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Extrapancreatic insulin gene expression in the fetal rat

(yolk sac/liver/proinsulin/mRNA quantitation)

LOUIS MUGLIA* AND JOSEPH LOCKER†‡§

Departments of †Pathology, ‡Biochemistry, and *Biophysics and Theoretical Biology, The University of Chicago, Chicago, IL 60637

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ABSTRACT **Analysis of gene expression in rat yolk sac, a primitive endodermal structure, revealed a low level of developmentally regulated insulin production. At 14 days of gestation, a 2.4-kilobase (kb) RNA species hybridized to cloned insulin gene probes. This species increased throughout gestation. At 16 days, a second transcript of 0.72 kb became visible and, by 18 days, the 0.72-kb transcript predominated. In the pancreas, the fully processed insulin mRNA is 0.55 kb long. Over the same time period in the fetal liver (also a tissue of endodermal origin, as is the pancreas), only the 2.4-kb transcript was detected; no hybridizing transcripts were detected in adult liver RNA. Gel filtration studies and insulin radioimmunoassay of acid/ethanol-soluble peptides showed approximately equal amounts of proinsulin and insulin in 18-day yolk sac, a result suggesting that the transcripts in this tissue are translated. On the other hand, a lower level of insulin and the lack of proinsulin in fetal liver were compatible with a pancreatic origin of hepatocyte insulin by receptor binding rather than intrahepatic insulin synthesis.**

Insulin gene structure, and the structure of insulin itself, is highly conserved in higher animals (1, 2). Insulin genes usually contain two introns, one in the untranslated leader sequence at the 5′ end of the gene and the other near the boundary between the COOH terminal of the insulin B chain and the NH_2 terminal of the C peptide (1). The rat is unusual in that it has two nonallelic insulin genes, which encode insulin 1 and insulin 2 (1). The products of these genes vary at three amino acids in the signal peptide, two in the B chain, and two in the C peptide (1, 2). The two genes have recently diverged (1, 2) and are normally expressed at approximately equal levels (3). The rat insulin 1 gene, however, lacks the second intron (1), an anomaly indicating that this gene did not arise simply by a duplication event.

The insulin genes are part of an evolutionarily related gene family, since proinsulin is similar in structure and amino acid sequence to at least two other insulin-like growth factors (IGF-I and IGF-II). This homology has been defined for insulin-like growth factor I at both the amino acid and nucleotide levels (4, 5) and for insulin-like growth factor II at the amino acid level (5). The insulin-like growth factors, unlike insulin, are primarily synthesized in the liver and are actively produced by many fetal tissues (6).

Insulin biosynthesis in the islets of Langerhans has been extensively studied in adult animals (for review, see ref. 7) as well as during normal development (8) and during growth of islet cell tumors (9, 10). Some reports have suggested that insulin is also produced in the brain (11), the liver (12), and perhaps other tissues (12). Insulin gene expression has been characterized *in vivo* under normal conditions, in islet cell lines, and in nonislet cell lines transformed with recombinant DNA containing the insulin gene (13–15), but extrapancreatic insulin synthesis *in vivo* remains controversial (16).

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In the course of studies of expression of the rat albumin and α-fetoprotein genes in various fetal tissues (ref. 17; unpublished results), we also studied insulin gene expression on the assumption that it would occur only in pancreas. Instead, we found evidence of insulin gene expression in fetal liver and yolk sac. The yolk sac, the liver, and the pancreas have a common embryologic origin, from the primitive endoderm (18), and all have a major function in the regulation of nutritional metabolism (18). These considerations led us to investigate the presence of both insulin gene transcripts and insulin in these tissues. Our findings are reported in this paper.

MATERIALS AND METHODS

Animals. Timed pregnant female and other adult Sprague–Dawley rats were obtained from Holtzmann (Madison, WI). Gestational age of the rats was determined from data provided by the supplier and from developmental landmarks (19).

DNA Clones. cDNA and genomic clones of the rat insulin genes were provided by Donald F. Steiner (University of Chicago). Clone pDN9 (15) consists of an ≈600-base-pair segment of the insulin 2 gene cloned into the *lacZ′* expression vector pMC1403 (20). It includes sequences in front of the gene, the 5′ nontranslated region, the first intron, and the first three amino acid codons of preproinsulin (15). Clone pRI-7 (21) is a rat insulin 1 cDNA cloned into pBR322. It contains 540 base pairs of inserted DNA, including the entire coding region of the gene and ≈200 additional bases of flanking sequences.

RNA Purification. *Cytoplasmic RNA purification.* Tissue was homogenized with 20 strokes of a Dounce homogenizer in 100 mM NaCl/10 mM Tris·HCl, pH 7.6/10 mM EDTA containing aurin tricarboxylic acid (ATA) at 100 μg/ml (22) using 20 ml of buffer per g of tissue. Nuclei were sedimented for 10 min at 3000 × *g*. Sodium dodecyl sulfate was added to the postnuclear supernatant to a final concentration of 1%. This mixture was vigorously extracted three times with phenol/$CHCl_3$/isoamyl alcohol (50:48:2). RNA was precipitated with 2 vol of ethanol at −20°C for 16 hr and stored as an ethanol precipitate. Aliquots were dissolved in water and treated with orcinol (23) for quantitation.

Total RNA purification. Total cellular RNA from adult pancreas was purified by the method of Noyes *et al.* (24) except that we used 20 ml of extraction buffer per g of tissue.

Poly(A)$^+$ RNA purification. Poly(A)$^+$ RNA was purified from total cellular or cytoplasmic RNA using poly(U)-Sepharose 4B (Pharmacia, Uppsala, Sweden). Ethanol-precipitated RNA was dissolved at 25–50 μg/ml in concentrated salt (CS) buffer (0.7 M NaCl/50 mM Tris·HCl, pH 7.6/10 mM EDTA containing ATA at 100 μg/ml). Ten milliliters of this RNA solution was passed twice over 0.5 ml of poly(U)-Seph-

Abbreviations: ATA, aurin tricarboxylic acid; kb, kilobase(s); μU, microunit(s).

§To whom reprint requests should be addressed at present address: Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261.

arose (previously allowed to swell in 1 M NaCl containing ATA at 100 μg/ml and adjusted to pH 7.6 and then equilibrated with CS buffer) in a Pasteur pipette. The column was then washed with 5 ml (10 vol) of CS buffer. Poly(A)$^+$ RNA was eluted with 2 ml of elution buffer (90% formamide/10 mM EDTA/0.2% Sarkosyl containing ATA at 100 μg/ml). If the column was to be reused, it was washed with 20 vol of elution buffer and then with 10 vol of CS buffer. Poly(A)$^+$ RNA was precipitated from the formamide solution with 3 vol of ethanol for 16 hr at −20°C.

Gel Electrophoresis, Blotting, and Hybridization. RNA was dissolved in 75% formamide to prevent aggregation, resolved on 1.5% agarose/6 M urea gels (25), and blotted to nylon membranes (Genatran, D and L Filter Corporation, Woburn, MA) according to the method of Thomas (26). Hybridization was carried out with nick-translated (27) [^{32}P]-DNA at 50 ng/ml, at an average specific activity of 6×10^7 dpm/μg of DNA, as described in Kunnath and Locker (28). Autoradiograms were quantitated with a scanning densitometer.

Insulin Extraction and Chromatography. For insulin extraction according to Davoren (29), tissue was rapidly homogenized in a Dounce homogenizer in cold 65% ethanol/0.1 M HCl, using at least 1 ml/50 mg of tissue. The extract was incubated for 2 hr at 4°C and debris was sedimented at 2000 × *g* for 10 min. pH was adjusted to 5.5 with NH_4OH, and 2 vol of ethanol and 4 vol of diethyl ether were added. This mixture was incubated overnight at 4°C, and the precipitate was sedimented at 4000 × *g* for 15 min. The pellet was drained and then suspended in 1.5 ml of 3 M acetic acid, and the solution was adjusted to pH 2.5. This mixture of dissolved peptides was chromatographed on Bio-Gel P-10 (Bio-Rad) that had been equilibrated with degassed 3 M acetic acid containing bovine serum albumin at 10 μg/ml as described by Robbins *et al.* (30).

Insulin Radioimmunoassay. Column fractions were lyophilized and dissolved in 0.113 M borate buffer (pH 8.0) containing bovine serum albumin at 5 mg/ml. Radioimmunoassay was carried out in the laboratory of Arthur Rubenstein according to the method of Starr *et al.* (31).

RESULTS

Detection of Insulin Gene Transcripts. Hybridization of a radiolabeled probe for the rat insulin 2 gene to total cytoplasmic RNA from fetal liver and yolk sac detected two specific transcripts. Hybridization to a series of yolk sac RNAs from 12, 14, 16, and 18 days of gestation is shown in Fig. 1*A*. A 2.4-kilobase (kb) transcript is apparent at 14, 16, and 18 days, and a 0.72-kb transcript is weakly detectable at 16 days and prominent at 18 days. There is a marked increase in the 0.72-kb transcript in late gestation, while the 2.4-kb transcript increases more gradually over a longer period of time. In fetal and 1-day neonatal liver (Fig. 1*B*), we detected the 2.4-kb transcript but not the 0.72-kb transcript. For further analysis, we purified poly(A)$^+$ RNA, which enabled increased loading of mRNA on the gel before blotting, and repeated the hybridization experiments (Fig. 1*C*). To facilitate identification of insulin gene transcripts, we also included poly(A)$^+$ RNA purified from total pancreas. As expected, a single insulin mRNA band at 0.55 kb was predominant in the pancreatic preparation; the transcripts previously detected in liver and yolk sac total cytoplasmic RNA showed augmented hybridization, indicating that they had been enriched in the poly(A)-containing fraction. They were clearly of different size than pancreatic insulin mRNA.

The three hybridization experiments described above were carried out under stringent conditions (i.e., 50% formamide and 37°C), and the results indicate a high degree of homology between the labeled probe and the transcripts. Under these conditions, pDN9 hybridizes only to insulin 1- and insulin 2-specific bands in DNA digests (ref. 2 and unpublished results). However, we felt it necessary to evaluate hybridization under even more stringent conditions (i.e., 50% formamide and 50°C) (Fig. 1*D*). In this experiment, we used pRI-7, which contains only DNA sequences from within the insulin structural gene, as the probe to rule out cross-hybridization to a sequence flanking the insulin gene. In addition,

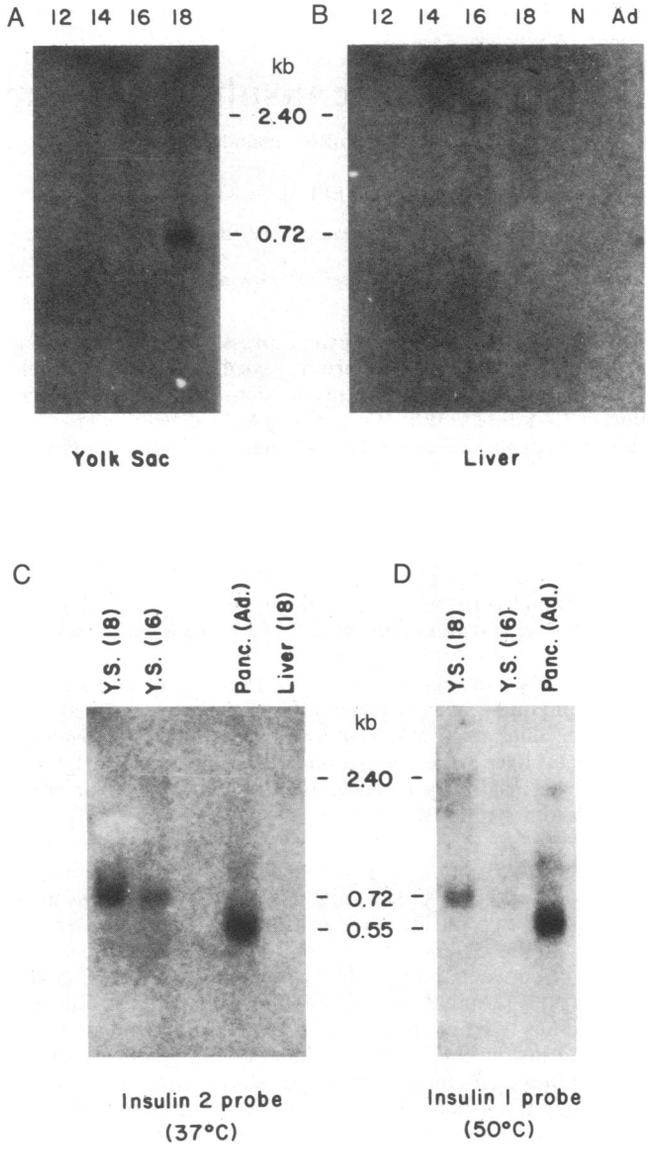

FIG. 1. Hybridization to insulin-specific transcripts. All hybridizations were carried out using RNA resolved on 1.5% agarose/6 M urea gels (25) and blotted to nylon membranes. RNA was dissolved in 75% formamide to prevent aggregation. Approximate transcript size was calculated from the relative migration of 28S and 18S rRNA and pancreatic insulin mRNA (3). Hybridizations used plasmid pDN9 at 37°C (*A–C*) or pRI-7 at 50°C (*D*). (*A*) Hybridization to total cytoplasmic RNA preparations from yolk sacs of 12, 14, 16, or 18 days of gestation. Each gel lane contained 10 μg of RNA. (*B*) Hybridization to total cytoplasmic RNA preparations from livers of 12, 14, 16, or 18 days of gestation as well as a 1-day-old neonate (lane N) and an adult (lane A). Each gel lane contained 10 μg of RNA. (*C*) Hybridization to purified poly(A)$^+$ RNA. An amount of poly(A)$^+$ RNA equivalent to 50 μg of total RNA was loaded in each lane. Poly(A)$^+$ RNA from pancreas, equivalent to 10 μg of total RNA, was included as a control. (*D*) Hybridization to purified poly(A)$^+$ RNA, equivalent to 50 μg of total RNA, was carried out using pRI-7 at 50°C. Y.S., yolk sac; Panc., pancreas; Ad., adult.

this probe originates from insulin 1 mRNA. The same transcripts at 0.72 and 2.4 kb were readily detected, indicating that these transcripts contain base sequences almost totally homologous to the insulin gene (Fig. 1*D*). It should be pointed out that the insulin 1 and insulin 2 genes are 93% homologous in their coding regions (1) and we observe them to cross-hybridize even under these conditions and that the two 0.55-kb mature insulin messages are not resolved from one another in this gel system.

Despite the low intensity of these hybridizations due to low levels of transcripts, we were able to compare them to the hybridization of a labeled probe for albumin mRNA (17). We used this albumin mRNA hybridization as a standard to determine the absolute amounts of insulin gene transcripts in these preparations, from which we calculated the number of molecules per cell of each transcript (Table 1). It was assumed that all cells contain the same amount of total RNA and, further, that the tissues are homogeneous. Thus, these values provide a useful perspective as a reasonable first approximation to the actual insulin mRNA levels. A day 18 yolk sac cell has about 1/60th the level of insulin mRNA (0.72 kb) of a pancreatic islet cell. This level is attained following a 5-fold increase between 16 and 18 days of gestation. The 2.4-kb transcript, also found in fetal liver, is present at significantly lower levels. From these hybridizations, we estimate a lower limit of detection of about 50 molecules per cell.

Detection of Insulin. Once we had established that insulin gene transcripts were present in yolk sac, we analyzed the tissue for the presence of insulin. In this analysis, we extracted acid/ethanol-soluble peptides, chromatographed this peptide mixture on Bio-Gel P-10, and carried out an insulin radioimmunoassay on the fractions. The results are shown in Fig. 2. The yolk sac analysis (Fig. 2*A*) showed an immunoreactive peak that migrated to the same position as porcine insulin (Fig. 2*E*). Another immunoreactive peak of similar proportion eluted at the position characteristic of proinsulin. Peptides from fetal pancreas (Fig. 2*B*) showed two peaks in the same positions, although in this case the insulin peak was much larger than the peak of proinsulin and related conversion intermediates. We last evaluated the fetal liver (Fig. 2*D*) and found a small amount of insulin but no proinsulin. This small peak of hormone could not have been a contaminant

Table 1. Quantitation of insulin-specific transcripts

Tissue	Gestational age, days	RNA species,* kb	Molecules per cell†
Yolk sac	16	2.40	110
		0.72	960
	18	2.40	560
		0.72	2,800
Liver	18	2.40	450
		0.72	ND‡
	Adult	2.40	ND‡
		0.72	ND‡
Pancreas§	Adult	0.55	1,600
Islets‖		0.55	160,000

*Normal insulin message in pancreas is 0.55 kb long (9). The transcripts observed in liver and yolk sac are 0.72 and 2.40 kb long (Fig. 1).

†Calculated from our hybridization data, controlled with hybridization to liver albumin mRNA (17), using the measurement of albumin mRNA per liver cell of Nahon *et al.* (32) and assuming a constant amount of RNA per cell in different tissues.

‡Hybridization not detected.

§Calculated from the value 15 pg of insulin mRNA per μg of total RNA given by Kakita *et al.* (8) and the value 36.9 pg of RNA per cell given by Nahon *et al.* (32).

‖Calculated from the above values assuming that islets comprise 1% of the pancreas.

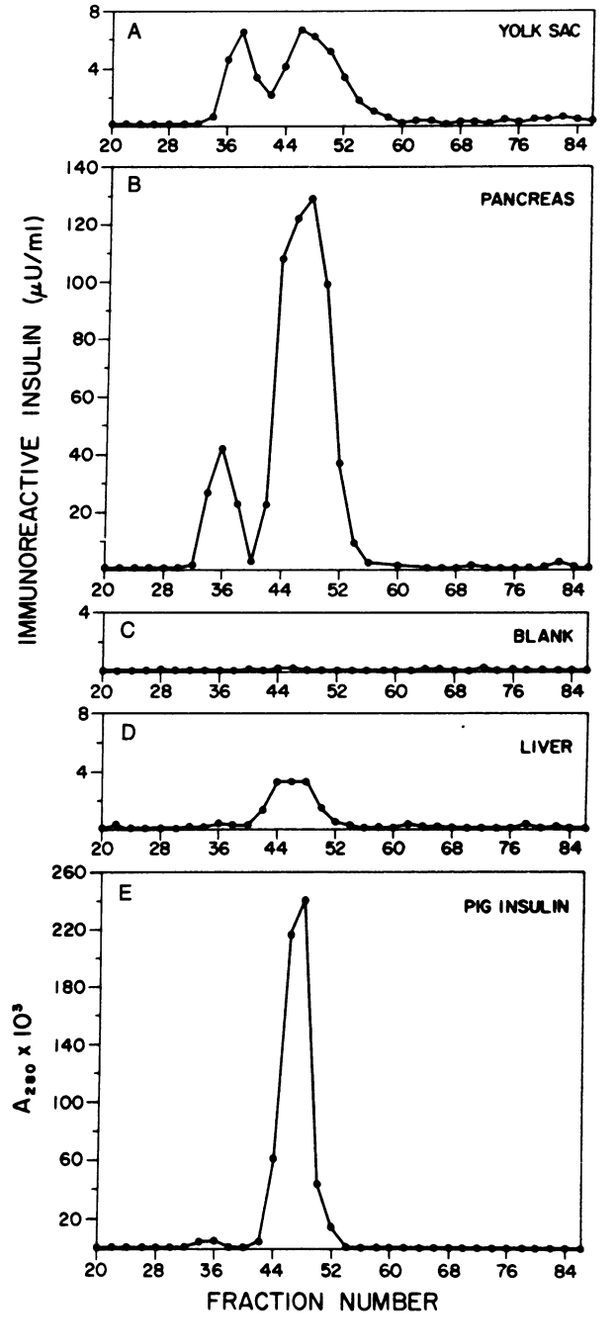

FIG. 2. Chromatographic resolution of immunoreactive insulin. Acid/ethanol-soluble peptides from 10 day 18 fetal rats were resolved on a column of Bio-Gel P-10 equilibrated with 3 M acetic acid containing bovine serum albumin at 10 μg/ml; the column was 55 × 1.5 cm, and 1-ml fractions were collected. The excluded volume peak is in fraction 31. Fractions were lyophilized and then dissolved in radioimmunoassay buffer. All runs were carried out on the same column in the order shown. The column was extensively washed between runs. The blank was included to ensure that the column was not contaminated with pancreatic insulin. As a standard, 1 mg of porcine insulin was chromatographed and detected by absorbance at 280 nm.

from previous runs on the column because the liver analysis was preceded by running a blank column without any tissue extract (Fig. 2*C*).

The quantitative analysis of results obtained from gel filtration of extracted yolk sac and from day 18 fetal rat serum is presented in Table 2. Since fetal serum contains only 0.030 microunit (μU)/mm^3, neither the yolk sac nor the liver insu-

Table 2. Quantitation of immunoreactive insulin in day 18 fetal tissues

Tissue	Weight,* mg	Insulin, ng/g of tissue†
Yolk sac	32	8.0
Liver	59	1.6
Pancreas	6	667.0
Serum	—	1.2‡

*Average values determined in this laboratory.
†Calculated from total areas of the chromatographic peaks of Fig. 2, using the conversion 1 ng of insulin = 25 μU.
‡Assuming a serum density of 1 g/ml.

lin represent simple serum contamination: 30 mg of serum would contain 0.9 μU, while a 30-mg yolk sac contained 6.4 μU. The 2.3 μU detected in the fetal liver, however, is only slightly more than the amount contained in an equivalent weight of serum (≈1.8 μU).

***In Vivo* Labeling Experiment.** We felt it desirable to demonstrate synthesis of insulin and proinsulin in the yolk sac, to confirm our findings based on RNA hybridization and tissue analysis. A day 18 yolk sac was incubated with 100 μCi of [^{3}H]leucine (1 Ci = 37 GBq) in 0.5 ml of modified Eagle's medium for 2 hr at 37°C. Acid/ethanol-soluble peptides were extracted and pooled with the extracts from nine unlabeled yolk sacs. The region of the insulin and proinsulin peaks was pooled, immunoprecipitated with rabbit anti-insulin serum (provided by Donald F. Steiner, University of Chicago) and staphylococcal protein A (Pansorbin, Calbiochem), and rechromatographed after dissociation of the precipitated complexes. Rechromatography yielded three peaks, one in the excluded volume, one at the position of proinsulin, and the last at the position of insulin. However, the level of radioactivity was so low, 0.007% of total incorporation into acid/ethanol-soluble peptides, that we were unable to characterize this material further.

DISCUSSION

Presence of Insulin in the Fetal Yolk Sac and Liver. Our data indicate not only that a significant amount of insulin is present in the fetal yolk sac but also that insulin is synthesized there. We detected not only insulin, but also proinsulin, and an RNA species likely to be insulin mRNA. These latter observations indicate that insulin synthesis occurs in these cells. The fetal liver seems to represent a different situation. It contains less insulin than the yolk sac and, although it contains a small amount of a high molecular weight transcript from the insulin gene, there is no species likely to be a functional insulin mRNA. Further, the absence of proinsulin makes it unlikely that insulin is synthesized and processed in this tissue.

We think it likely that the low level of insulin present in day 18 fetal liver represents receptor-bound or internalized insulin. An adult liver contains 1.69×10^8 hepatocyte nuclei per ml (33), and a fetal liver, with its hematopoietic cells, has about half that many. From our observations, the concentration of insulin we measured amounts to about 1700 insulin molecules per hepatocyte. At the serum level we measured, 30 μU/ml (185 pM), 1700 insulin molecules per hepatocyte could be accounted for by 20,000 insulin receptors per hepatocyte and a K_d of 2×10^{-9}. We did not measure insulin in the adult liver, although this has been reported elsewhere (12). The fact that we could not detect any form of insulin gene transcript in adult liver makes it unlikely that insulin is synthesized in this tissue.

Variety of Insulin Gene Transcripts. In rat pancreas, normal transcription and RNA processing from the insulin genes should produce several discrete transcripts, most of which have been characterized (9). Mature insulin 1 and insulin 2 mRNA are each 0.55 kb long. An ≈1.2-kb species represents an unprocessed insulin 2 transcript containing both introns, and an ≈0.65-kb species may be either an unprocessed insulin 1 transcript or an insulin 2 transcript from which the large intron has been excised. An ≈1.1-kb species, an insulin 2 precursor from which the small intron has been excised, is also expected. Lomedico *et al.* (1) have localized the 5′ end of the mature message; this result would indicate only this series of transcripts at 1.2, 1.1, 0.65, and 0.55 kb. However, a low level of transcription from other promoters has not been ruled out, and one study, an analysis of insulin gene transcripts in human pancreas and islet cell tumors, showed a small amount of hybridization to high molecular weight RNA in addition to the known insulin gene transcripts (10).

From our data, we discerned two insulin gene transcripts not readily defined by the expected processing scheme. The 0.72-kb transcript found in yolk sac appears to be a functioning mRNA. There are several possible explanations for the existence of this transcript. (*i*) The intron in the nontranslated leader sequence, common to both insulin genes, may not have been excised from this transcript. The predicted size would be 0.67 kb, consistent with the 0.72 kb we measured on partially denaturing agarose/urea gels, which give only approximate molecular weights (25). From sequence data (1), such a transcript would have an appropriately located binding site for the small ribosomal subunit and would initiate translation from the same AUG codon as the normal message. (*ii*) The 0.72-kb transcript may contain the same coding region as the 0.55-kb transcript, but with a longer poly(A) tail. Hyperpolyadenylylation has been noted in some islet cell tumors (J. Michael Welsh, University of Chicago, personal communication). (*iii*) Transcription may begin at a new promoter, 5′ to the normal insulin gene promoter or transcription may terminate at a new sequence. (*iv*) Finally, and least likely, the 0.72-kb transcript may result from an alternative splicing pathway and may contain additional base sequences not found in the usual insulin gene transcript.

The origin of the 2.4-kb transcript is even more problematic. Because of its large size, this transcript is unlikely to be the mRNA for a protein the size of normal proinsulin nor does its presence seem to correlate with proinsulin synthesis. Possibilities *iii* and *iv* (above) could be modified to account for the 2.4-kb transcript, but this transcript might also have arisen from incomplete processing of the 0.72-kb transcript (assuming a new promoter) or from transcription of the other DNA strand. Further analysis should elucidate the nature of both the 2.4- and 0.72-kb transcripts.

Physiologic Significance. Synthesis of insulin by the yolk sac may have a role in fetal development. There is a marked rise in pancreatic insulin and serum insulin late in gestation; the peak is not seen until shortly after birth (8). The rise in yolk sac insulin is similar to that in pancreas between days 16 and 18; we did not sample later times during gestation. The common rise might imply a common developmental mechanism or a humoral control. It is noteworthy that, although most of the insulin in the pancreas is probably stored for postnatal secretion (34), the yolk sac involutes at birth. In addition, the yolk sac is believed to have an absorptive and nutritional regulatory role in the fetus (20), and it is closely related embryologically to the placenta, which has known endocrine function during gestation. It remains possible, however, that, when very sensitive detection methods are used, low levels of expression of genes may be found in many tissues, possibly resulting simply from transient partial accessibility of their promoters to RNA polymerase II as development progresses.

In conclusion, we have detected definite extrapancreatic insulin gene expression during fetal development, in the yolk sac, and what may well be abortive insulin gene expression

in the fetal liver. Both show alterations in RNA processing or transcriptional promoter selection compared with pancreatic islets. These alternative modes of expression may be especially relevant to DNA-mediated insulin gene transfer experiments (13–15), since, to produce high levels of insulin, transfected cells must have mechanisms for correct transcription, processing, sequestration, and secretion control. Finally, these RNA processing or transcription controls are likely to be important mechanisms in differentiation.

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