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Ontogeny of Growth Hormone Releasing Hormone and Insulin-Like Growth Factors-I and -II Messenger RNA in Rat Placenta[1]

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ABSTRACT. Hypothalamic growth hormone releasing hormone (GHRH) stimulates pituitary growth hormone secretion, which is essential for normal postnatal growth. Reports of an immunoreactive and biologically active GHRH-like peptide in placenta led us to investigate placental expression of GHRH mRNA. Placentas from d 19 gestation fetal rats were assayed for GHRH-like peptide by ELISA and for GHRH mRNA. Placenta GHRH-like peptide levels averaged 3.7 ± 0.2 ng/g tissue. Dot-blot hybridization revealed the presence of GHRH mRNA in rat placenta in quantities greater than those of the message in rat hypothalamus. Northern gel analysis of poly-A enriched RNA was used to evaluate the specificity of GHRH mRNA hybridization and to determine the size of the placental mRNA. Placental and hypothalamic GHRH mRNA were of nearly identical size, although placental RNA had a broad band of hybridization that extended below that seen in hypothalamus. Further confirmation of homology between placental and hypothalamic GHRH mRNA was determined by an RNAse protection assay, in which a placental protected fragment was identical in size to that resulting from protection of the hypothalamic complementary RNA. The ontogeny of GHRH mRNA in rat placenta was determined by dot-blot hybridization. The message was detected at the earliest date examined, d 7, and increased more than 2-fold by d 14 and 5-fold by d 17. The ontogeny of IGF-I and IGF-II mRNA in placenta was also determined. IGF-I mRNA was detected at all gestational dates examined, but was highest on d 10, whereas IGF-II mRNA was not detectable on d 7 or 10 but was present on d 14, 17, and 20. We conclude that GHRH mRNA is present in rat placenta at least from d 7 of gestation. Placental expression of GHRH mRNA is concurrent with maximal expression of placental IGF-II, but not IGF-I mRNA. The similar time course of expression for GHRH mRNA and IGF-II leads us to speculate that they may have related functions in the last week of rat gestation. (*Pediatr Res* 29: 510–516, 1991)

Abbreviations

GHRH, growth hormone releasing hormone
cRNA, complementary RNA
TFA, trifluoroacetic acid
tRNA, transfer RNA
PLP, prolactin-like protein
PL, placental lactogen
GH, growth hormone
UTP, uridine triphosphate

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The placenta is a rich source of hypothalamic neuropeptides including corticotrophin releasing hormone (1), gonadotropin releasing hormone (2), thyrotropin releasing hormone (3), and proopiomelanocortin (4). Two reports have also documented the presence of immunoreactive (5, 6) and biologically active GHRH in rat placenta. Because the placenta has mechanisms for concentrating circulating substances for subsequent delivery to the fetus, these findings are only inferential proof of a placental origin for GHRH. GHRH mRNA has also recently been reported in placenta (7–9). We now confirm the presence of immunoreactive GHRH and GHRH mRNA in rat placenta. To begin to explore a physiologic role for GHRH *in utero*, we examined the ontogeny of GHRH mRNA in rat placenta. To determine if GHRH expression correlates with that of other growth factors, the ontogeny of IGF-I and IGF-II mRNA in placenta was also studied.

MATERIALS AND METHODS

Antiserum generation. Antibodies were generated in New Zealand White rabbits as previously described (10). After the booster, the animals were bled weekly and the serum assayed in modifications of a previously described ELISA for the detection of anti-GHRH antibodies (11, 12).

Animals. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Timed-pregnant Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY). Eight timed-pregnant rats were studied for determination of the ontogeny of placental mRNA. Two pregnant rats from each of the following days of gestation yielded placentas for study: d 7 (31 fetuses/placentas); d 10 (22 fetuses/placentas); and d 14 (30 placentas). One pregnant rat yielded 12 placentas at d 17 of gestation and one rat yielded 12 placentas at d 20 of gestation. On gestational d 7 and 10, we did not achieve complete separation of the fetus from the placenta.

Tissue extraction and GHRH ELISA. Placentas were extracted for GHRH ELISA using modifications of previously described methodology (6, 13). In brief, they were homogenized with ice-cold 50% acidified methanol (0.1 N HCl-CH_3OH) with the addition of phenyl-methylsulfonylfluoride (10 μg/mL) and pepstatin (10 μg/mL). The homogenate was spun at 48 000 × *g* for

30 min at 4°C. The supernatant was further extracted over a C-18 Sep-pak cartridge (Waters Associates, Milford, MA) that had been treated by successive 4-mL washes of 0.01 M TFA, 80% acetonitrile/20% 0.01 M TFA (vol/vol), and 0.01 M TFA. Acidified samples were applied to the cartridge and eluted with 80% acetonitrile/20% 0.01 M TFA. The extract was dried and reconstituted in 1 mL PBS buffer. After a 15-min incubation at 37°C, samples were centrifuged at 1500 × *g* for 10 min and the supernatant assayed for GHRH. A direct-binding rat GHRH ELISA was used to measure GHRH immunoreactivity in placenta. Details of this assay have previously been described (10).

RNA extraction. Extraction of total RNA from placenta and hypothalamus was by guanidine-HCl (14). Aliquots of tissue (100 mg) were homogenized in 10 volumes of 7 M guanidine-HCl, pH 5.5, 20 mM sodium acetate, 240 mM ammonium acetate, 1 mM DTT, 10 mM iodoacetate. Homogenates were adjusted to 0.5% sodium sarcosyl and centrifuged, and the supernatant precipitated at −20°C for 48 h with 1/2 volume of ethanol. Precipitates were dissolved in 7 M guanidine-HCl, pH 7.0, 20 mM sodium acetate, 1 mM DTT, 20 mM sodium EDTA, 10 mM iodoacetate and precipitated with 1/20 volume 2 M sodium acetate, pH 5.0, and 1/2 volume of ethanol. These RNA pellets were dissolved and precipitated two additional times, then washed with ethanol:0.1 M sodium acetate, pH 5.0 (2:1), and dissolved in 10 mM Tris HCl, 0.1 mM EDTA, pH 7.6. The dissolved RNA was extracted three times with chloroform:isoamyl alcohol (24:1) and precipitated. Poly-A enriched RNA were obtained by one cycle of oligo-(dT) cellulose. One mg of total RNA from placenta (extracted from ~400 mg of placental tissue fragmented from a single 0.5-g, d 20 gestation placenta) and 800 μg of total RNA obtained from extraction of 16 pooled hypothalami (yielded 925 μg of total RNA) were chromatographed, yielding 33 and 38 μg of poly-A enriched RNA, respectively.

Preparation of cRNA for hybridization. Rat hypothalamic GHRH cDNA (gift of Ron Evans, Salk Institute, San Diego, CA) and IGF-I and IGF-II cDNA (15, 16) were subcloned into plasmid vector pSP-72 (Promega, Madison, WI). RNA was generated using either T7- or SP6-RNA polymerases. Polymerase reactions were performed in 40 mM Tris-HCl, pH 8.0, 5 mM DTT with 1 mM ATP, cytidine triphosphate, and guanosine triphosphate, in the presence of 1 U/μL of RNA polymerase for 30 min at 37°C (17). Cold sense-strand RNA was prepared for GHRH, IGF-I, and IGF-II standards and for demonstration of GHRH RNAse protection by addition of 1 mM cold UTP only. Antisense-[^{32}P]-labeled RNA was prepared as a probe for GHRH hybridizations by addition of 40 μM UTP and 50 μCi [^{32}P]-UTP. Sp act of labeled probes was 1 to 8 × 10^8 cpm/μg cDNA.

Preparation of IGF-I and IGF-II cDNA for hybridization. For hybridization of IGF-I and IGF-II cDNA to placental total RNA, IGF-I (14) and IGF-II (gift of Matthew Rechler, NIH, Bethesda, MD) (16) cDNA were used. The cDNA were excised by digestion with the appropriate restriction endonucleases and labeled by random primer extension (18, 19) with 50 μCi [^{32}P]-deoxycytidine triphosphate using a kit from Pharmacia Inc. (Piscataway, NJ). Sp act of probes ranged from 7 to 10 × 10^8 cpm/μg cDNA.

Preparation of RNA for hybridization. Dot blots were prepared as previously described using concentrations of 4, 2, 1, and 0.5 μg of total RNA (14). For standards, 0.5–32 pg of cold sense strand GHRH, 4–96 pg IGF-I cRNA, or 2.5–100 pg IGF-II cRNA were also applied to dot blots. To control for nonspecific hybridization, equal quantities of yeast tRNA were placed on each filter. For comparison of hypothalamic and placental GHRH mRNA, 10 μg of placenta and hypothalamic poly-A RNA were electrophoresed in a 1.8% agarose gel in 2.2 M formaldehyde-20 mM phosphate. For Northern blots of the ontogeny studies, 10 μg of total RNA from representative samples at each gestation date were electrophoresed in a 1.5% agarose gel. Sample buffers included ethidium bromide to facilitate visualization of ribosomal RNA bands, evaluate transfer of the samples, ensure the absence of degradation, and assess the relative quantities applied. The RNA was transferred to Zetaprobe membrane (Bio-Rad Laboratories, Richmond, CA) by electrotransfer in 7.5 mM Tris, pH 7.8, 3.75 mM sodium acetate, 37.5 μM EDTA at 80 V for 6 h.

Hybridization and washing. Blots used in RNA-RNA hybridizations (GHRH) were prehybridized and hybridized in 50% formamide, 2.5 × Denhardt's solution, 50 mM sodium phosphate, pH 7.5, 1 mM EDTA, 0.8 M NaCl, 10 μg/mL poly-A, 200 μg/mL sheared salmon sperm DNA, 100 μg/mL yeast tRNA. Northern blots were prehybridized in 50% formamide, 5 × Denhardt's solution, 0.5% SDS, 5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM sodium phosphate, pH 7.5, 1 mM EDTA), 200 μg/mL sheared salmon sperm DNA. Hybridization solution was the same, except the concentration of Denhardt's solution was decreased to 2.5×. Prehybridization was for 4–12 h and hybridization was for 18–24 h at 60°C. Filters were washed in 1 × SSPE, 0.1% SDS for 15 min at room temperature, three times in 1 × SSPE, 0.1% SDS at 65°C for 20 min each, and in 0.1 × SSPE, 0.1% SDS at 60°C for 50 min.

For RNA-DNA hybridizations (IGF-I, IGF-II), prehybridization and hybridization were carried out in 50% formamide, 5 × Denhardt's solution, 50 mM sodium phosphate, pH 7.5, 0.2% SDS, 200 μg/mL sheared salmon sperm DNA, 100 μg/mL yeast tRNA. Times of prehybridization and hybridization were as for RNA-RNA hybrids, but were at 42°C. Filters were washed for 10 min at room temperature in 2 × SSC, 0.1% SDS four times, and in 0.2 × SSC, 0.2% SDS at 60°C two to three times. All filters were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for times varying from 12 h to 10 d.

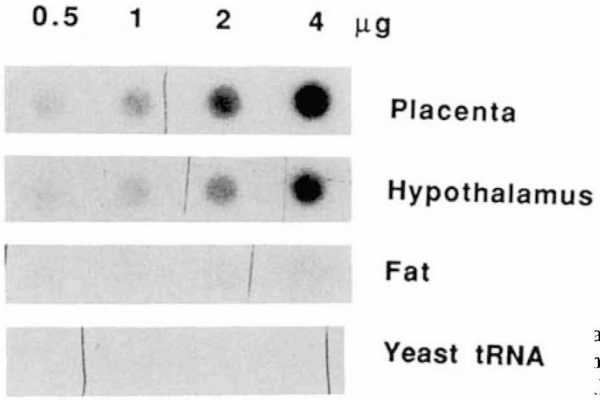
RNAse protection assay. Aliquots of *in vitro* generated GHRH (10–100 pg) or placental RNA (25 μg) were hybridized in solution to antisense-[^{32}P]-labeled GHRH RNA (20). Hybridizations were performed at 80°C in 10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 2 mM EDTA, 20% formamide for 16–18 h. After hybridization, the hybrids were treated with RNAses A and T1, followed by proteinase K digestion and extraction with phenol-chloroform. Samples were precipitated and applied to an 8% acrylamide, 8 M urea gel in 89 mM Tris, 89 mM borate, 2 mM EDTA and subjected to electrophoresis at 110 V for 3 h. The gel was dried and exposed to Kodak XAR-5 film for 24 h.

Data analysis. The data are expressed as the mean ± SEM. The significance of changes during gestation was determined using analysis of variance (Statview 512+; Brainpower, Inc., Calabasa, CA). Dot blots were quantitated by videodensitometry using a modification of the method of Mariash *et al.* (21). Imaging was performed with a JVC model GX-N8U color videocamera with the program "Quick Capture" and a Macintosh II computer.

RESULTS

Immunoreactive GHRH in placenta. GHRH immunoreactivity in rat placenta was confirmed in the ELISA. GHRH concentrations averaged 3.7 ± 0.2 ng/g of wet weight (n = 5; 0.1 g tissue/initial well) from gestational d 20 placentas. No GHRH was detected in any plasma samples. GHRH content of hypothalami from 60-d-old male rats served as a positive control and averaged 0.32 ng GHRH/hypothalamus. Recovery of exogenous GHRH after extraction averaged 80%. The least detectable dose was 40 pg per well. The intraassay and interassay coefficients of variation at the ED_{50} were 8.3 and 14.9%, respectively.

GHRH mRNA in placenta. Dot-blot hybridization of placental total RNA resulted in a hybridization signal that was more intense than that generated by hybridization to total hypothalamic RNA (Fig. 1). Because the distribution of GHRH in hypothalamus is limited to a small region, comparison of amounts of RNA may be affected significantly by the size of the tissue block used for extraction. The more intense signal in placenta suggests that GHRH in placenta is more generally distributed



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as described in Materials and Methods. No nonspecific hybridization signal is evident in fat total RNA or yeast tRNA dots.

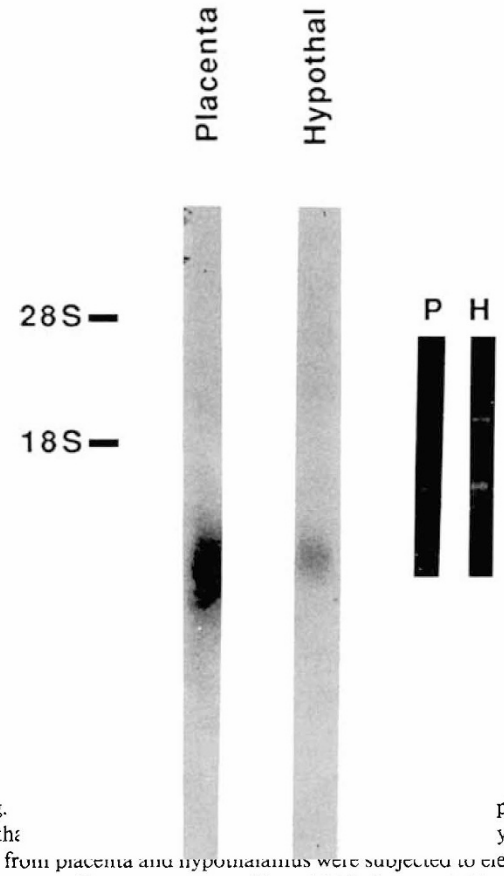

Fig. placenta and
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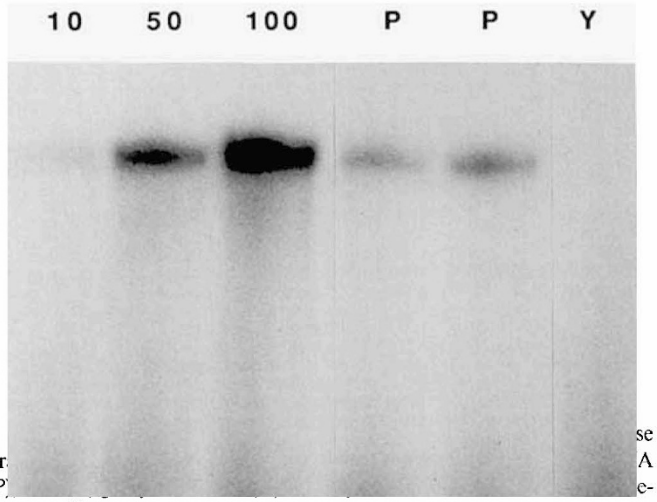
RNA from placenta and hypothalamus were subjected to electrophoresis in each lane. Size markers are 18S and 28S ribosomal RNA. Migration of the placental band is slightly farther than that seen in hypothalamus. *Inset* shows the gel lanes from which these blots were transferred. Intensities of residual ribosomal RNA bands in each lane were approximately equal. The lane designated *P* contains placental poly-A enriched RNA and lane *H* contains hypothalamic poly-A enriched RNA.

and/or more abundant than in hypothalamus. No signal was seen in similar amounts of total RNA from white fat, or when yeast tRNA was applied, suggesting that the signal generated is specific to GHRH. On Northern analysis of poly-A enriched RNA, placental rat GHRH mRNA was ~700 nucleotides in length (Fig. 2), and the band of hybridization migrated slightly farther than the signal observed in hypothalamus. The magnitude of difference is difficult to assess because the only basis for comparison is the much larger 18S and 28S ribosomal RNA species, but it averaged ~50 to 100 nucleotides.

To assess the degree of homology between placental hypothalamic GHRH mRNA, we used the hypothalamic cDNA to perform an RNAse protection assay of placental total RNA. *In vitro* generated hypothalamic GHRH sense-cRNA and placental total RNA were hybridized in solution to labeled antisense GHRH-cRNA. The fragment protected by the full-length, *in vitro* generated hypothalamic cRNA is identical in size to that seen in placental total RNA after digestion with RNAses A and T1 (Fig. 3). No hybridization signal is seen when yeast tRNA is used instead of placental RNA.

Ontogeny of GHRH mRNA in placenta. To determine the ontogeny of GHRH in placenta, placentas were harvested from timed-pregnant females at varying times of gestation. GHRH mRNA in placenta measured by dot-blot hybridization was detectable (0.18 ± 0.04 pg GHRH cRNA/μg placental total RNA) ($n = 4$ at all time points) at the earliest date examined (d 7), but increased more than 2-fold (0.46 ± 0.13 pg/μg total RNA) at d 14 and 5-fold by d 17 (1.31 ± 0.14 pg/μg total RNA). Quantities were unchanged at d 20 of gestation (Fig. 4*A* and *B*). On a Northern blot of total RNA (Fig. 4*C*), a hybridization signal was apparent by d 14 and increased in a fashion similar to that noted in the dot blots. Only one major RNA species was seen on d 14 and 17 (~825 nucleotides); however, in d 20 placenta, two additional species were apparent, one slightly smaller (~700 nucleotides) and one larger (~3550 nucleotides).

Ontogeny of IGF-I and IGF-II mRNA in placenta. Dot blot and Northern hybridizations of IGF-I and IGF-II mRNA were also performed to compare the ontogeny of these placentally expressed growth factors with placental GHRH. In contrast to GHRH, IGF-I mRNA in placenta was highest at d 10 of gestation (19.67 ± 2.67 pg IGF-I mRNA/μg total RNA) and decreased by d 14 to levels below the detection limit (Fig. 5), whereas IGF-II mRNA was not seen in d 7 and 10 samples, but was readily detected at d 14, 17, and 20 of gestation (323.9 ± 26.7, 417.7 ± 2.76, and 411 ± 40.6 pg IGF-II mRNA/μg total RNA, respectively) (Fig. 6). On Northern blots, IGF-I mRNA was faintly present at d 7 of gestation and visible only on prolonged exposures (data not shown). The pattern of RNA species seen was identical to that seen at d 10. The predominant IGF-II mRNA



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GHRH [^{32}P]-labeled cRNA, followed by RNAse A and T1 digestion. After PAGE, the dried gel was radiographed for 24 h. A protected fragment in placenta is identical in size to that protected by hybridization of sense and antisense strands of full-length hypothalamic GHRH cRNA.

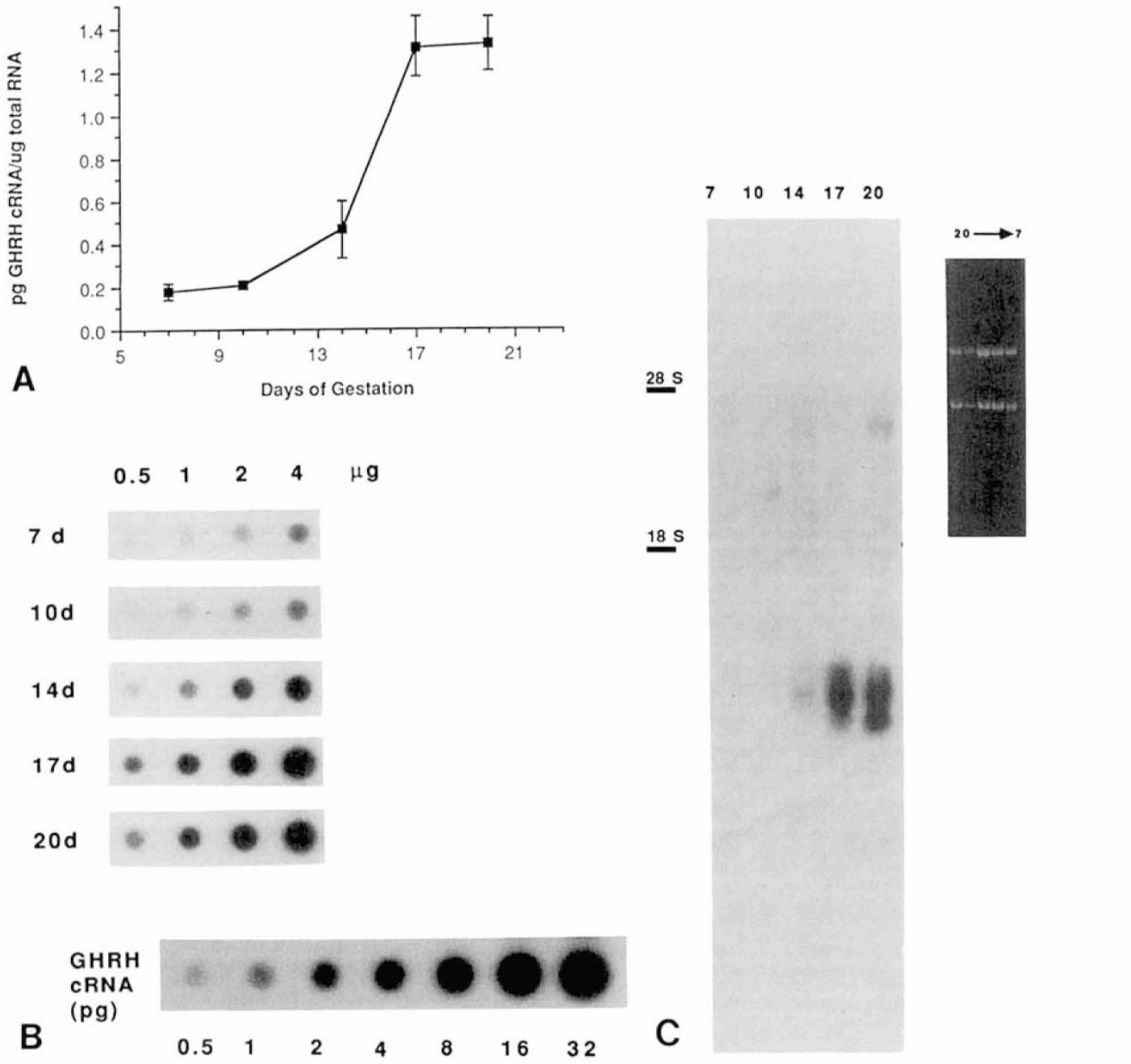

Fig. 4. Ontogeny of GHRH mRNA in placenta. *Panel A*, amounts of GHRH cRNA per µg placental total RNA at varying ages. Shown are mean values ± SEM, $n = 4$ in each group. *Panel B*, representative dot-blot hybridization signal for each age (*upper*) and GHRH cRNA standard (*lower*). *Panel C*, Northern gel of representative sample at each gestational age (*shown on top*). Size markers are 18S and 28S RNA. *Inset* shows ethidium bromide staining of the Northern gel (lanes are reversed, as noted above the *inset*). Intensity of the d 17 RNA is slightly less than that for other days due to a pipetting error. Ten µg was applied to each lane.

band was at 4.0 kb, with additional faint bands visualized on prolonged exposures.

DISCUSSION

Previous reports have documented the presence of immunoreactive (5, 6) and biologically active GHRH in rat placenta (5). In the most recent of these reports, GHRH concentrations in placenta did not change from d 13 through term, although GHRH content did increase from d 13 to d 20 of gestation. Although some substances such as large polypeptides seem to be excluded from transfer across the placenta, flux of other substances across placental tissue can occur by a number of mechanisms: *1*) simple diffusion (*i.e.* anesthetic gases), *2*) restricted diffusion (*i.e.* hydrophilic substances), *3*) facilitated diffusion (*i.e.* glucose), *4*) active transport (amino acids), and *5*) receptor-mediated endocytosis (IgG) (22). Thus, the finding of GHRH in placental extracts is not proof of placental origin.

The finding of a GHRH mRNA sequence in placenta that hybridizes to GHRH cRNA under stringent conditions lends strong support to its placental origin. The GHRH mRNA present in placenta is similar in character to that produced in the hypothalamus, as determined by the presence of a protected fragment of identical size after RNAse digestion using a hypothalamic GHRH cRNA in the protection assay. However, at d 20 we noted three apparent RNA species. One band is of the size of hypothalamic GHRH mRNA and is present in all lanes of the Northern blot. Although the smaller species may be artifactual, we have observed a broad band at d 20 of gestation in all previous gels, including in our poly-A hybridization. The larger band was also not seen previously in examination of rat placental RNA (7), and is not evident in our sample of poly-A enriched placental RNA. It is larger than the major GHRH-like RNA in testis, but is the same size as a lighter band seen in testis at postnatal d 65 and 90 (23). The significance of the alternate species is not known. The larger RNA could be a precursor, or the differences in the bands could be secondary to alternative splicing or processing. Whether the alternate bands are of physiologic significance and are differentially regulated is still unknown.

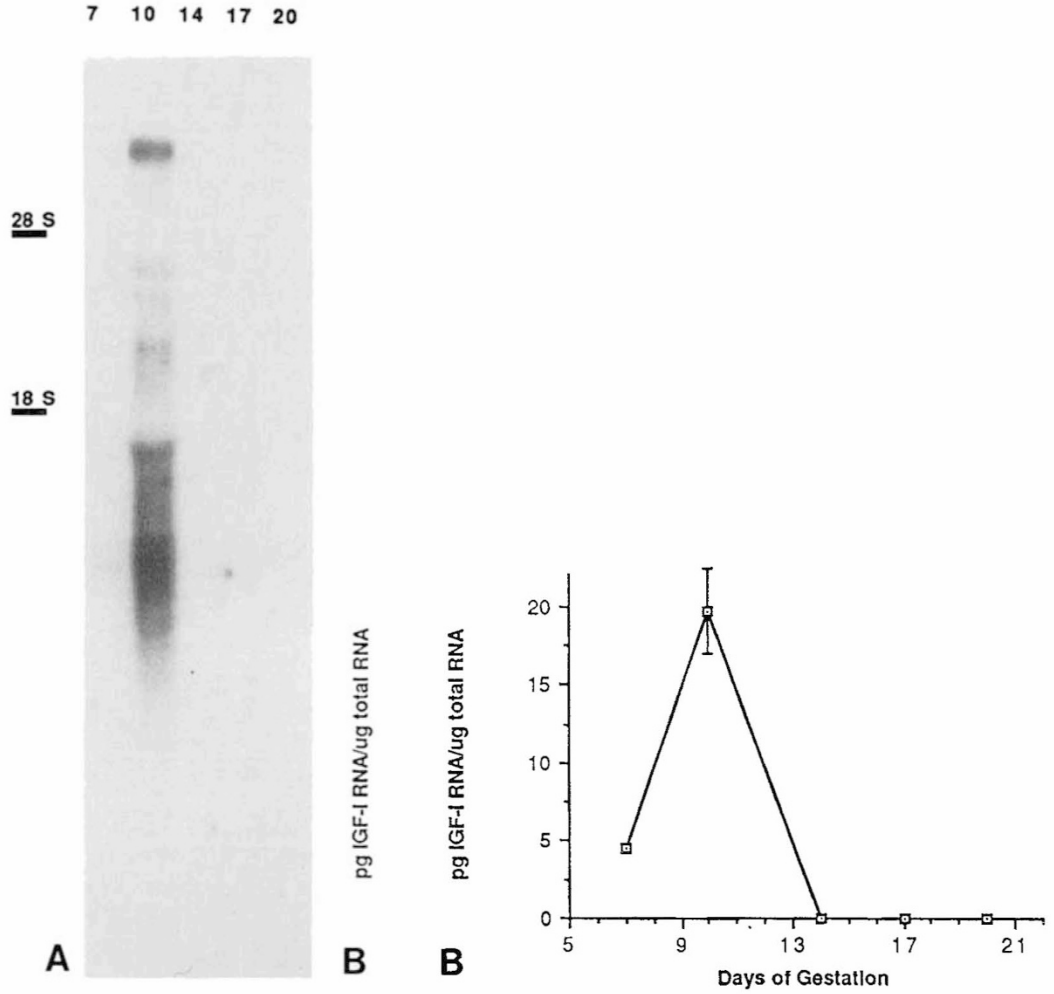

Fig. 5. Ontogeny of IGF-I mRNA in placenta. The Northern blot used in Figure 4*C* was stripped and reprobed with IGF-I cDNA (*A*). Size markers are 18S and 28S ribosomal RNA. Four individual placentas at each time point were examined by dot blot for quantitation of the changes in all IGF-I mRNA species, and compared to fixed quantities of an *in vitro*-generated sense strand IGF-I cRNA (*B*). Values shown are means ± SEM.

Three other groups have also documented the presence of GHRH mRNA in placenta (8, 9, 24). Suhr *et al.* (8) cloned and sequenced GHRH in mouse using a d 13 gestation placental cDNA library. They found that GHRH mRNA is present at d 14 of mouse gestation and increases in the latter half of gestation, much as we observed in rat. The band of hybridization for the major RNA species in placenta in their Northern gel is also broader, and possibly smaller, than the hypothalamic signal. A larger species is also present on their Northern blot. Using *in situ* hybridization, they showed that GHRH is expressed in trophoblast giant cells. Margioris *et al.* (9) made the same observation in rat placenta, and also saw a strong placental signal in a Northern blot of total RNA. They found that secretion of placental GHRH seemed to be regulated by both adenyl cyclase and protein kinase C pathways (9). These studies strongly support the hypothesis of local production of GHRH by the placenta.

Like other hypothalamic-like peptides found in placenta, the function of GHRH in placenta is unknown. Placental releasing hormones may regulate placental pituitary-like hormones, as has been suggested for both placental corticotrophin releasing hormone and gonadotropin releasing hormone (2, 4, 25, 26). Similarly, the placenta could have a growth axis analogous to the hypothalamic-pituitary-target tissue axis. The placenta expresses all the components of such an axis, including GHRH, GH-like hormones, and IGF.

A number of GH gene family members are expressed in rat placenta, including PLP-A, PLP-B, PL-I, and PL-II. The physiologic role of these GH-like peptides remains unknown, although they have distinctive ontogenetic changes during gestation. There is a rapid increase in PL-I during midgestation (d 10–13), followed by a fall in peptide levels (27). PL-II, PLP-A, and PLP-B mRNA increase significantly beginning at d 12, 14, and 15 and are expressed until term (28–30), not unlike our observation of placental GHRH mRNA.

The physiologic significance of the GH-like peptides in placenta is unknown. PL has been proposed as the fetal GH by many investigators, but the evidence supporting this hypothesis is still controversial. Although production of PL is massive, appears early in human pregnancy, and increases in parallel with placental weight, its absence in humans does not seem to be detrimental to growth. Infants with complete deletions of the PL genes have normal prenatal growth, despite extremely low levels of this hormone in the maternal circulation (31, 32). Although it has both lactogenic and somatogenic properties (33), it is not potent as a postnatal GH (34). Although PL may not be essential in human fetal growth, much of the existing evidence suggests that it is more important in the fetal rat. In rats, it is an anabolic hormone that promotes amino acid transport into tissues, as GH does in postnatal life (35). PL can stimulate IGF-I production (36–38) and is detectable in fetal serum, and there are unique high-affinity PL receptors in fetal tissues (39).Furthermore, in the rat, administration of human PL by implantable minipump during late gestation results in accelerated fetal weight gain (40), and reverses the effects of low-protein diet on fetal somatomedin levels (41).

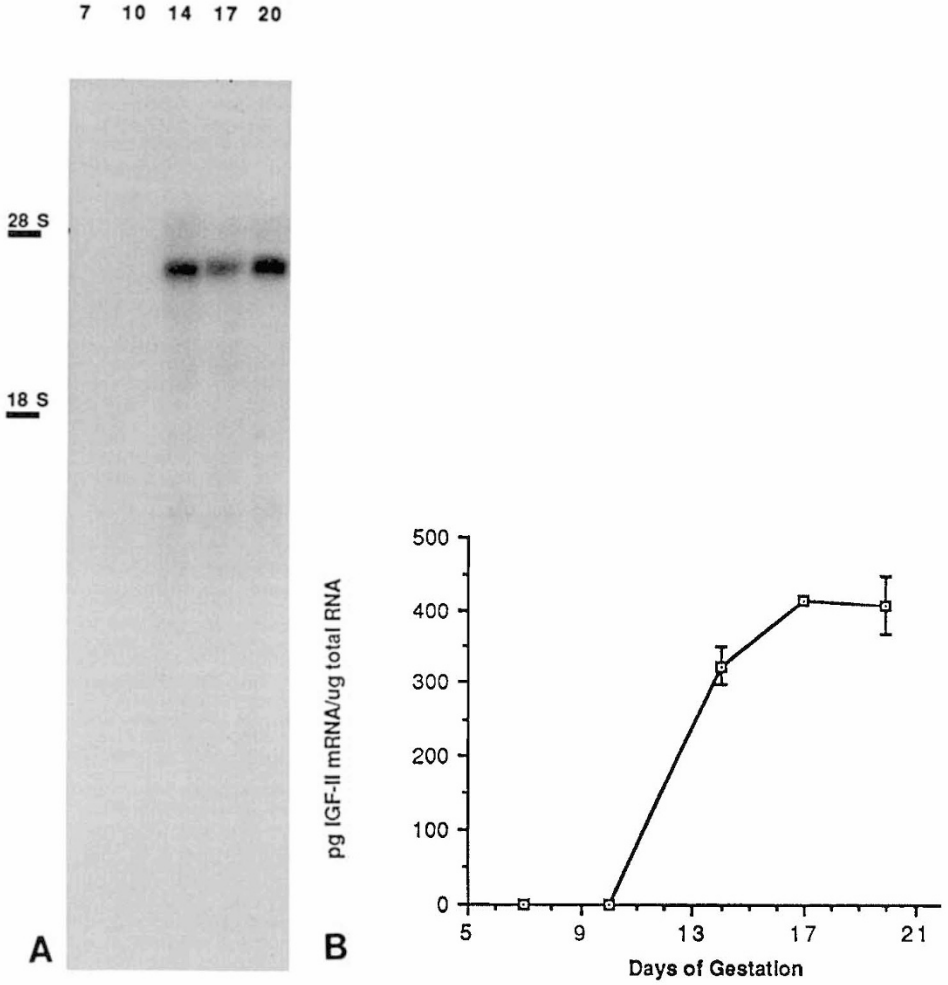

Fig. 6. Ontogeny of IGF-II mRNA in placenta. The Northern blot in Figures 4 and 5 was reprobed with IGF-II cDNA (*A*), and the individual placental samples (four per time point) were evaluated by dot blot. Size markers are 18S and 28S ribosomal RNA. The individual data points were compared to fixed quantities of an *in vitro*-generated sense strand IGF-II cRNA (*B*). Values shown are means ± SEM.

Both IGF-I and IGF-II have been found in human placenta, where IGF-I mRNA is higher in 1st and 2nd trimester placentas than in term placentas (42). Changes in IGF-I in developing rat placenta have not been examined. Shen *et al.* (43) found that IGF-II mRNA in human placenta was highest in the 2nd trimester (43). In rodents, an IGF-II cDNA was found in a mouse d19 placental library (44), and term rat placentas express IGF-II mRNA (45), but no studies have examined the ontogeny of placental IGF-II. Most investigators believe that the contribution of IGF-I to fetal growth is limited in comparison to other growth factors. This conclusion is based on the finding that fetal serum IGF-I and hepatic IGF-I mRNA levels are considerably lower than in normal adult animals (46). In contrast, IGF-II is relatively abundant in fetal life in comparison to its postnatal levels (47, 48), and is not significantly influenced by GH (38). For these reasons, it has been proposed that IGF-II could be the primary fetal somatomedin (38). Both IGF are induced by PL in specific tissues: PL induces IGF-I expression in cultured human fetal pancreas (36) and induces IGF-II in rat fetal fibroblasts (38).

In this study, we demonstrated that the expression of IGF-I mRNA in rat placenta is highest in midgestation around d 10, essentially concomitant with published reports of peak expression of PL-I, and before increases in embryonic IGF-I expression (49). The RNA pattern seen is like that previously described in liver (50). Although we were not able to completely separate fetal tissues from d 7 and 10 placental samples, the low levels of IGF-I expression in d 11 embryos seen by Rotwein *et al.* (49) suggest that the peak in IGF-I expression seen in our samples is probably due to placental expression of IGF-I. IGF-II mRNA expression in placenta is not detected at d 7 and 10 and is maximal by d 14. The predominant IGF-II mRNA band was at 4.0 kb, with additional faint bands visualized on prolonged exposures, as described by others (45, 50). Inasmuch as we did not examine d 11–13 placentas, we cannot precisely time the day at which IGF-II mRNA expression begins to increase. Rotwein *et al.* (49) further found that IGF-II was readily demonstrable in d 11 rat embryonic tissue, whereas even on prolonged exposures of IGF-II dot blots, no apparent expression was seen in d 10 placenta. Unless a marked increase in placental IGF-II expression occurs on d 11, our observations suggest that the regulation of expression of IGF in placenta and embryo is temporally discordant.

The interaction of placental hormones is likely to be complex and to result in a changing hormonal environment for both mother and fetus. If placental GHRH serves as a regulator of placental GH-like hormones, it is likely to do so only in the latter half of gestation. By temporal association, the most likely GH-like targets for placental GHRH action in the rat might be PL-II, PLP-A, or PLP-B. Our data are consistent with, although not proof of, placental GHRH having an interactive role with placental IGF-II during the last week of rat gestation. They do not support a role for placental GHRH in placental IGF-I regulation. Further examination of the regulation of placental GHRH should provide insight about the function of placental hormones.

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