Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 527–531, January 1993
Developmental Biology

Differential expression of the two nonallelic proinsulin genes in the developing mouse embryo

(PCR/immunocytochemistry)

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Communicated by Donald F. Steiner, October 12, 1992

ABSTRACT **In the mouse, insulin is produced from two similar but nonallelic genes that encode proinsulins I and II. We have investigated expression of these two genes during mouse embryonic development, using a PCR to detect the two gene transcripts and immunocytochemistry to visualize the two corresponding proteins. At appearance of the dorsal pancreatic anlage at day 9.5 of gestation, both mRNAs could be detected in the embryos, and both proteins were present together in the same cells of the developing pancreas. At days 9.5 and 10.5, when the ventral anlage appears, there were fewer proinsulin II mRNAs than proinsulin I mRNAs. At day 12.5 this ratio was reversed. Proinsulin II mRNA, but not proinsulin I mRNA, could be detected at day 8.5 in the prepancreatic embryo. Proinsulin II mRNA, but not proinsulin I mRNA, was also found in the heads of embryos at day 9.5 and at all later stages studied. These results indicate that the two proinsulin genes are regulated independently, at least in part. They also suggest that insulin might play a role as a growth factor in the developing mouse brain.**

Insulin, a key hormone in metabolic homeostasis, is synthesized, stored, and secreted by beta cells of the pancreatic islets. The protein is synthetized in the form of a precursor, preproinsulin, which is highly conserved among animal species. Unlike most mammals, mice and rats express two nonallelic genes that encode proinsulins I and II. In the mouse these two proteins differ by two amino acids in the B chain and three amino acids in the C peptide. Mouse C peptide I also lacks the Gly-Ala residues present in positions 17 and 18 of C peptide II (1, 2). The corresponding genes in mouse and rat are highly homologous, and their organization is similar, except that the preproinsulin I gene possesses only the first of two introns present in the preproinsulin II gene (1, 3).

On previous studies concerning the regulation of these two nonallelic genes, mRNAs were reported to be present in nearly equal quantities in adult pancreas of mouse (1, 4), as well as of rat (5), suggesting that the two genes are regulated similarly. However, other results indicate that their regulation might be, at least partly, different. (*i*) The proinsulins I and II synthesized and the corresponding insulins stored in pancreatic islets isolated from mouse pancreas were found in a 1:2 ratio. (*ii*) Transient expression experiments with the chloramphenicol acetyltransferase-coding sequence placed under control of 5′-flanking sequences of the rat proinsulin genes with systematic block mutations gave somewhat different results for proinsulins I and II (6–8). Even less is known about the regulation of expression of each of these genes during development. Studies in the rat have indicated that both proinsulin I and II mRNAs are present at the 15th to 16th day of development, and their abundance increases in constant proportion to maximum values measured 16 weeks after birth (5).

Here we have investigated regulation of the expression of the two proinsulin genes during mouse development. In the mouse, the pancreas is derived from the duodenum as two evaginations evolving at days 9.5 (dorsal) and 10.5 (ventral) of the gestation. The evaginations coalesce at day 11, and insulin has been first detected in previous studies at day 11.5 (9, 10). We have used a reverse-transcriptase–PCR (RT–PCR) assay, which allows identification and estimation of the relative amounts of mRNA transcribed from each of the two proinsulin genes. This RT–PCR, using a single pair of primers and a single probe for the two transcripts, allowed comparison of the relative amounts of proinsulin I and II mRNAs in the samples (11). Immunocytochemistry with antibodies specific for each of the two proinsulins allowed us to distinguish the products of the two genes in embryo sections. We have, thus, been able to detect insulin mRNAs and proteins much earlier, to distinguish the two forms, and to trace them within the developing embryo body. Our results indicate that regulation of the two genes is, at least in part, independent and, in addition, suggest that proinsulin II might play a role of growth factor in the developing mouse brain.

MATERIALS AND METHODS

Embryos. Pregnant (C57BL/6 × DBA/2)F_1 females were killed by cervical dislocation on the indicated days; the day of the vaginal plug was considered as 0.5 day postcoitum at noon, as described (12). For blastocyst stage, zygotes were flushed from oviducts, freed from follicle cells by hyaluronidase treatment, and allowed to develop in M2 medium in microdrop cultures at 37°C for 3 days (12). Pools of five developed blastocysts were collected in 2 μl of M2 medium; the tissues, disrupted by one cycle of freezing and thawing, were assayed directly by either PCR or RT-PCR. Later embryonic stages (days 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, and 14.5) were also collected. For day 8.5 (8–12 somites), embryos from a litter were pooled and treated together. For day 9.5 (21–29 somites), embryos were decapitated, and heads and bodies were each pooled for analysis. Individual 8.5- and 9.5-day-old embryos were further dated by morphological criteria, as described by Rugh (13). For day 10.5 and thereafter, embryos were decapitated, and each head and body was analyzed individually. Adult pancreases were dissected from mice of the same F_1 cross.

RNA Analysis. RNA was prepared from the fresh tissues by the guanidium thiocyanate procedure and then centrifuged

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Abbreviations: hprt, hypoxanthine–guanine phosphoribosyltransferase; RT, reverse transcriptase.

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through CsCl, as described (14). Sixty micrograms of total RNA obtained from each sample was treated with DNase, and 2.5 μg was then submitted to RT–PCR, as described (11). A single pair of oligonucleotide primers, 5′(5′-GGCTTCT-TCTACACACCCA-3′) and 3′(5′-CAGTAGTTCTCCAGC-TGGTA-3′) were used because they were homologous to both proinsulin I and II genes. After RT and PCR, each cycle consisting of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C, a single heterogeneous DNA fragment was obtained. To distinguish between proinsulins I and II, this DNA fragment was digested with *Msp* I, which cuts at different sites in each of the two genes, thus generating subfragments of 34, 71, and 77 bp for proinsulin I and subfragments of 76 and 112 bp for proinsulin II. These subfragments were separated by agarose gel electrophoresis, transferred onto GeneScreen*Plus* or Hybond membranes and analyzed by Southern hybridization. A single oligonucleotide probe (5′-ACAATGCCACGCTTCTG-3′) 5′-end-labeled with [γ-^{32}P]-ATP to a specific activity of 2×10^9 cpm/μg was used, and the membranes were hybridized overnight at 42°C, washed [final wash in 0.1× standard saline citrate (SSC)/1% SDS, at 42°C], and exposed to Amersham Hyperfilm-MP. The probe hybridized to a characteristic subfragment from each proinsulin mRNA. An aliquot of each RNA preparation was also submitted to PCR without RT to demonstrate absence of DNA.

Hypoxanthine–guanine phosphoribosyltransferase (hprt) mRNA in the samples was also amplified as a control. The primers were 5′(5′-GATGATGAACCAGGTTATGA-3′) and 3′(5′-ATGTCCCCCGTTGACTGA-3′), and the probe was 5′-TTATCAGACTGAAGAGCTAC-3′. Because there was no interference between amplification of proinsulins and of hprt, both amplifications were done in the same tube. In each RT–PCR series, a control tube was included containing all elements of the reaction except the RNA.

The results of amplification were quantitated by submitting the hybridized filters to either direct analysis of beta emission with a Betascope 603 blot analyzer (Betagene) or analysis by cutting out the labeled bands and counting their radioactivity in a scintillation counter.

Histological Preparation. The cellular localization of proinsulins I and II was studied in embryos and tissues that had been immediately fixed in Bouin-Hollande sublimate after their removal from the animals. They were fixed overnight to 3 days, depending on size, and embedded in Paraplast. Serial 3-μm sections were cut and mounted onto gelatinized slides, as described (15). For the 18.5-day embryos, small fragments of the pancreas were fixed separately.

Immunocytochemistry. Indirect immunoperoxidase staining was done on these sections, as described (16). For proinsulins I and II, antisera 657 and 660 were raised against synthetic C peptide I and II sequences, respectively, as described (17). Briefly, the synthetic sequences Pro-Gln-Val-Glu-Gln-Leu-Glu-Leu-Gly-Gly-Ser-Pro-Gly-Asp-Leu-Cys (C peptide I, positions 5–19) and Pro-Gln-Val-Ala-Gln-Leu-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Asp-Leu-Cys (C peptide II, positions 5–21) were coupled to keyhole limpet hemocyanin and used for immunizing rabbits. These antisera exhibit no cross-reactivity with the heterologous antigen (17). For immunocytochemistry, antisera 657 and 660 were used at a dilution of 1:2000 and 1:500, respectively. To confirm the specificity of the staining pattern seen, synthetic C peptide I and II sequences and insulin were used in preadsorption experiments (25 μg/ml of undiluted antiserum).

The following specific antisera were also used: guinea pig polyclonal antibovine insulin 8309 (Novo Industries, Bagsvaerd, Denmark), diluted 1:1000; peroxidase-labeled goat anti-guinea pig immunoglobulin G (IgG) (Nordic Immunology, Tilburg, The Netherlands) diluted 1:200, and peroxidase-labeled goat anti-rabbit IgG (Dako Immunoglobulins, Copenhagen) diluted 1:200.

RESULTS

To characterize the kinetics and reproducibility of the RT–PCR assay, the procedure was first applied to mRNA isolated from adult mouse pancreas. Fig. 1 shows that total pancreatic mRNA was reverse-transcribed and submitted to increased numbers of amplification cycles. The two DNA fragments characteristic of proinsulins I and II, respectively, were then visualized by digestion of the products with *Msp* I, electrophoresis, and hybridization with the labeled probe. Amplified products were first detected at 20 cycles and became clearly visible at 25 cycles. The intensity of the bands then increased only slightly after 30 or more cycles, and the standard cycle number for the RT–PCR assay was set at 25 cycles (Fig. 1). As the percentages given below the gel indicate, the ratio of intensities of proinsulin I and II bands remained 1:2 at all amplification cycles. This same 1:2 ratio was found for four different animals (data not shown). These results indicated that the comparison between proinsulin I and II transcripts was reproducible, and the relative intensities of the radiolabeled fragments were taken to reflect the relative amounts of proinsulin I and II mRNAs in an RNA preparation. The procedure was then used to study proinsulin expression in embryos.

Detection of Proinsulin mRNAs in Embryos. RNAs prepared from decapitated embryo bodies between 9.5 and 14.5 days of gestation were submitted to RT–PCR, as shown in Fig. 2*A*. From appearance of the pancreatic anlage at day 9.5, both mRNAs were detected in embryos. At least two embryos of each age were examined, and the relative proportions of proinsulin I and II RNAs were determined, as indicated below the gel. At days 9.5 and 10.5, there was less proinsulin II than proinsulin I mRNA. At day 11.5, the difference was smaller, and, starting at day 12.5, the ratio was reversed: there was more proinsulin II than proinsulin I mRNA, as in adult pancreas. The amount of mRNA increased significantly starting at day 13.5.

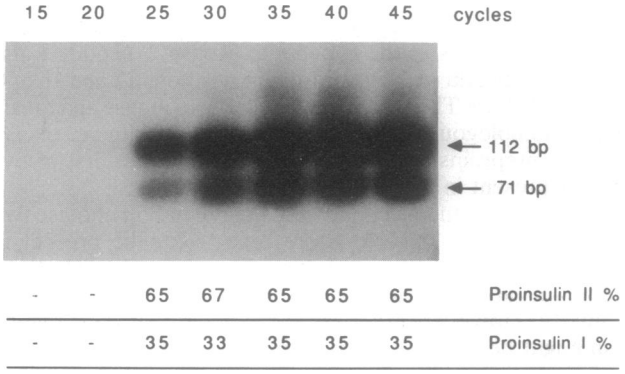

FIG. 1. Increased numbers of PCR cycles for proinsulin I and II mRNAs from adult pancreas. Ten nanograms of total pancreatic mRNA was reverse-transcribed and submitted to PCR by using a single pair of primers common to the two proinsulin mRNAs. Seven reaction tubes were run in parallel, and every fifth cycle one tube was transferred to 72°C for a final elongation lasting 10 min. The amplified DNAs were then ethanol-precipitated and solubilized in water. A portion (20%) of each sample was digested with *Msp* I; the fragments were then submitted to gel electrophoresis and hybridized to a probe homologous to both mRNAs. The resulting autoradiogram obtained after 2 hr of exposure shows hybridization to a 112-bp fragment characteristic for proinsulin II and a 72-bp fragment characteristic for proinsulin I. Percentages given below the gel indicate the radioactivity measured for each band with a Betascope 603 blot analyzer.

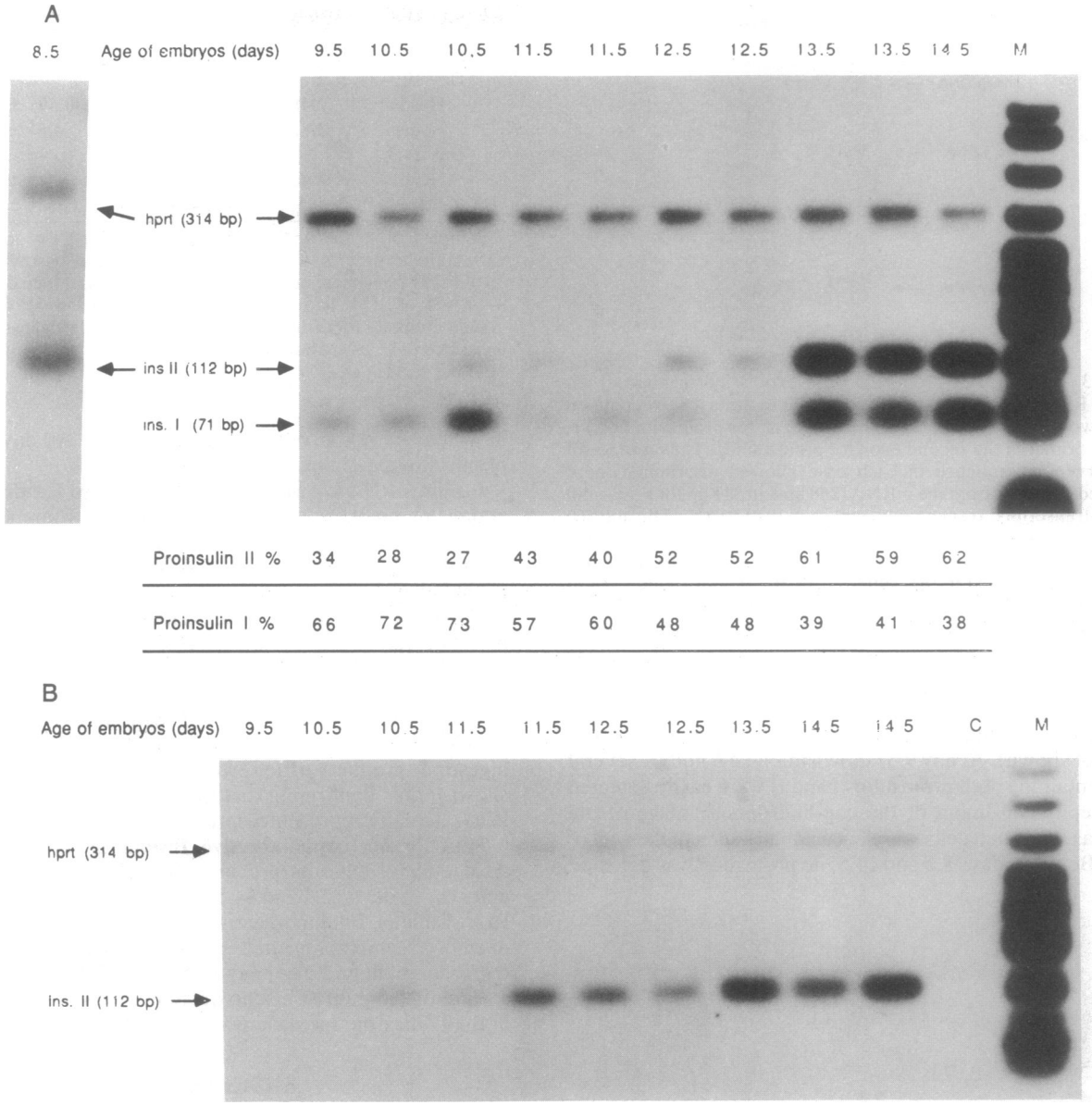

FIG. 2. (*A*) Detection of proinsulin (ins.) I and II mRNAs in embryos. Total mRNA (250 ng) from decapitated embryos of the indicated age was subjected to RT–PCR and Southern blot analysis. Two additional experiments gave identical results. Numbers given below the gel indicate the percentage of proinsulins I and II, as determined by direct quantification of beta emission. (*B*) Detection of proinsulin II transcripts in RNA preparations from embryo heads at different gestation ages. Only proinsulin II RNA could be detected. Normalization with reference to intensity of the hprt band in each sample indicated no significant difference between proinsulin II mRNA levels in the heads of different embryos of the same age. M, size markers from a *Hpa* II digest of pBR 322; C, control RT–PCR reaction with no added RNA.

Whole embryos pooled at day 8.5 were also examined, as shown in Fig. 2*A*. Surprisingly, in these prepancreatic embryos, only proinsulin II mRNA was found. This same result was found with three different primer pairs amplifying exclusively either proinsulin I or proinsulin II mRNA (data not shown). In total, 10 pools each consisting of eight day-8.5 embryos were examined. In eight pools, proinsulin I transcripts were not detected. In the other two, traces of proinsulin I transcripts were visible and were interpreted as resulting from the presence of slightly more advanced embryos in these two pools. This finding suggests that the developmental shift from proinsulin II to proinsulin I+II transcription occurs just after 8.5 days.

Pools of five blastocysts were also examined, as shown in Fig. 3. Because of the small amount of material, it was not possible to obtain DNA-free RNA preparations and, therefore, a different primer pair had to be used for each proinsulin gene. Each pair amplified a fragment of the gene including an intron and a fragment of the corresponding mRNA lacking the intron. Only the longer fragments corresponding to the genomic sequence were amplified. There was no proinsulin mRNA detected, whereas hprt mRNA was amplified as usual. Absence of expression of proinsulin in blastocysts has been reported by Heyner *et al.* (18).

Proinsulin I and II Are Detected in the Same Cells of the Developing Pancreas as Early as Day 9.5. The presence of proinsulins I and II was also examined at the protein level by immunocytochemistry. As seen in Fig. 4 *A–C*, C peptide I and II immunoreactivities were found in the central area of the islets of Langerhans at day 18.5. Competition with homologous peptide at 25 μg/ml completely abolished the immunostaining. Competition with heterologous antigen neither suppressed nor diminished immunoreactivity (data not shown). Between days 12.5 and 14.5, the staining was confined to the islet cell clusters. Also, as seen in Fig. 4 *D* and *E*, the same cells were immunoreactive for both C peptide I

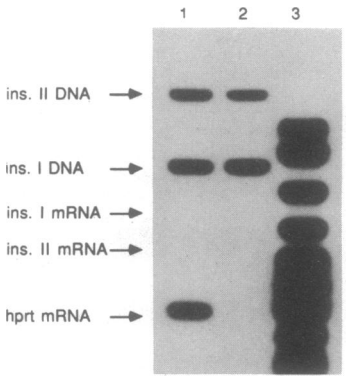

FIG. 3. Absence of proinsulin gene expression in blastocysts. Lane 1: RT–PCR was done with a 3′ primer in the last exon common to the two genes, a 5′ primer in the first exon for proinsulin (ins.) I, and a 5′ primer in the second exon for proinsulin II. The presence of an intervening sequence in each case allowed discrimination of amplified products from the mRNA (258 bp and 341 bp for proinsulin II and I transcripts, respectively) and from the DNA (747 bp and 460 bp for proinsulin II and I genes, respectively). Only amplified products from the proinsulin genes are visible. As a control, the hprt mRNA was amplified in the same RT–PCR (146 bp). Lane 2: as in lane 1 without RT. Only the DNA can be amplified. Lane 3: pBR 322 *Hpa* II. The primers used for proinsulin and hprt differed from those in Figs. 1 and 2.

and C peptide II. At day 11.5 of gestation, the same colabeling was seen in both the dorsal and ventral anlage of the pancreas (data not shown). At day 9.5, shown in Fig. 4 *F* and *G*, several cells containing both proinsulins I and II were easily detected in the epithelial lining of the cap-like dorsal anlage of the pancreas, and such cells were occasionally seen in the wall of ducts. Between days 8.5 and 14.5, no proinsulin was detected

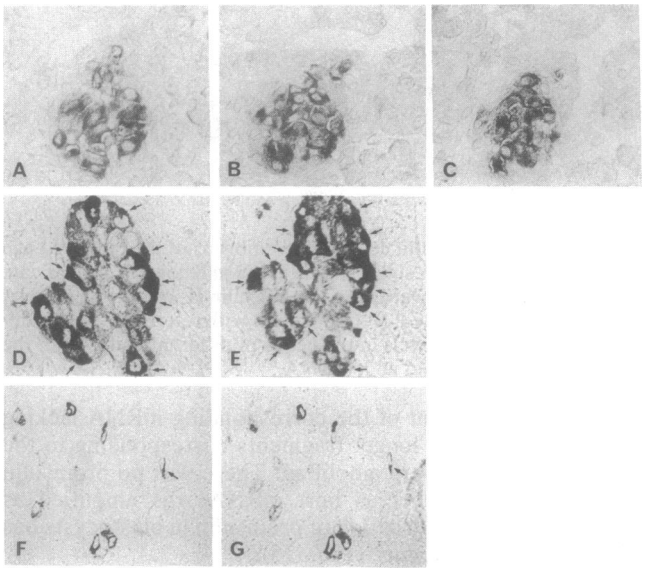

FIG. 4. Immunocytochemical localization of proinsulins I and II in developing mouse pancreas. Staining was done on 3-μm serial sections by the indirect immunoperoxidase method. (*A–C*) Day 18.5 of gestation. Beta cells, identified in a typical islet of Langerhans with anti-insulin antibodies (*B*), were also labeled with anti-C peptide I (*A*) and II (*C*) antibodies. (*D* and *E*) Day 12.5 of gestation. Proinsulin I (*D*) and proinsulin II (*E*) are coexpressed in the same cells (arrows) of primitive islets. (*F* and *G*) Day 9.5 of gestation. Proinsulin I (*F*) and proinsulin II (*G*) are detected in the same few epithelial cells of the dorsal anlage of the pancreas. Note that at this early stage, proinsulins I and II (arrow) are present in the wall of a duct. (*A–C*, ×200; *D* and *E*, ×215; *F* and *G*, ×170.)

Table 1. Immunoreactive insulin in heads of mouse embryos

	Insulin, μU	Mean ± SEM
Day 12.5		
Heads	0.48, 0.45, 0.82, 0.62	0.59* ± 0.14
Bodies	3.8, 5.7, 4.3	4.6† ± 0.8
Day 14.5		
Heads	1.4, 1.5, 1.3, 1.3	1.4* ± 0.1
Bodies	685, 504, 602, 469	565† ± 85

Samples of heads and bodies were prepared by acid/ethanol extraction, as described (19). Dried extracts were dissolved in 0.5–1 ml of 0.2 M phosphate buffer, pH 7.6/8 M urea and then diluted with 8–10 vol of RIA buffer before assays. RIAs were done by a double-antibody procedure with rat/mouse standards, essentially as described (20). μU, Microunits.
*μU per head.
†μU per body.

in whole embryos outside the pancreas. At day 8.5, no proinsulin-containing cells were detected.

Insulin mRNA Is Also Expressed in Embryo Heads. Starting at day 9.5, embryos were readily dissected from membranes and decapitated. RNA was then prepared from the heads and submitted to RT–PCR. Fig. 2*B* shows that proinsulin II mRNA was detected in the embryo heads at all ages; a total of 19 heads was tested. Transcripts for proinsulin I were never detected. This result was also found with primer pairs specific for either proinsulin I or proinsulin II (data not shown). The 188-bp-amplified fragment obtained from 13.5-day embryo head was digested with five restriction enzymes—*Alu* I, *Ava* II, *Msp* I, *Fnu*4HI, and *Hph* I. Sizes of digestion products fitted with expected sizes (data not shown), thus confirming that the transcripts detected in the heads actually represented proinsulin II mRNA.

Presence of Immunoreactive Insulin in Embryo Heads. Acid/ethanol extracts prepared from heads and bodies of embryos at days 12.5 and 14.5 of gestation were analyzed by RIA (Table 1). Immunoreactive insulin was detected in heads of embryos and represented ≈10% of the material detected in the bodies at day 12.5. At day 14.5, a 2- to 3-fold increase was seen in the amount of insulin detected in the heads, whereas in the bodies the increase was >100-fold (Table 1).

DISCUSSION

We have examined expression of the two nonallelic proinsulin genes of mouse during embryonic development. We found that both mRNAs are produced at 9.5 days of gestation when pancreas formation begins. Because, as discussed below, we also found extrapancreatic expression of the proinsulin II gene, it is significant that both proinsulin proteins were present in every insulin-producing pancreatic cell at day 9.5.

Starting at day 9.5, when the first pancreatic anlage is detected, the relative amounts of proinsulin I and II mRNAs varied with time. There was initially less proinsulin II, a finding agreeing with the results of Herrera *et al.* (10), but the proportion was reversed after 12.5 days of gestation, 1 day before proinsulin expression became high. The level of proinsulin II mRNA remained higher than that of proinsulin I, even in the adult. This latter observation in the adult is at variance with results obtained by S1 nuclease assay (1) but fit well, however, with the amounts of proinsulins I and II synthesized in isolated islets (2). The evolution of the proinsulin I/proinsulin II ratio in the embryo suggests at first glance a differential regulation of the two genes, the expression of gene II being lower than that of gene I, at the onset of expression in beta cells. Immunocytochemistry did not allow quantitative comparison between proinsulins I and II in the pancreatic cells.

The presence of proinsulin II transcripts at 8.5 days of gestation and also in the heads of embryos starting at day 9.5 was documented by RT–PCR with several different primer pairs. However, absence of proinsulin I transcripts by the RT–PCR with our single pair of primers for both genes rules out any contamination by pancreas extracts or extraneous PCR products. Also, the primers and probe sequences differed from known sequences of other members of the insulin gene family.

In the prepancreatic 8.5-day embryo, the site of expression of the proinsulin II gene is still unknown. Because proinsulin II transcripts were found in the heads starting at day 9.5, this early expression could be in neural tissue. It is also possible that proinsulin II is weakly expressed in a few cells of the primitive gut and is an early cell-lineage marker. However, we could not detect such cells by immunocytochemistry. Gittes and Rutter (21) first detected by RT–PCR proinsulin transcripts in the foregut of the mouse embryo at day 9 of gestation, only in the area of the duodenum from which the pancreas anlage will appear 10–12 hr later. Exclusive expression of only one of the proinsulin genes has already been reported for yolk sac extraplacental membranes of the rat (22–24). We found that the proinsulin II gene was expressed in the mouse yolk sac, at days 14.5 and 8.5 (L.D., unpublished data). Because of the difficulty in completely dissecting the 8.5-day-old embryo from the yolk sac, we cannot exclude that a few remaining yolk sac cells accounted for our results. Further investigation, particularly *in situ* hybridization, might be used to determine the expression site.

In the heads, expression of the proinsulin II gene is probably located in neural tissue because we found transcripts in RNA prepared from isolated brains of 15-day embryos (D.B., unpublished data). Immunoreactive insulin was detected in head extracts of embryos at days 12.5 and 14.5 of gestation, but we do not know yet whether this corresponds to insulin or proinsulin. No proinsulin-containing cells were detected in heads by immunocytochemistry. Further experiments will decide whether *in situ* hybridization allows localization of proinsulin II transcripts in a few cells of this heterogeneous population.

Proinsulin gene expression has been reported in fetal liver (24–26), hypothalamus (27), and pituitary gland (28) of rat. A chimeric gene containing the rat insulin I promoter was expressed in the ventral neural tube of transgenic mice (9). Spaventi *et al.* (29) have presented evidence that insulin is secreted by embryonic tissues during the prepancreatic stage of mouse development. Proinsulin-like immunoreactivity has been detected in cultures of fetal mouse brain cells (30). Insulin is also known to be expressed during very early embryogenesis in a number of nonmammalian species (31, 32). Shuldiner *et al.* (33) reported recently that the two nonallelic insulin genes of *Xenopus*, both expressed in pancreas, are differentially expressed during neurulation in prepancreatic embryos.

The role of proinsulin during embryonic development is poorly understood. Insulin and insulin-like factors are known to be required for rapid growth of fetal tissues, and the effect of insulin on cell proliferation in culture has been well-documented. For example, insulin added to the culture of embryonic cells from 8.5-day-old embryo stimulates their proliferation (29). Because maternal insulin does not cross the placental barrier (34, 35), synthesis of embryonic insulin might be necessary for cell growth and differentiation of neural and, possibly, other embryonic tissues before appearance of the endocrine pancreas.

Our thanks go to Don Steiner, Raymond Carroll, Diane Ostrega, and Marsha Jackson for the insulin immunoassays on mouse embryos. We also thank S. Elsevier-Saint-Just for critically reading the manuscript. L. Deltour was a fellow of the Ligue Nationale contre le Cancer and of the Association pour la Recherche sur le Cancer. This work was partly supported by the Association pour la Recherche sur le Cancer, the Ligue Nationale contre le Cancer, and the Fondation pour la Recherche Médicale.

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