ends of the ducts, acinar cells cease Glut2 expression. Insulin protein is first detected in small clusters at the interface between pancreatic epithelium and mesenchyme. These clusters disperse into the interstitial tissue between E13 and E17. At E17 a distinct, larger population of insulin-expressing cells arises in the Glut2-expressing ductal network. Insulin- and Glut2-coexpressing cells then appear to segregate into large aggregates to form the beta cells of the islets of Langerhans. These observations support the hypothesis that two biologically distinct populations of insulin-expressing cells arise during pancreas formation.**

The origin of the insulin-expressing beta cells of the pancreatic islets of Langerhans is an unresolved issue. Since the early work of Golosow and Grobstein (1) and Rutter and his colleagues (2, 3) on the morphogenesis of the pancreas, a number of cell lineage hypotheses have been put forward (4). The experimental inaccessibility of the embryonic pancreas has prevented direct cell lineage marking experiments. Studies using cell ablation (5, 6) and tissue transplantation (7) techniques and studies of gene expression patterns (8–16) have led to the general proposal that the four populations of endocrine cells found in the adult islet derive from a multipotent progenitor cell type present during embryogenesis. The biochemical identity of this progenitor has been controversial. The most recent work suggests that the primitive endocrine progenitor in the pancreas expresses the peptide PYY (16). This progenitor type is thought to give rise to immature endocrine cells expressing multiple islet hormones and finally to separate lineages expressing single hormones.

We sought to expand on the current model of islet endocrine cell development by describing the temporal and spatial expression patterns of another beta-cell gene product, the glucose transporter type 2 (Glut2). Glut2 is expressed in numerous fetal and adult tissues, including pancreas, liver, intestine, brain, and kidney (17, 18). Within the adult pancreas, however, Glut2 expression has been described only in the beta cells, where it may be essential in their glucose-sensing function (18).

We probed histological sections of the developing rat pancreas from 11 days of gestation (E11) through neonate stages with antibodies to insulin, glucagon, amylase, and Glut2. We observed that the endodermal epithelial cells of the entire pancreatic primordium express Glut2. This leads us to propose that two distinct populations of insulin-expressing cells arise during pancreas development.

MATERIALS AND METHODS

Tissue. Timed pregnant CD rats were obtained from Charles River Breeding Laboratories. Noon of the morning when plugs were discovered was designated as E0.5. Embryos were collected, cleaned of adherent membranes, and fixed whole or dissected to obtain gut organs. Tissue was fixed on ice in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hr and then processed for immunohistochemistry, using routine paraffin embedding procedures.

Reagents. The Glut2 antibody was obtained from East Acres Biologicals (Southbridge, MA). The guinea pig anti-insulin and rabbit anti-glucagon antibodies were obtained from Incstar (Stillwater, MN). Biotinylated secondary antibodies, avidin-conjugated horseradish peroxidase, avidin-conjugated fluorescein isothiocyanate (FITC), and Texas red-conjugated anti-rabbit and anti-goat antibodies were all obtained from Vector Laboratories.

Immunohistochemistry. Five micrometer sections of paraffin-embedded tissue were deparaffinized, hydrated, and treated with a 1% periodic acid solution to remove endogenous peroxidase activity. This step was omitted in the immunofluorescence experiments. The sections were incubated in a 3% solution of nonfat dry milk in 20 mM Tris·HCl pH 8.0/150 mM NaCl/0.1% Tween 20 (TTSB) for 30 min at room temperature. The primary antibody (dilutions: Glut2 1:500, insulin 1:5000, glucagon 1:1000, and amylase 1:250) in TTSB was added and incubated for 1 hr at room temperature. After washing in TTSB, slides were incubated in biotinylated secondary antibodies at a final dilution of 1:200 in TTSB for 30 min at room temperature. Slides were washed in TTSB and then incubated for 30 min with horseradish peroxidase-conjugated avidin. To visualize the immunoreactivity, slides were washed and incubated at room temperature in 20 mM Tris·HCl, pH 8.0/0.15 M NaCl containing diaminobenzidene (DAB) at 100 μg/ml and 0.01% hydrogen peroxide. Sections were counterstained with hematoxylin, dehydrated, and coverslipped with 50% (vol/vol) Canada balsam (Fisher) in methyl salicylate (Sigma). For immunofluorescence experiments the horseradish peroxidase-avidin was replaced with either avidin-FITC or Texas red-conjugated tertiary antibody. FITC and Texas red fluorescence was visualized by using 490-nm and 545-nm filters on an Olympus BH2-RFCA microscope.

RESULTS

Expression of Glut2 and Insulin in the Pancreatic Primordium and Early Pancreas. Parasagittal sections of early E11

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Abbreviations: Glut2, glucose transporter type 2; E*n*, embryonic day *n*; FITC, fluorescein isothiocyanate.

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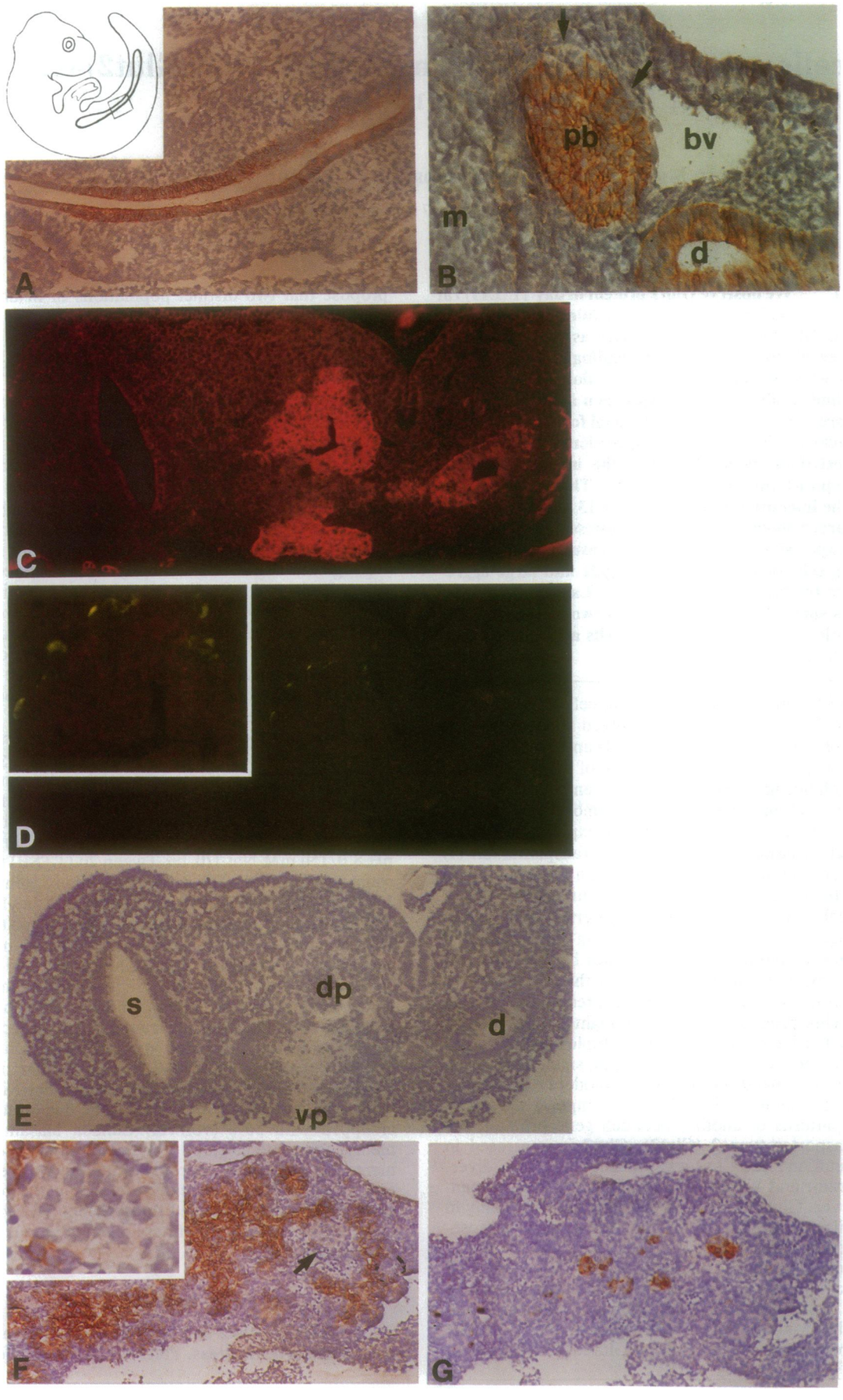

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

rat embryos, spanning the entire gut and containing the pancreatic primordium, were probed with anti-Glut2 antibodies, using immunoperoxidase techniques. The pancreatic diverticulum had not yet formed. We found that a broad band of endoderm posterior to the stomach, at the site of the pancreatic primordium, was immunoreactive for Glut2 (Fig. 1*A*). The surrounding mesodermal mesenchymal cells showed no immunoreactivity.

By E12 of rat gestation, the cells of the pancreatic primordium evaginate into the mesenchymal tissue to form the dorsal pancreatic diverticulum. Fig. 1*B* shows a parasagittal section of a rat pancreatic bud at E12 probed for Glut2. Immunoreactivity is evident throughout the pancreatic bud and adjacent duodenum. These unexpected observations showed that Glut2 is expressed prior to overt pancreas morphogenesis and continues to be expressed in the early pancreatic epithelial bud.

Sections of E12/E13 rat dorsal and ventral pancreas were also probed for Glut2 and insulin gene expression. Insulin immunoreactivity was observed in small clusters (about 10 cells per cluster) of histologically distinct cells located at the interface of pancreatic epithelium and mesenchyme (Fig. 1*D* and data not shown). In serial section analysis insulin and glucagon appear to coexist in some of the same cells (data not shown). These cell clusters may be, on the basis of their morphology, the putative endocrine cells first reported by Wessells and Evans (19) and later detected immunohistochemically by Teitelman and Lee (11) and Deltour *et al.* (20).

Patterns of Insulin and Glut2 Expression Are Mutually Exclusive in the Early Embryo. By early E13 the embryonic pancreas is evident as two epithelial buds posterior to the stomach on the dorsal and ventral planes (Fig. 1 *C*, *D*, and *E*). Sections of E13 foregut containing both the dorsal and ventral pancreatic buds were probed for Glut2 by immunoperoxidase or immunofluorescence techniques. Fig. 1*C* shows red fluorescence indicating Glut2 protein in both the dorsal and ventral pancreas and to a lesser extent the connecting duodenal epithelium. At higher magnification, the immunoreactivity appears to be restricted to the cell membranes, as expected for the location of mature Glut2 protein.

The same sections of E13 pancreas were probed for insulin protein expression. Fig. 1*D* shows faint green cytoplasmic fluorescence in a few cells. The insulin-immunoreactive cells are located at the interface between the pancreatic epithelium and mesenchyme and do not stain positively for Glut2.

From E13 to E16 both dorsal and ventral pancreatic epithelium proliferate to form a complex duct network with presumptive acini (1, 3). Fig. 1 *F* and *G* are serial sections of an isolated E14 dorsal pancreas probed for Glut2 and insulin. The branching ductal network of the E14 pancreas is apparent from hematoxylin staining and highlighted by immunostaining for Glut2 (Fig. 1*F*). It appears that most of the ductal network epithelial cells bind anti-Glut2 antibodies. An adjacent section (Fig. 1*G*) shows five clusters of cells containing insulin. A close examination of these clusters (Fig. 1*F Inset*) reveals a morphology similar to the glucagon- and insulin-positive cells first observed at the epithelial–mesenchymal interface of the E12 buds (Fig. 1*B*). These cell clusters do not appear to be part of the ductal network but rather appear to be dispersed in the interstitial tissue.

From stages E14 to stage E16 the embryonic pancreas continues its growth to form a complex ductal network with distinct acini. Serial section immunoperoxidase and double immunofluorescence analyses show that pancreatic Glut2 and insulin staining patterns are mutually exclusive from E12 to E16 (Fig. 1 *C* and *D* and *F* and *G* and 2 *A* and *B*). The pancreatic ductal network continued to stain strongly for Glut2, while insulin staining was restricted to small interstitial cell clusters. The ratio of Glut2-positive to insulin-positive cells is estimated to be at least 100.

Insulin Expression Arises in the Glut2-Positive Epithelium at E17. By E17 the pancreas has undergone a number of dramatic morphological changes. The stroma is more prominent and acinar structures are more distinct. The acini are strongly immunoreactive for amylase (data not shown) but not for Glut2 (Fig. 2*D* and data not shown). There is a striking increase in the number of cells simultaneously staining for both Glut2 (Fig. 2*D*) and insulin (Fig. 2*E*). These cells are not interstitial but appear to comprise substantial areas of the ductal network. The ratio of Glut2- to insulin-immunoreactive cells at this stage is now about 1 (Fig. 2*F*).

Immunohistochemical analysis of E18 and E19 rat embryo pancreas reveals numerous clusters (100–300 cells) of cells expressing both insulin and Glut2 (data not shown). These large aggregates appear distinct from the ductal network and have formed spherical "islet-like" shapes during their apparent progression into the interstitial space.

DISCUSSION

We have observed Glut2 expression in the endoderm prior to pancreatic bud formation and subsequently in the epithelial cells of both dorsal and ventral pancreatic primordia. We have documented continued Glut2 expression in the branching pancreatic epithelium through E18 of rat gestation. Forming acini lose Glut2 as they begin to express differentiation-specific genes—e.g., amylase. By E19, the ductal epithelium also lacks detectable Glut2. Instead, anti-Glut2 antibodies label large dispersed aggregates of cells which coexpress insulin.

The first insulin-positive cells detected in this study reside in small clusters with endocrine-cell-like morphology. The majority of cells in these clusters express glucagon. Given the level of resolution in our immunohistochemistry, we cannot resolve whether the cells coexpress insulin and glucagon. These clusters persist throughout pancreas morphogenesis, apparently dispersing throughout the stroma. We do not

FIG. 1 (*on opposite page*). Expression of Glut2 and insulin from E11 to E14. (*A*) Parasagittal section of an early E11 rat embryo foregut probed with antibodies to Glut2. The staining is restricted to a discrete stripe of endodermal cells at the site from which the pancreatic bud will form at late E11. (×150.) (*Inset*) Schematic of an E11 rat embryo with the boxed area indicating the foregut. (*B*) Glut2 immunostaining in a parasagittal section of an E12 dorsal pancreas. The dorsal pancreatic bud (pb) is a bulb of endodermal epithelial cells connected to the duodenum. The pancreatic epithelium is surrounded by mesenchyme (m) and is adjacent to a blood vessel (bv). The duct connecting the pancreatic epithelial bud to the duodenum is not in the plane of the section, although the duodenum (d) is apparent. Staining is present on cells throughout the pancreatic epithelium and adjacent duodenum. Immunoreactivity is absent from the pancreatic mesenchyme and the clusters of histologically distinct cells, marked by the arrows, situated between the pancreatic epithelium and mesenchyme. (×150.) (*C–E*) Parasagittal sections of an E13 gut region, showing both the dorsal and the ventral pancreatic buds. (×150.) Both buds and the adjacent duodenum stain positive for Glut2 (*C*). Insulin staining is found only in cells at the mesenchymal–epithelial interface and not in the Glut2-expressing cells of the buds or duodenum (*D*). *Inset* in *D* shows a ×300 magnification of insulin staining at the interface. (*E*) Bright-field photograph; d, duodenum; dp, dorsal pancreas; s, stomach; vp, ventral pancreas. (*F* and *G*) Serial sections of an E14 pancreas probed with antibodies to Glut2 and insulin. The branched E14 pancreatic ductal network stains densely for Glut2 (*F*) but not insulin (*G*). Clusters of insulin-immunoreactive cells are contiguous to, but separate from, the ductal network. Arrow in *F* indicates a histologically distinct cluster of Glut2-negative cells (enlarged to ×300 in *Inset*) similar to that seen in the E12 pancreas bud.

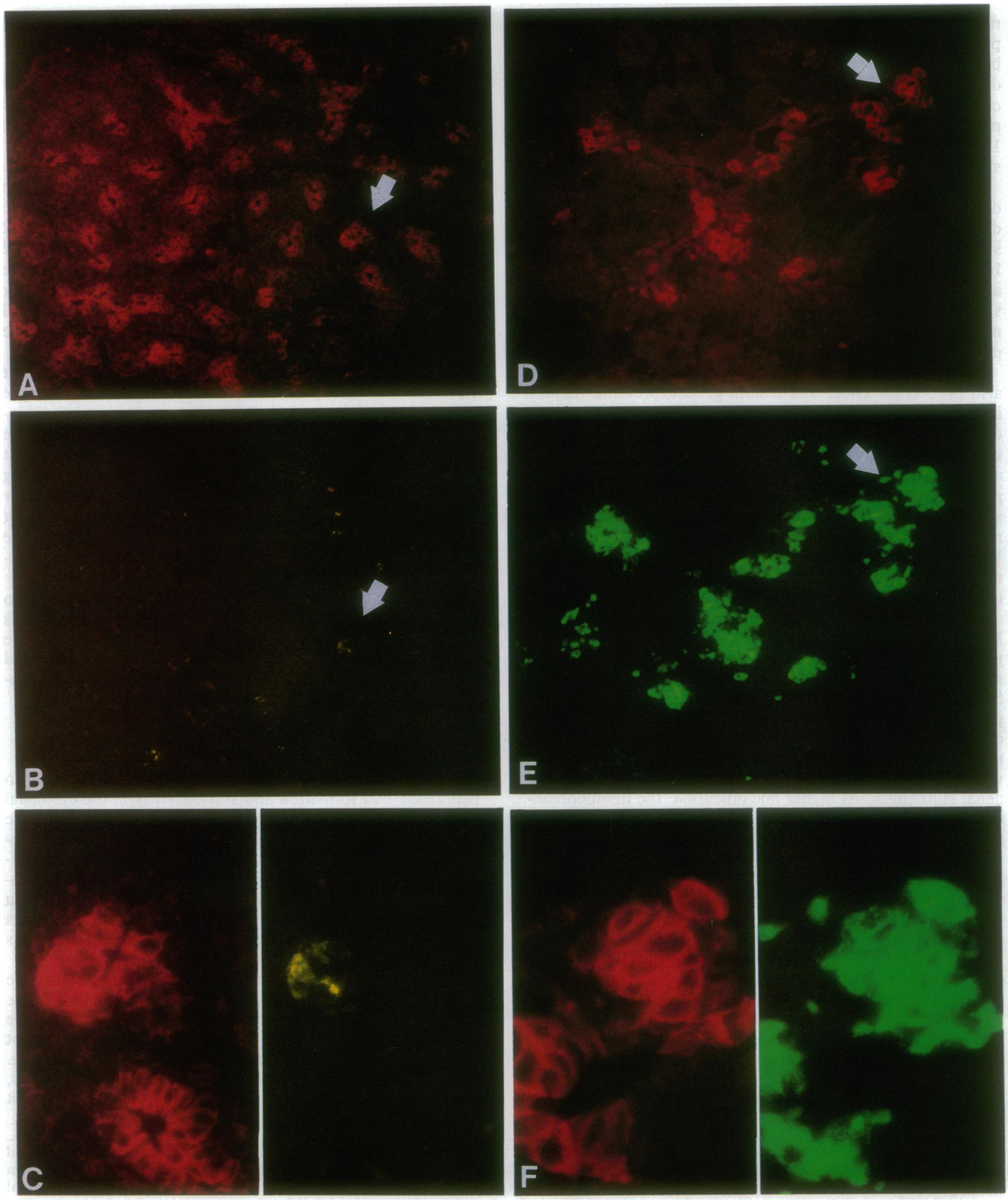

FIG. 2. Expression of Glut2 and insulin at E16 and E17. Sections were probed with antibodies to Glut2 and insulin and immunoreactivity was visualized by fluorescence. (*A–C*) Section of an E16 pancreas stained for both Glut2 and insulin. (*A* and *B*, ×80; C, ×320.) Ductal network cells are positive for Glut2, as revealed by the Texas red fluorescence (*A*), but not for insulin (*B*). Insulin immunoreactivity is sparse and found predominantly in the interstitium. Arrows in *A* and *B* indicate area of enlargement shown in *C* comparing Glut2 and insulin expression. Weak cytoplasmic staining for insulin is seen in a few cells, but the vast majority of Glut2-positive ductal cells remain insulin negative. (*D–F*) Section of an E17 pancreas probed simultaneously for Glut2 and insulin. (*D* and *E*, ×80; *F*, ×320.) Ductal network cells positive for Glut2 (*D*) are now also immunoreactive for insulin (*E*), as apparent from the now intense green cytoplasmic fluorescence. Arrows in *D* and *E* indicate area of enlargement shown in *F* comparing Glut2 and insulin expression. All Glut2-expressing cells now coexpress insulin.

detect a great increase in the number of insulin-immunoreactive cells until about E17. The vast majority of insulin-positive cells at E17–18 reside not in interstitial clusters but in the Glut2-positive branched epithelium. By E19, the re-

sidual ductal epithelium is largely devoid of Glut2 and insulin-positive cells, which now appear in large, dispersed, nonepithelial structures, as described above.

The simplest interpretation of these observations is that two populations of insulin-expressing cells arise in pancreas development. The first, a relatively minor population, is closely associated with glucagon-positive cells histologically similar to those in mature islet endocrine tissue. The second, which ultimately represents the vast majority of insulin-positive cells, develops as a Glut2-coexpressing population within or closely associated with the branching endodermal epithelium. We propose that these cells leave the epithelium to form the beta cells of the first islets of Langerhans. *De novo* expression of insulin in many epithelial cells at E17–19 is consistent with the concomitant 1000-fold increase in pancreatic insulin content previously documented (21) and with the rapid increase in insulin-expressing cells at the equivalent stages in the mouse (20). An alternative, though more complicated, model would have insulin expression abruptly increase in the endocrine-cell-like cell population which originates at E12. This would require a dramatic increase in the number or size of endocrine-cell-like cell clusters; we do not detect this histologically. There would also have to be coincident activation of Glut2 expression in this population and inactivation in the branched epithelium. Finally, these cells would have to physically associate with the branching epithelium at E17 and subsequently return to the stroma at E18. Direct cell lineage marking experiments should allow us to test these models definitively.

One prediction of our hypothesis is that the majority of islet beta cells derive from a different lineage than the nonbeta cells. Independent experimental observations have led others to make the same prediction. Two homeodomain-encoding genes, *Prox-1* (22) and *IPF-1* (23), show patterns of expression similar to the pattern of Glut2. *IPF-1* gene product, a nuclear protein capable of transactivating insulin gene expression, is largely beta cell specific in the adult mouse and rat pancreas (23, 24). Like Glut2, this protein is expressed in all the epithelial cells of the pancreatic rudiment at the equivalent of rat E12, and it is not detected in associated endocrine-cell-like cells which express glucagon and other nonbeta-cell endocrine markers (23). If the assumption is made that *IPF-1* expression marks clones, some of which continue to express it through development, then the alpha- and beta-cell lineages are distinguishable from one another at the earliest stages of pancreas morphogenesis.

On the other hand, detection of insulin in some of the earliest endocrine-cell-like cells leaves open the possibility that dual beta-cell lineages exist. These early insulin-immunoreactive cells are typically found to coexpress other islet hormones such as peptide PYY and glucagon (14). Indeed a common PYY-expressing progenitor has been postulated to give rise to the earliest endocrine-cell-like cells (16).

The physiological and evolutionary significance of dual insulin-expressing cell lineages is unknown. One other precedent exists for dual endocrine cell lineages expressing the same hormone. In the development of the pituitary gland, a transient population of thyrotropes develops in the rostral tip at E12 in mice (25). A second population arises in the caudomedial part of the anterior pituitary at E15.5, which is distinguishable from the first by its dependence on the expression of the wild-type POU domain transcription factor Pit-1. In dwarf mice with mutant Pit-1, only the transient early thyrotropes arise. It is similarly possible that different molecular mechanisms serve to generate the early pancreatic insulin-expressing cells and the later fetal population of Glut2- and insulin-coexpressing endocrine cells.

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1. Golosow, N. & Grobstein, C. (1962) *Dev. Biol.* **4**, 242–255.
2. Pictet, R. L., Clark, W. R., Williams, R. H. & Rutter, W. J. (1972) *Dev. Biol.* **29**, 436–467.
3. Rutter, W. J. (1980) *Monogr. Pathol.* **21**, 30–38.
4. LeDouarin, N. M. (1988) *Cell* **53**, 169–171.
5. Andrew, A. (1976) *J. Embryol. Exp. Morphol.* **35**, 577–593.
6. Fontaine, J. & Le Douarin, N. M. (1977) *J. Embryol. Exp. Morphol.* **41**, 209–222.
7. Fontaine-Perus, J., Le Lievre, C. & Dubois, M. P. (1980) *Cell Tissue Res.* **213**, 293–299.
8. Alpert, S., Hanahan, D. & Teitelman, G. (1988) *Cell* **53**, 295–308.
9. Gittes, G. K. & Rutter, W. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1128–1132.
10. Teitelman, G. (1991) *Recent Prog. Hormone Res.* **47**, 259–297.
11. Teitelman, G. & Lee, J. K. (1987) *Dev. Biol.* **121**, 454–466.
12. Herrera, P. L., Huarte, J., Sanvito, F., Meda, P., Orci, L. & Vassalli, J. D. (1991) *Development (Cambridge, U.K.)* **113**, 1257–1265.
13. De Krijger, R. R., Aanstoot, H. J., Kranenburg, G., Reinhard, M., Visser, W. J. & Bruining, G. J. (1992) *Dev. Biol.* **153**, 368–375.
14. Teitelman, G., Alpert, S., Polak, J. M., Martinez, A. & Hanahan, D. (1993) *Development (Cambridge, U.K.)* **118**, 1031–1039.
15. Teitelman, G., Lee, J. K. & Alpert, S. (1987) *Cell Tissue Res.* **250**, 435–439.
16. Upchurch, B. H., Aponte, G. W. & Lecter, A. B. (1994) *Development (Cambridge, U.K.)* **120**, 245–252.
17. Thorens, B., Sarkar, H. K., Kaback, H. R. & Lodish, H. F. (1988) *Cell* **55**, 281–290.
18. Thorens, B., Weir, G. C., Leahy, J. L., Lodish, H. F. & Bonner-Weir, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6492–6496.
19. Wessells, N. K. & Evans, J. (1968) *Dev. Biol.* **17**, 413–446.
20. Deltour, L., Leduque, P., Blume, N., Madsen, O., Dubois, P., Jami, J. & Bucchini, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 527–531.
21. Rall, L. B., Pictet, R. L. & Rutter, W. J. (1979) *Endocrinology* **105**, 835–841.
22. Oliver, G., Sosa-Pineda, B., Geisendorf, S., Spana, E. P., Doe, C. Q. & Gruss, P. (1993) *Mech. Dev.* **44**, 3–16.
23. Ohlsson, H., Karlsson, K. & Edlund, T. (1993) *EMBO J.* **13**, 4251–4259.
24. Miller, C. P., McGehee, R. E. & Habener, J. F. (1994) *EMBO J.* **13**, 1145–1156.
25. Lin, S.-C., Li, S., Drolet, D. W. & Rosenfeld, M. G. (1994) *Development (Cambridge, U.K.)* **120**, 515–522.