Insulin-Gene Expression in Extrafetal Membranes of Rats

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We quantified fetal rat extrapancreatic insulin-gene expression by measuring mRNA in the yolk sac and placenta. Yolk sac makes a significant contribution to the total fetal insulin stores. The placenta contains a much smaller amount of insulin mRNA. Yolk sac insulin mRNA is barely detectable at 16 days gestation but increases markedly to a maximum at 21 days, 1 day before birth. In contrast to the pancreatic 550-nucleotide (n) insulin mRNA, yolk sac has a 720-n mRNA. However, on removal of the terminal poly(A), both transcripts produce a 440-n RNA, the size predicted for a fully processed insulin mRNA. *Diabetes* 38:39–43, 1989

Despite the absence of insulin transport across the placenta, high-level insulin biosynthesis is found only in late gestation. In the fetal rat, this late-gestational insulin synthesis occurs not only in the islets of Langerhans but also in the yolk sac (1). Pancreatic insulin is first detectable at ~15 days gestation and increases 100-fold to approach the adult level by day 20 (2). The developmental regulation of yolk sac insulin-gene expression is the subject of this study.

Insulin has several important effects in late gestation. One striking effect is a massive insulin-dependent hepatic uptake of glucose followed by hepatic glycogen synthesis (3–6). Hepatic glycogen stores peak on day 21 of gestation and break down rapidly after birth (3–6). Similarly, hydroxymethylglutaryl-CoA reductase is stimulated by insulin during late gestation, apparently resulting in the very high hepatic cholesterol synthesis of late gestation (7). Insulin also appears to be one of the major anabolic factors that regulates both fetal size and the amount and distribution of fetal adipose tissue (8).

We have previously described a new source of insulin in the rat fetus (1). The yolk sac, an extrafetal organ believed to have a role in the absorption of nutrients, contains insulin mRNA, immunoreactive insulin, and a proinsulin-like species. The yolk sac persists as a viable tissue throughout the entire gestation of the rat. Yolk sac insulin biosynthesis differs from pancreatic insulin biosynthesis in at least two ways. The major yolk sac insulin-gene transcript is 720 nucleotides (n), not 550 n as in pancreas. Also, yolk sac contains an unusually high proportion of proinsulin-like material (1).

In our previous study, we showed qualitative data indicating the presence of insulin-gene products in the rat yolk sac. Liu et al. (9) have reported the presence of a probable insulin mRNA in human placenta. The level of this transcript was elevated in a case of maternal diabetes, suggesting that it is metabolically regulated. In this study, we quantified insulin mRNA in the yolk sac, placenta, and other extrafetal membranes. This enabled us to define the developmental pattern of insulin-gene expression in these tissues and to show that there is much more extrafetal insulin mRNA than our previous study indicated. We also determined the structure of the 720-n insulin-gene transcript.

MATERIALS AND METHODS

Timed pregnant KGH rats were provided by H. Kunz (Department of Pathology, University of Pittsburgh, Pittsburgh, PA). KGH rats ($RT1^9$) were chosen because this inbred strain has a high pregnancy rate and consistently large litter size (10). KGH rats have a gestation time of 22 days (conception occurs on day 0) and usually have 12–14 offspring per litter. Two to three litters each from gestation days 16, 18, 20, 21, and 22 were studied. Organs from each litter were pooled into yolk sac and placenta preparations. Each preparation was then analyzed separately. We compared KGH preparations with RNA from Sprague-Dawley rats obtained in our previous studies (1). There were no significant developmental differences in insulin biosynthesis between the strains.

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Dissection procedures. Rats were dissected under ether anesthesia and subsequently killed by transection of the aorta. The uterus was carefully opened, and only fetuses within intact amniotic sacs were used. The yolk sac was removed intact; it was fully extended with a forceps and cut off at the umbilicus after carefully removing any loops of bowel. Other extrafetal membranes (predominantly amnion) were picked up with a forceps and added to the yolk sac. These additional membranes added 20–40% to the yolk sac mass and were included because they sometimes contained yolk sac fragments. A layer of yolk sac was carefully stripped from the fetal surface of the placenta (11), although some small fragments probably remained. The tissues were drained of excess liquid, chilled on ice, immediately weighed, and homogenized for RNA purification. From opening the uterus, it took 5–10 min to process a litter through homogenization. No RNA degradation was observed on stained electrophoretic gels.

RNA purification and quantification. Our cytoplasmic RNA purification, quantification, electrophoresis, and blotting protocols have previously been described (1,12). Briefly, tissues were homogenized in >20 ml/g of 100 mM NaCl, 10 mM Tris, pH 7.6, 50 mM EDTA, 100 μg/ml aurin tricarboxylic acid. Nuclei were sedimented out, and the solution was made 1% sodium dodecyl sulfate and extracted several times with phenol/chloroform/isopentyl alcohol. RNA was precipitated with ethanol, redissolved in H_2O (2 cycles), and quantified with the orcinol reaction.

Recombinant DNA and hybridization conditions. Plasmid prI2c is a subclone of the rat insulin 2 gene in the vector pGEM-2 (Promega Biotech, Madison, WI; Fig. 1). We derived this subclone from a λ-genomic clone of the rat insulin 2 gene (provided by A. Efstratiadis, Harvard University, Cambridge, MA; 13).

Riboprobe transcripts of prI2c were used for quantitative hybridization to RNA prepared from the developmental stages. To make an antisense RNA probe, prI2c was cut with *Hae*III and transcribed with T7 RNA polymerase via α-[^{32}P]CTP. The 20-μl reaction mix contained 200 ng cleaved DNA, 40 mM Tris, pH 7.6, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 2.5 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 50 μCi α-[^{32}P]CTP (400 Ci/mmol), and 10 U T7 RNA polymerase. After 60 min of incubation at 37°C, the entire reaction mix was loaded on an agarose-urea gel and resolved for 1 h. A 450-n transcript band was detected by autoradiography, excised from the gel, and extracted from the crushed gel slice by freeze-thaw followed by 10 min centrifugation at 13,000 × *g*. The extruded liquid was used directly as a hybridization probe. We modified our standard conditions to obtain specific hybridization with this probe (14). Hybridization at 60°C in 50% formamide followed by washes at 60°C detected only insulin mRNA in pancreatic preparations. Lower hybridization temperatures gave nonspecific hybridization to rRNA bands. Hybridization was quantified with a densitometer.

In poly(A) cleavage experiments, we used nick-translated DNA from cDNA clone pRI-7 (provided by D.F. Steiner, University of Chicago, Chicago, IL; 15), which was hybridized at 37°C in 50% formamide.

Poly(A) removal. For poly(A) removal, we modified the method of Vournakis et al. (16). Ten-microgram aliquots of total RNA were annealed to 10 μg of oligo-dT (Bethesda Research, Bethesda, MD) for 30 min at room temperature in 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 100 mM KCl. The mixtures were then incubated with RNase H (Bethesda Research) followed by phenol extraction and ethanol precipitation. RNA pellets were redissolved, resolved on agarose-urea gels, blotted, and hybridized to ^{32}P-labeled pRI-7 DNA.

RESULTS

Analysis of mRNA structure. We compared the 720-n yolk sac insulin-gene transcript to the 550-n transcript found in adult and fetal pancreas. After poly(A) removal, the 550- and 720-n transcripts produced the same 440-n RNA band (Fig. 2). Thus, both RNAs have contiguous 440-n regions with high homology to the probe and poly(A) regions of 110 and 280 n, respectively.

mRNA quantification by blot hybridization. We extracted total RNA from late-gestational yolk sac and placenta and recorded the tissue weights and the yield of RNA. This analysis extended our earlier study to the end of gestation, when pancreatic insulin increases markedly.

RNA recovery from yolk sac was ~1.4 mg RNA/g tissue. The placenta had a lower RNA content (0.8 mg/g tissue). These values did not change significantly after 16 days of gestation. In a previous study, we quantified extraction of yolk sac and liver mRNA. Extraction was incomplete in small

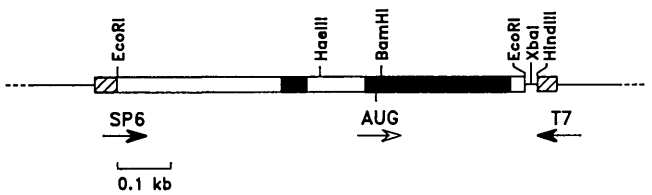

FIG. 1. Map of plasmid prI2c. *Eco*RI segment (0.8 kb) of rat insulin 2 gene (11) was cloned into *Eco*RI site of plasmid vector pGEM-2. Map shows insulin-gene exons (*solid area*), noncoding regions (*open area*), and SP6 and T7 promoters of vector (*hatched area*). Direction of transcription from each promoter is indicated by *solid arrow*. AUG, initial methionine codon in coding portion of insulin gene. *Open arrow* indicates direction of insulin-gene transcription in vivo. For synthesis of antisense strand probe, DNA was cleaved with *Hae*III and transcribed with T7 RNA polymerase.

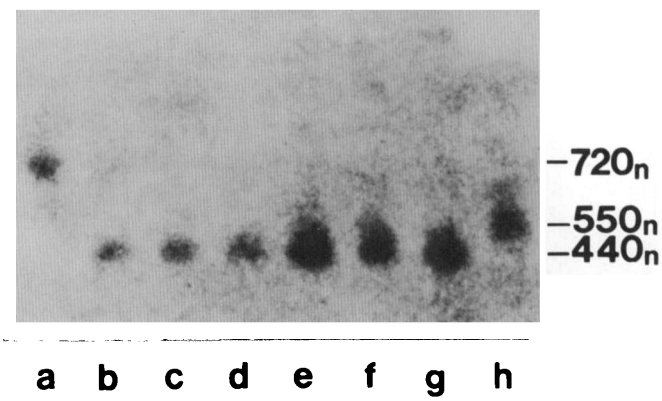

FIG. 2. Effect of poly(A) removal on insulin mRNA size. Total 18-day yolk sac (*lanes a–d*) or pancreatic (*lanes e–h*) RNA was annealed to oligo-dT and treated with varying concentrations of RNase H (0 U, *lanes a* and *h*; 0.1 U, *lanes b* and *g*; 0.5 U, *lanes c* and *f*; 1 U, *lanes d* and *e*), as described in MATERIALS AND METHODS. Both 720-n yolk sac and 550-n pancreatic mRNA cleave at all enzyme concentrations to 440 n.

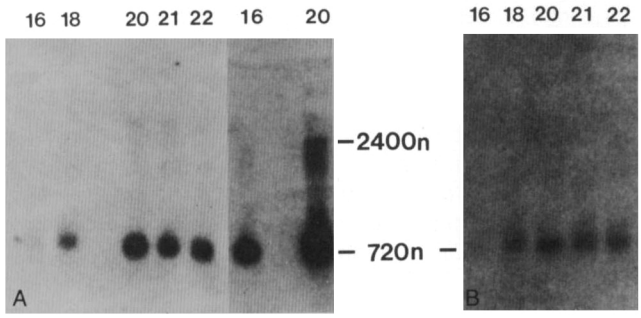

FIG. 3. Blot hybridization of insulin RNA. *A*: hybridization to yolk sac RNA. Five micrograms of yolk sac RNA from 16, 18, 20, 21, and 22 days gestation was resolved on agarose-urea gel, blotted to nylon membrane, and hybridized for 16 h to 1 × 10^6 cpm/ml of antisense riboprobe. Autoradiogram was exposed for 16 h. Two lanes on *right* (16 and 20 days) show hybridization to 10 μg RNA with 2 × 10^6 cpm/ml of probe and 40 h autoradiogram exposure. *B*: hybridization to placental RNA. Five micrograms of placental RNA from 16–22 days gestation was analyzed as in *A*. Autoradiogram was exposed for 64 h.

volumes (e.g., 3 ml/g tissue) but complete if volumes >10 ml/g tissue were used (12). For the preparations in this study, we used ≥20 ml extraction buffer/g tissue, a condition determined to give complete mRNA recovery. We did not further analyze recovery in the preparations used for this study.

We made a special attempt to quantify mRNA in all extrafetal membranes to provide an estimate of total insulin stores. We had previously found low levels of insulin mRNA in the placenta and no insulin mRNA in amnion (unpublished observations). Because of the large size of the placenta, the observation of insulin mRNA suggested substantial insulin stores. In this study we were careful to separate yolk sac from placenta. However, we added all additional extrafetal membranes, largely amnion, to the yolk sac preparation so that measurements would be quantitative.

A riboprobe hybridization system improved detection of insulin mRNA. Representative hybridizations are illustrated in Fig. 3. We used these and similar hybridization experiments to quantify the 720-n transcript.

A 2400-n transcript band was also detected. It was much weaker than the 720-n band and difficult to quantify. Higher-resolution electrophoresis experiments indicated that the 2400-n band actually represents several closely spaced bands (not illustrated). The blur between the 2400- and 720-n bands suggests that some of the higher-molecular-weight transcript was degraded or that other transcripts were present but poorly resolved (Fig. 3). The 2400-n band was detectable (on long exposures) in almost all of the RNA preparations, but it was usually too weak to quantify.

We used the hybridization experiments and total yields of RNA to determine relative 720-n mRNA levels and amounts. These were compared to the growth of yolk sac and placenta. The three analyses are presented in Fig. 4. Relative mRNA levels increased 10-fold from day 16 to day 20 of gestation and then moderately decreased to the end of gestation (day 22). The yolk sac continued to grow until day 21, and thus total mRNA was greater on day 21 than day 20. Day 22 showed a reduction in mRNA level, total mRNA, and tissue mass. The latter probably reflects the beginning of parturitional involution. Placenta had much lower mRNA lev-

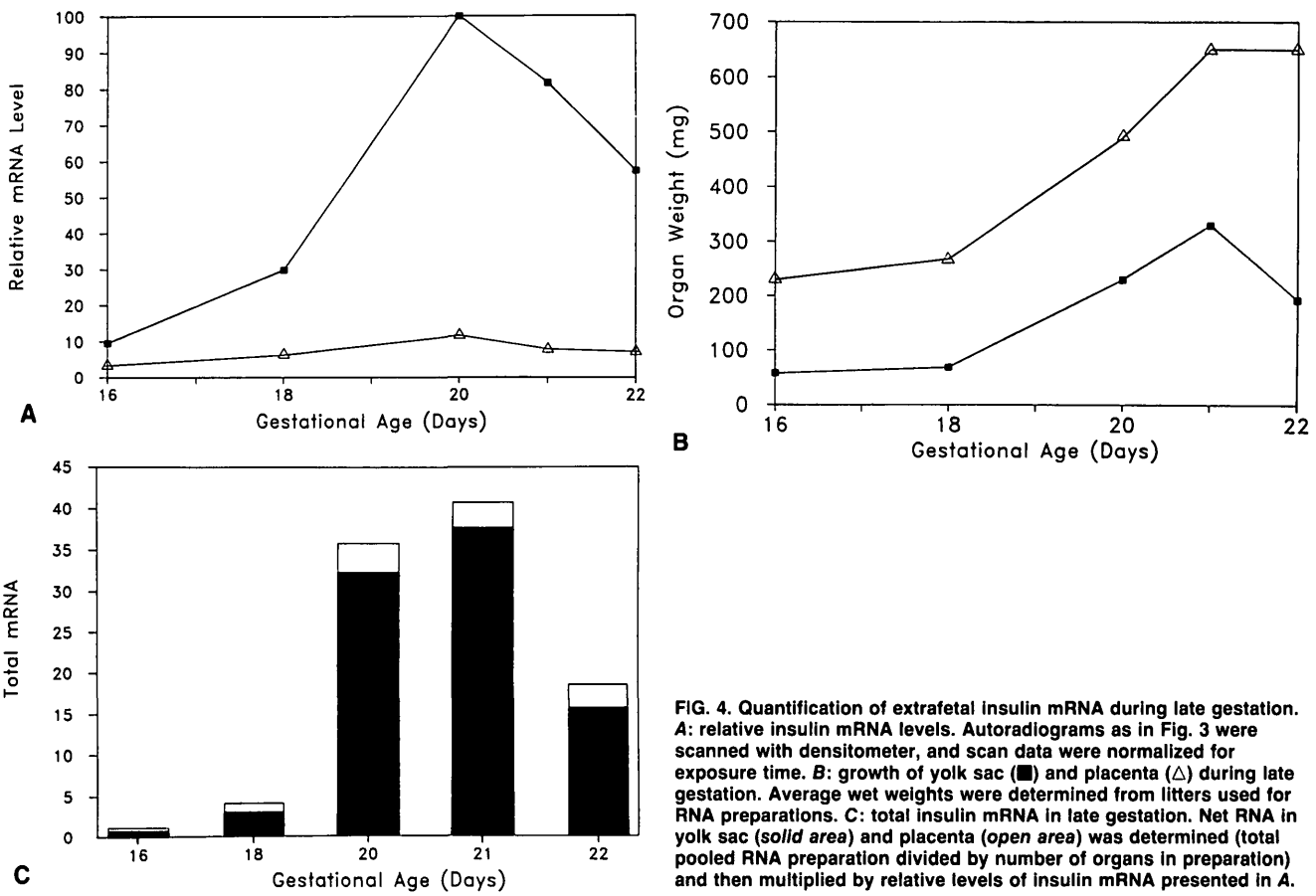

FIG. 4. Quantification of extrafetal insulin mRNA during late gestation. *A*: relative insulin mRNA levels. Autoradiograms as in Fig. 3 were scanned with densitometer, and scan data were normalized for exposure time. *B*: growth of yolk sac (■) and placenta (△) during late gestation. Average wet weights were determined from litters used for RNA preparations. *C*: total insulin mRNA in late gestation. Net RNA in yolk sac (*solid area*) and placenta (*open area*) was determined (total pooled RNA preparation divided by number of organs in preparation) and then multiplied by relative levels of insulin mRNA presented in *A*.

els (Fig. 4*A*), and despite its relatively large size (Fig. 4*B*), it contributed only a small amount of the total mRNA (Fig. 4*C*).

DISCUSSION

mRNA structure. The 720- and 550-n insulin-gene transcripts differ in the length of their poly(A) segments. Both appear to be fully processed mRNAs containing the 440-n coding region predicted by the insulin 1– and insulin 2–gene sequences (13). The insulin 1– and insulin 2–gene coding regions have such high homology that RNase H cleavage under the conditions we used would not distinguish their transcripts. The 720-n transcript could thus be the product of either insulin gene. Because the poly(A) tail shortens as mRNA persists in the cytoplasm (17,18), a longer poly(A) region could indicate an mRNA that turns over more rapidly before the poly(A) has had a chance to be shortened, or an mRNA with a longer half-life with more poly(A) to be removed. Alternatively, mRNA may be protected from poly(A) processing if it is not translated. We observed immunoreactive insulin in yolk sac (1), so the 720-n transcript appears to be a functional mRNA. Muschel et al. (19) reported insulin mRNA with poly(A) tails 120–140 n longer than normal in insulinoma cell lines. They found that high levels of glucose increased both the level of insulin mRNA and the length of the poly(A) segment. The significance of longer poly(A) is unclear but must indicate that yolk sac has posttranscriptional controls that differ from pancreas.

In addition to the 720-n transcript, we demonstrated higher-molecular-weight transcripts, particularly at 2400 n. The latter transcript(s) is present at very low levels, and we did not try to quantify it. Our previous study showed 2400-n transcripts as early as 16 days and possibly even at 14 days in yolk sac, but these hybridizations were very weak (1). This study (and unpublished observations) suggests that the 2400-n transcript level changes in parallel with the 720-n transcript. The 2400-n transcript is polyadenylated (1). Also, hybridization to total cellular, nuclear, and cytoplasmic RNA from yolk sac showed that the 2400-n transcript is a cytoplasmic RNA not a contaminating nuclear RNA (L.M. and J.L., unpublished observations). Because the 2400-n transcript is demonstrated in very high stringency hybridizations, it seems to represent an insulin-gene transcript. However, insulin-gene sequences indicate that an unprocessed RNA transcribed from the usual initiation site and terminated at the usual polyadenylation site should be 1200 n (13). We conclude that this long transcript results from an upstream insulin-gene promoter or cleavage at an alternate downstream polyadenylation site. This transcript appears to represent a processed RNA that is transported to the cytoplasm. However, the low levels of 2400-n transcript makes its significance questionable.

Tissue localization. Our data demonstrate that the 720-n transcript is predominantly in the yolk sac. We included amnion and other extrafetal membrane fragments in the yolk sac preparation so that the total levels would be accurately determined. Preliminary studies did not show insulin-gene transcripts in amnion, but we cannot exclude insulin mRNA in other minor extrafetal structures. The 18-day levels are consistent with our previous study in which we used only yolk sac segments (1). We were careful to exclude intra-abdominal structures to prevent contamination of the preparations with fetal pancreas, which can be excluded by the absence of 540-n transcripts in our hybridizations. However, the yolk sac usually has an intra-abdominal portion, and we have probably underestimated the total of 720-n transcripts.

Our data demonstrate placental 720-n transcripts. We tried to remove as much yolk sac as possible from the preparations. If the placental transcripts represent contaminating yolk sac fragments, then the placental preparations would have to be 5–10% yolk sac fragments by weight, a level that is very unlikely. Putative insulin-gene transcripts have been observed in the term human placenta (9), an organ vastly larger than the human yolk sac. However, the rat placental levels or insulin mRNA are low and represent only a small proportion of the total extrafetal stores.

Developmental expression of extrapancreatic insulin. It must be stressed that the data presented herein do not demonstrate insulin secretion or insulin synthesis, only developmental regulation and high levels of an insulin mRNA in yolk sac. Our earlier study did demonstrate both insulin and proinsulin in 18-day yolk sac, and the high proinsulin-to-insulin ratio strongly suggests insulin synthesis in the yolk sac (1). Together, these studies suggest that the level of 720-n transcripts predicts the level of yolk sac insulin synthesis. Secretion of yolk sac insulin remains hypothetical.

Our mRNA determinations predict a late-gestational role for yolk sac insulin. The fetal pancreas, of course, synthesizes substantial amounts of insulin in late gestation, but pancreatic insulin could be stored for the postnatal period. The yolk sac mRNA level peaks on day 20, and the total mRNA reaches a maximum on day 21. Hepatic glycogen increases at the same time. Compared with the data of Ballard and Oliver (4), this glycogen accumulation occurs ~1 day later than the insulin mRNA accumulation of the yolk sac. It is tempting to speculate that yolk sac insulin regulates the massive hepatic glycogen synthesis that appears to provide a special accumulation of nutrients for the immediate postnatal period. If the yolk sac is a source of circulating insulin, then it could contribute to the regulation of fetal growth, fetal adipose tissue, and fetal cholesterol biosynthesis as well as fetal hepatic glycogen synthesis.

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