Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 5137–5140, May 1997
Developmental Biology

Phenotypic alterations in insulin-deficient mutant mice

BERTRAND DUVILLIÉ*, NATHALIE CORDONNIER*, LOUISE DELTOUR, FRANÇOISE DANDOY-DRON, JEAN-MICHEL ITIER, ELIANE MONTHIOUX, JACQUES JAMI†, RAJIV L. JOSHI, AND DANIELLE BUCCHINI

Institut Cochin de Génétique Moléculaire, Institut National de la Santé et de la Recherche Médicale U257, 24 rue du Faubourg Saint Jacques, 75014 Paris, France

Communicated by Donald F. Steiner, University of Chicago, Chicago, IL, March 7, 1997 (received for review January 10, 1997)

ABSTRACT **Two mouse insulin genes, *Ins1* and *Ins2*, were disrupted and *lacZ* was inserted at the *Ins2* locus by gene targeting. Double nullizygous insulin-deficient pups were growth-retarded. They did not show any glycosuria at birth but soon after suckling developed diabetes mellitus with ketoacidosis and liver steatosis and died within 48 h. Interestingly, insulin deficiency did not preclude pancreas organogenesis and the appearance of the various cell types of the endocrine pancreas. The presence of *lacZ* expressing β cells and glucagon-positive α cells was demonstrated by cytochemistry and immunocytochemistry. Reverse transcription-coupled PCR analysis showed that somatostatin and pancreatic polypeptide mRNAs were present, although at reduced levels, accounting for the presence also of δ and pancreatic polypeptide cells, respectively. Morphometric analysis revealed enlarged islets of Langherans in the pancreas from insulin-deficient pups, suggesting that insulin might function as a negative regulator of islet cell growth. Whether insulin controls the growth of specific islet cell types and the molecular basis for this action remain to be elucidated.**

Insulin is synthesized, stored, and secreted by the pancreatic islet β cells in a highly regulated manner and plays a vital role in glucose homeostasis. Insulin action also results in several other pleiotropic effects that are less well documented. Embryonic insulin synthesis begins early in gestation, but fetal glycemia closely follows maternal blood glucose levels. The question, therefore, arises as to what function embryonic insulin might fulfill during development. For instance, one might ask whether insulin plays an autocrine or paracrine role in pancreatic islet cell growth and differentiation, since insulin is synthesized with other hormones in developing islet cell types (1–3). Recently, this question has been addressed in a few transgenic studies. For instance, the gene encoding PDX-1 (4, 5), a homeodomain transcription factor synthesized in adult β cells and capable of transactivating insulin gene expression, has been inactivated by targeted disruption (6, 7). Agenesis of pancreas resulting from PDX-1 deficiency precluded from addressing the question of the possible role of insulin in islet cell growth and differentiation. Similarly, mice lacking the LIM homeodomain transcription factor ISL1, synthesized in all classes of islet cells in the adult, were arrested in development soon after embryonic day 9.5 (8). The requirement of ISL1 in pancreatic epithelium for the differentiation of all islet cell types was, however, demonstrated by *in vitro* culture of explants from ISL1-deficient embryonic day 9.5 embryos that gave rise to cells that were negative for glucagon, insulin, and somatostatin. In another study, transgenic mouse embryos expressing the gene encoding the diphteria toxin A chain under control of the rat *Ins2* promoter were generated (9). The resulting genetic ablation of the insulin-producing cells did not appear to alter the development of the nontargeted islet cell types, but an incomplete penetrance of the toxigene was a limitation in this approach.

The present work describes the generation of mice carrying null mutations in the two nonallelic insulin genes, *Ins1* and *Ins2*, and reports, to our knowledge, the first phenotypic description of double nullizygous insulin-deficient mice with particular emphasis on the effects of insulin deficiency on the endocrine pancreas.

MATERIALS AND METHODS

Engineering of the Targeting Vectors. All bacterial, phage, and DNA manipulations were performed as described (10). Mouse strain 129 genomic DNA libraries in phage λ were screened with probes corresponding to mouse *Ins1* or *Ins2* cDNA. The restriction maps of the inserts in recombinant phages were established and several fragments were subcloned.

For *Ins1*, 8.7-kb *Hin*dIII–*Sma*I and 8.2-kb *Apa*I–*Xho*I fragments (5′ and 3′ sides of *Ins1*) were subcloned into pSK$^+$ using *Hin*dIII/*Sma*I and *Apa*I/*Xho*I sites to produce p34 and p4, respectively. A 4-kb *Bam*HI–*Hin*dIII fragment (5′ side of *Ins1*) was also subcloned into pSK$^+$ using *Bam*HI/*Hin*dIII sites to produce pR1. The vector used to engineer the targeting vector was pJorg (obtained from J. Hamm, European Molecular Biology Laboratory, Heidelberg), a pKS$^+$ derivative containing *neo* at the *Bam*HI site and *tk* at *Xho*I/*Hin*dIII sites. The 2.5-kb *Not*I–*Pvu*II fragment from pR1 corresponding to the 5′ homology was cloned into pJorg at *Not*I/*Xba*I sites to produce pR2, in which the 6.5-kb *Hin*dIII fragment from p4 corresponding to the 3′ homology was cloned at the *Hin*dIII site leading to the targeting vector for *Ins1*. The probes 1 and 2 were synthesized with the *Bam*HI fragment from p34 and the *Hin*dIII–*Xho*I fragment from p4, respectively.

For *Ins2*, 5-kb and 7-kb *Eco*RI fragments (5′ and 3′ sides of *Ins2*) were subcloned into pSK$^+$ at the *Eco*RI site to produce p11 and p12, respectively. A *Nsi*I site present in the 7-kb insert in p12 was destroyed to produce p12ΔNsiI. A 5-kb *Xba*I fragment containing *Ins2* was also subcloned into pSK$^+$ at *Xba*I site to produce p13. *Ins2* region (positions −950 to +20) was PCR-amplified with the primers 5′-CGCTCTAGACCCTCCTCTTGCATTTCAAA-3′ and 5′-CGCATGCATGTAGCGGATCACTTAGGGT-3′ that provide *Xba*I and *Nsi*I sites and cloned upstream of *lacZ* coding sequence in pGN (obtained from P. Brûlet; ref. 11) at *Xba*I/*Nsi*I sites; the *Nsi*I site was destroyed and this resulted in p15. The *Xba*I/*Sfi*I fragment in p15 was replaced by a 2.5-kb *Xba*I–*Sfi*I fragment from p13 to produce p16. To engineer the targeting vector, pJorg was first modified by inserting a *Nsi*I linker at *Hin*dIII

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/945137-4$2.00/0
PNAS is available online at **http://www.pnas.org**.

Abbreviations: ES, embryonic stem; RT–PCR, reverse transcription-coupled PCR; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; IGF, insulin-like growth factor.

*B.D. and N.C. contributed equally to this work.
†To whom reprint requests should be addressed.

site to produce p10. The *Xba*I–*Xho*I fragment from p16 containing the 5′ homology (2.7 kb) and *lacZ* were cloned into p10 using *Xba*I/*Spe*I sites to produce p17, in which the 5.5-kb *Sma*I–*Eco*RI fragment from p12ΔNsiI corresponding to the 3′ homology was cloned at the *Nsi*I site using *Nsi*I linkers to produce the targeting vector for *Ins2*. The probe 3 was synthesized with the *Xho*I–*Eco*RI fragment from p12 and the probe 4 was synthesized with the *Bam*HI fragment generated from pJorg.

Generation of Mutant Mice. All embryonic stem (ES) cell cultures and mouse embryo manipulations were performed as described (12–14). The targeting vector DNAs were linearized with *Not*I prior to electroporation into D3 ES cells (obtained from P. Chambon, Laboratorie de Genetique Moleculaire des Eucaryotes, Illkirch, France). After G418 and ganciclovir (a gift from Syntex, Palo Alto, CA) selection, 3 and 12 recombinant clones of *Ins1* and *Ins2*, respectively, were identified. Recombinant cells (10–15 cells) were microinjected into B6 blastocysts that were transferred into the oviduct of pseudopregnant females. Several male germ-line chimeras were obtained that were crossed with B6 females. The various crosses that resulted in double homozygous mice are described in the text. At least two clones were used for each construct. Southern blot analyses using cellular or mouse-tail DNAs were performed as described (12) with the probes indicated.

Reverse Transcription-Coupled PCR (RT–PCR) Analysis. Total pancreatic RNA (1 μg), prepared as described (15), was subjected to a combined RT–PCR using a Promega kit under the conditions specified. *Ins1* and *Ins2* expression was analyzed by using the strategy previously described (16). Briefly, a unique primer pair (5′-GGCTTCTTCTACACACCCA-3′/5′-CAGTAGTTCTCCAGCTGGTA-3′) was used for the PCR amplification step (40 cycles). The *Msp*I digestion of PCR products analyzed by Southern blot using a single 5′-^{32}P-labeled oligonucleotide (5′-ACAATGCCACGCTTCTG-3′) revealed a fragment of 71 and 112 bp for *Ins1* and *Ins2*, respectively. Glucagon mRNA was amplified with the primers (5′-GGTGCAAGGCAGCTGGCAGC-3′/5′-CACGGCGGGAGTCGAGGTAT-3′) and the probe was 5′-[^{32}P]-ACAGGGCACATTCACCAGCG-3′. For somatostatin mRNA the primers were (5′-CAGAAGTCTCTGGCGGCTGC-3′/5′-GGCAGACCTCTGCAGCTCCA-3′) and the probe was 5′-[^{32}P]-CAGAGCTGCTGTCCGAGCCC-3′. For pancreatic polypeptide mRNA the primers were (5′-TGCTGCCTCTCCCTGTTTCT-3′/5′-TCTGCGGAGCTGAGTTTCAT-3′) and the probe was 5′-[^{32}P]-TGCTGCTGCAGCCCCTGCAG-3′. β-actin mRNA was amplified with the primer pair (5′-CGTGGGCCGCCCTAGGCACCA-3′/5′-TTGGCCTTAGGGTTCAGGGGGG-3′) and a 5′-^{32}P-labeled oligonucleotide (5′-AAGGACTCCTATGTGGGTGACG-3′) as probe.

Histological, Immunocytochemical, Histochemical, and Skeletal Analyses. Liver and pancreas samples from *Ins1*$^{-/-}$ *Ins2*$^{-/-}$ and normal littermates were fixed by immersion in 10% formaldehyde. After dehydration and embedding in paraffin, 5-μm sections were performed and stained with hematoxylin and eosin for direct microscopic examination. Immunocytochemistry was performed on paraffin sections with primary rabbit antibodies against C-peptide 1 and C-peptide 2 (provided by O. D. Madsen, ref. 16) reacting with proinsulin 1 and proinsulin 2, respectively, and glucagon (Euro-diagnostica, Malmö, Sweden). For C-peptide 1 and C-peptide 2, goat anti-rabbit serum coupled to peroxidase (Immunotech, Westbrook, ME) was used as secondary antibody. For glucagon immunostaining, incubation with swine anti-rabbit serum (Dako, Glostrup, Denmark) was followed by incubation with rabbit peroxidase-anti-peroxidase (Dako). The reactions were revealed with diaminobenzidine and slides were counterstained with hematoxylin. For 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining, pancreas were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline at 4°C before incubation with X-Gal solution overnight. Specimens were then post-fixed in 4% paraformaldehyde, embedded into paraffin, sectioned, and counterstained with nuclear red.

For skeleton analysis, embryos were dissected, eviscerated, and fixed overnight in 95% ethanol at room temperature. After a 1-week incubation in alcian blue at 37°C, skeletons were incubated with 2% KOH for 24 h and then with alizarin red for 12 h. Tissues were then cleared in 20% glycerol/1% KOH for 1 week and skeletons were transferred to 50% glycerol/50% ethanol at room temperature for storage as described (17) and photographed.

RESULTS AND DISCUSSION

Generation of Insulin-Deficient Mice. The targeting vectors that we used to inactivate *Ins1* and *Ins2* are presented Fig. 1 *A* and *B*. These vectors were designed to delete most of the *Ins1* and *Ins2* coding sequences. Moreover, the *Ins2* vector was

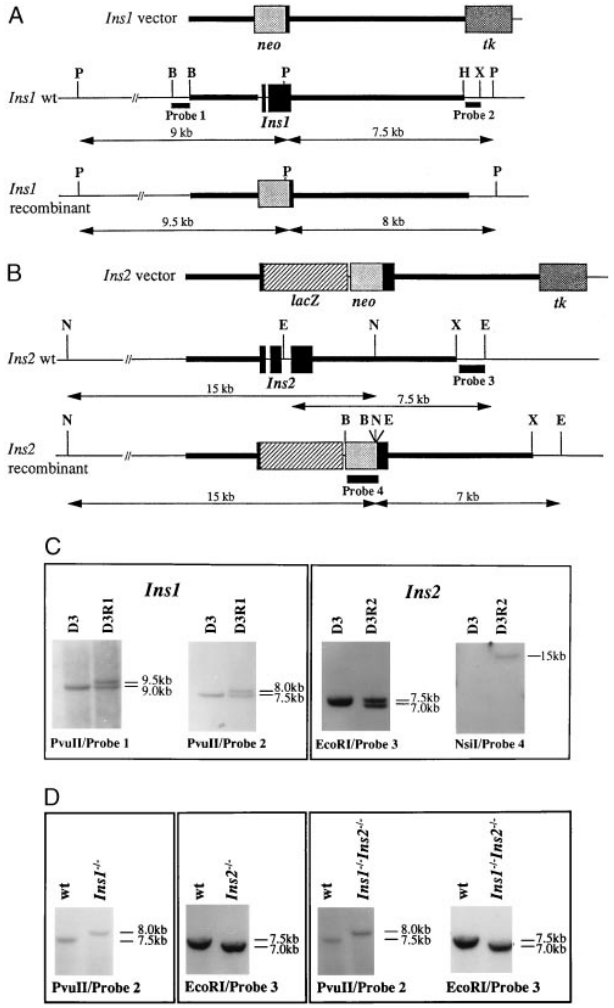

FIG. 1. Targeted disruption of *Ins1* and *Ins2*. Structures of the targeting vectors as well as of the wild-type (wt) and recombinant alleles are shown in *A* for *Ins1* and in *B* for *Ins2*. The restriction enzymes and probes used for genotyping of cellular and mouse DNAs are indicated. Autoradiograms of Southern blot analyses confirming the presence of ES cells (D3) carrying recombined alleles for *Ins1* (D3R1) or *Ins2* (D3R2) and shown in *C* and of *Ins1*$^{-/-}$, *Ins2*$^{-/-}$ and double homozygous mutant mice are shown in *D*. *neo*, Neomycin phosphotransferase gene; *tk*, thymidine kinase gene from herpes simplex virus type 1; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; P, *Pvu*II; X, *Xba*I.

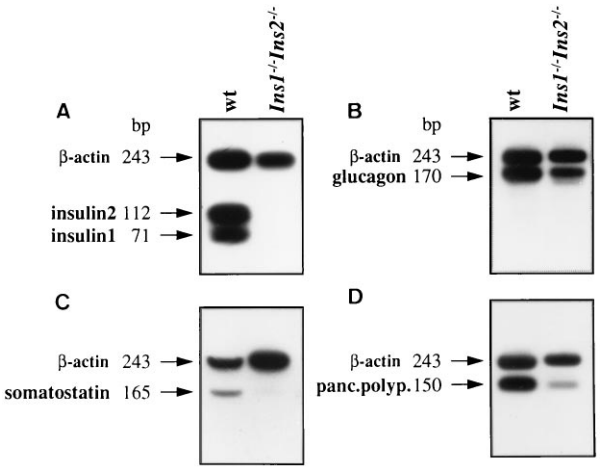

FIG. 2. RT–PCR amplification of mRNAs for insulin 1/insulin 2 (*A*), glucagon (*B*), somatostatin (*C*), and pancreatic polypeptide (*D*) in the pancreas of wild-type (wt) mice and double homozygous mutants. β-actin mRNA was amplified as control. pBR322/*Hpa*II was used as size marker.

engineered to put *lacZ* under the control of the *Ins2* promoter. Both vectors contained *neo* and *tk*, which allowed positive and negative selection, respectively, of transfected ES cells. After electroporation of vector DNAs into D3 ES cells, G418- and ganciclovir-resistant clones were screened by Southern blot analysis using the restriction enzymes and probes indicated in Fig. 1 *A* and *B* and recombinant ES cell clones for each mutation were identified (Fig. 1*C*). Such cells were used to generate male germ-line chimeras for each mutation. Heterozygous animals were identified and crossed to obtain homozygous $Ins1^{-/-}$ or $Ins2^{-/-}$ animals (Fig. 1*D*). These single homozygous mutants were viable and fertile. The intercross of $Ins1^{-/-}$ and $Ins2^{-/-}$ animals generated double heterozygotes that were then mated, and the offspring were genotyped. Double homozygous mutant pups were identified as illustrated in Fig. 1*D* and were present among the progeny in a ratio of approximately 1:16 (15 double homozygotes among 235 pups born). Absence of *Ins1* and *Ins2* transcripts in total RNA from the pancreas of double homozygous pups as evidenced by RT–PCR analysis (Fig. 2*A*) confirmed that these animals carry a null mutation for both *Ins1* and *Ins2*. Thus, the lack of embryonic insulin did not result in any embryonic lethality of double nullizygous embryos.

Growth Retardation and Metabolic Disorders in Insulin-Deficient Mice. Double homozygous mutant pups appeared morphologically normal except that they were smaller and their mean body weight at birth or within a few hours of birth was 22% less than that of their siblings (Fig. 3). The growth difference between insulin-deficient and normal pups was also obvious when comparing their skeletons (Fig. 3). The growth retardation occurred during fetal life, since the few insulin-deficient embryos examined after cesarean around day 18 of gestation weighed 15–20% less than the other embryos. At that time, the liver appeared normal. Examination of the time course of the embryonic growth retardation to determine how it correlates with insulin accumulation requires the sacrifice of a large number of pregnant females and must await the generation of a larger colony of these mutant mice.

Insulin deficiency resulted in the rapid development of diabetes mellitus and ketoacidosis. Glycosuria was detected as soon as the mutant pups had suckled, and the appearance of ketone bodies followed within a day. The triglyceride levels in plasma were judged to be high from its milky appearance. Anatomical examination of the mutant pups revealed a marked hepatomegaly with liver steatosis (Fig. 4 *A* and *B*). Finally, insulin-deficient mice showed early neonatal lethality since the mutant pups died on average within 48 h.

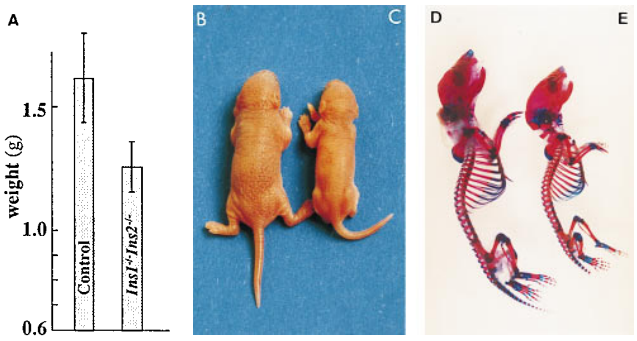

FIG. 3. Growth retardation of double nullizygotes. (*A*) Weights of double nullizygous ($n = 11$) neonates compared with control animals, which include wild-type mice and various heterozygotes ($n = 69$). Size and skeletons of control (*B* and *D*) and insulin-deficient (*C* and *E*) pups.

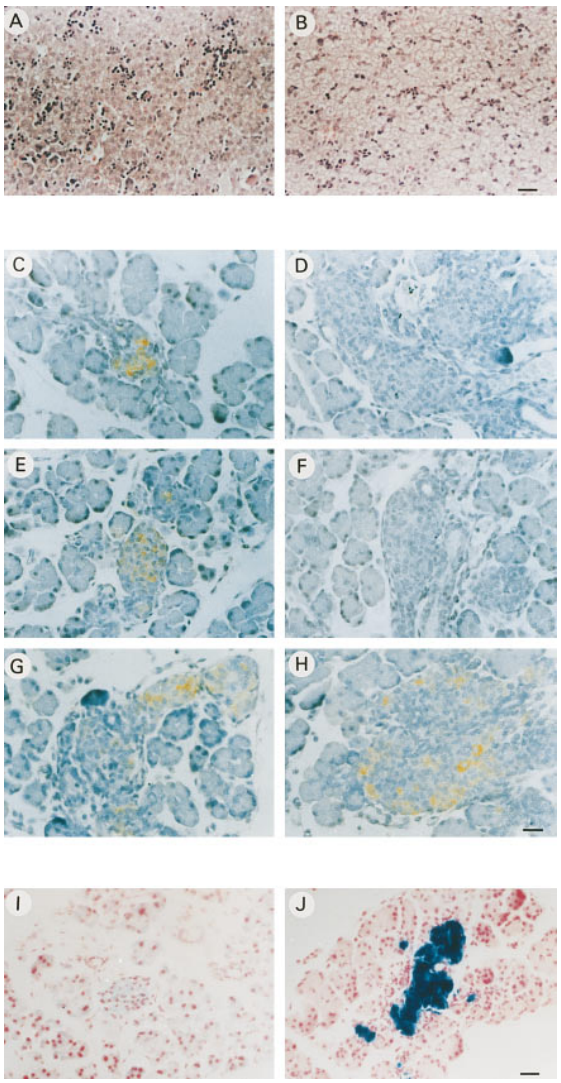

FIG. 4. Analysis of the liver and pancreas of wild-type and double nullizygous mice. Histological analysis of liver sections from wild-type (*A*) and insulin-deficient mice (*B*). Immunocytochemical staining of pancreas sections of wild-type (*C, E,* and *G*) and insulin-deficient mice (*D, F,* and *H*) with antibodies against C-peptide 1 (*C* and *D*), C-peptide 2 (*E* and *F*), and glucagon (*G* and *H*). *lacZ* expression in pancreatic sections of insulin-deficient mice visualized by X-Gal staining (*J*) and background staining obtained with sections from wt animals (*I*) are shown. [Bars = 10 μm (*A* and *B*) and 8 μm (*C–J*).]

Insulin action is mediated through the insulin receptor, a receptor with tyrosine kinase activity widely distributed in the body. Insulin and its receptor are structurally very similar respectively to insulin-like growth factors (IGF-1 and IGF-2) and the IGF-1 receptor. Insulin and IGFs lead to some common and some specific effects on cellular metabolism, growth, and differentiation. These different ligands can bind to and activate the heterologous receptor. The role that IGFs play during embryonic development has been investigated by targeted disruption of the genes encoding IGF-1, IGF-2, or the IGF-1 receptor (18–20). The phenotype of insulin-deficient mice described herein was comparable to that of the insulin-receptor-deficient mice that have been obtained recently (21, 22), except that the disorders resulting from the lack of insulin were more severe and appeared more rapidly. This finding suggests that in the insulin-receptor-deficient mice that become hyperinsulinemic, some effects of insulin might be achieved through its interaction with the IGF-1 receptor. More recently, it has been reported that growth retardation in embryos lacking both the insulin and IGF-1 receptors was more pronounced than in IGF-1-receptor-deficient embryos, in agreement with a role of the insulin receptor in embryonic development (23).

Pancreatic Islet Cell Growth and Differentiation in the Absence of Insulin. In our analysis of insulin-deficient animals, we have focused our efforts on examining the pancreas, which was clearly visible by anatomical observation. Histological analysis indicated that both endocrine and exocrine components were present. As expected, immunocytochemical staining of pancreatic sections using anti-C-peptide 1 and anti-C-peptide 2 antibodies specific for proinsulin 1 and proinsulin 2, respectively, was negative (Fig. 4 *D* and *F*). Since *lacZ* was inserted at the *Ins2* locus under the control of the *Ins2* promoter, the pancreas was assayed for cytochemical detection of β-galactosidase activity. As shown in Fig. 4*J*, most of the cells in the islets were stained blue when X-Gal was used as substrate and, therefore, represent β cells. Immunocytochemical staining of pancreatic sections using antibodies against glucagon revealed the presence of α cells in the islets (Fig. 4 *G* and *H*). The glucagon mRNA was readily detected by RT–PCR analysis (Fig. 2*B*). Somatostatin and pancreatic polypeptide mRNAs were also detected by RT–PCR, accounting for the presence also of δ and pancreatic polypeptide cells. These mRNAs, however, were present at very reduced levels (Fig. 2 *C* and *D*) and we do not yet know whether this is due to a down-regulation of the transcription of these two genes or whether the number of δ and pancreatic polypeptide cells in the islets is reduced.

Thus, embryonic insulin appears to be dispensable for pancreas organogenesis and the appearance of various islet cell types. It can be concluded from the present study that the agenesis of pancreas observed in PDX-1-deficient mice (6, 7) cannot be accounted for by the absence of insulin gene activity. Rather, it came as a surprise in the present study that the absence of embryonic insulin could result in hyperplastic islets as observed in microscopic examination (Fig. 4, compare *C, E,* and *G* with *D, F,* and *H*). In line with this observation, the surface occupied by the islets in pancreatic serial sections appeared to be $\sim$1.48 times larger in diabetic nullizygous (n = 5) than in normal littermates (n = 4) in preliminary morphometric analysis. An interesting question raised by this observation is whether insulin functions as a negative regulator of islet cell growth in pancreas organogenesis, and it will be interesting to further explore the underlying molecular mechanisms for this action of insulin.

Finally, insulin-deficient mice represent interesting tools for addressing many other basic issues related to insulin action and for testing different therapeutic strategies (i.e., injection of insulin analogues, transplantation of insulin producing cells, or gene therapy) for insulin-dependent diabetes. In this respect, first attempts of injecting insulin into insulin-deficient pups showed that glycosuria, ketoacidosis, and liver steatosis can be corrected within 1 day. This opens the possibility of obtaining fertile insulin-deficient adult mice by conventional insulin therapy, which would be useful for many other studies.

We thank L. Lamotte for excellent assistance in genotyping mouse mutants, J. M. Chirgwin for kindly providing us with cDNAs corresponding to *Ins1* and *Ins2*, O. D. Madsen for anti-C-peptide antibodies, P. Chambon for the D3 ES cells, P. Brûlet for pGN, J. Hamm for pJorg, and Syntex Laboratory for ganciclovir. B.D. is recipient of a fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. This work was partly supported by grants from the Association pour la Recherche sur le Cancer and the Ligue Nationale contre le Cancer.

1. Teitelman, G. (1993) *Tumor Biol.* **14,** 167–173.
2. Slack, J. M. W. (1995) *Development (Cambridge, U.K.)* **121,** 1569–1580.
3. Madsen, O. D., Jensen, J., Blume, N., Petersen, H. V., Lund, K., Karlson, C., Andersen, F. G., Jensen, P. B., Larsson, L. I. & Serup, P. (1996) *Eur. J. Biochem.* **242,** 435–445.
4. Ohlsson, H., Karlsson, K. & Edlund, T. (1993) *EMBO J.* **12,** 4251–4259.
5. Guz, Y., Montminy, M. R., Stein, R., Leonard, J., Gamer, L. W., Wright, C. V. E. & Teitelman, G. (1995) *Development (Cambridge, U.K.)* **121,** 11–18.
6. Jonsson, J., Carlsson, L., Edlund, T. & Edlund, H. (1994) *Nature (London)* **371,** 606–609.
7. Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L. M. & Wright, C. V. E. (1996) *Development (Cambridge, U.K.)* **122,** 983–995.
8. Ahlgren, U., Pfaaf, S. L., Jessell, T. M., Edlund, T. & Edlund, H. (1997) *Nature (London)* **385,** 257–260.
9. Herrera, P. L., Huarte, J., Zyfferey, R., Nichols, A., Mermillod, B., Philippe, J., Muniesa, P., Sanvito, F., Orci, L. & Vassalli, J. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12999–13003.
10. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
11. Le Mouellic, H., Lallemand, Y. & Brûlet, P. (1990) *Proc. Natl Acad. Sci. USA* **87,** 4712–4716.
12. Bradley, A., Evans, M., Kaufman, M. H. & Robertson, E. J. (1984) *Nature (London)* **309,** 255–256.
13. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336,** 348–352.
14. Joyner, A. L., ed. (1993) *Gene Targeting: A Practical Approach* (IRL, Oxford).
15. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
16. Deltour, L., Leduque, P., Blume, N., Madsen, O., Dubois, P., Jami, J. & Bucchini, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 527–531.
17. McLeod, M. J. (1980) *Teratology* **22,** 299–301.
18. DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. (1990) *Nature (London)* **345,** 78–80.
19. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. & Efstratiadis, A. (1993) *Cell* **75,** 59–72.
20. Powell-Braxton, L., Hollingshead, P., Warburton, C., Dowd, M., Pitts-Meek, S., Dalton, D., Gillett, N. & Stewart, T. A. (1993) *Genes Dev.* **7,** 2609–2617.
21. Accili, D., Drago, J., Lee, E. J., Johnson, M. D., Cool, M. H., Salvatore, P., Asico, D., José, P. A., Taylor, S. I. & Westphal, H. (1996) *Nat. Genet.* **12,** 106–109.
22. Joshi, R. L., Lamothe, B., Cordonnier, N., Mesbah, K., Monthioux, E., Jami, J. & Bucchini, D. (1996) *EMBO J.* **15,** 1542–1547.
23. Efstratiadis, A. (1996) *Exp. Clin. Endocrinol. Diabetes* **104,** Suppl. 2, 4–6.