Prenatal expression of the growth hormone (GH) receptor/binding protein in the rat: a role for GH in embryonic and fetal development?

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Summary

Although fetal growth is generally considered to be independent of pituitary growth hormone (GH), it is possible that pituitary GH plays a modulatory role in organ development or that a GH-like substance of non pituitary origin may influence fetal growth through the GH receptor. Accordingly, we have used immunohisto-chemisty, northern blot analysis, the reverse transcriptase-polymerase chain reaction and solution hybridiz-ation to study the ontogeny of the GH receptor/binding protein (BP) from the 12-day-old embryo (E12) to the E18 rat fetus.

GH receptor/BP immunoreactivity was observed in all major organ systems of the E18 rat fetus and was not preferentially associated with any germ layer derivative. A general increase in GH receptor/BP immunoreactivity was evident from E12 to E18, with a marked increase occurring between E16 and E18. Hemangioblastic tissue was, however, strongly or intensely immunoreactive at all stages of development, as was the placenta. Most noteworthy of the other tissues expressing GH receptor/BP immunoreactivity by day 18 were skeletal and smooth muscle, chondroprogenitor cells, epithelial lin-ing cells, neuronal ganglia, ependymal cells and the adrenal cortex. In the placenta, the most prominent immunoreactivity was associated with decidual cells.

Total RNA was isolated from E12 to E18 rat fetuses and adult rat liver. Northern hybridization with a ^{35}S-labelled rat GH receptor cRNA probe revealed that 3.9 kb and 1.2 kb transcripts complementary to the rat GH receptor ribopobe are present from at least E16. The existence of GH receptor mRNA at E12 and E14 was demonstrated by the polymerase chain reaction. Sol-ution hybridization analysis demonstrated an exponen-tial increase in the total embryonic GH receptor/BP mRNA during fetal development. However even by E18 levels were only 8% those of adult liver.

In conclusion, we have demonstrated the presence and defined the ontogeny of the GH receptor/BP in the rat foetus from mid gestation. These results, when com-bined with earlier studies of GH action on fetal tissues suggest a reevaluation of the role of GH in prenatal growth.

Key words: growth hormone receptor, binding protein, rat.

Introduction

Fetal growth and development is a complex process involving both cellular proliferation and differentiation. These processes are regulated through an interaction of the endocrine systems of the fetus, mother and placenta (Gluckman, 1986; Browne and Thorburn, 1989). Unlike postnatal development, where growth is clearly under central control through pituitary growth hor-mone (GH) and insulin-like growth factor-1 (IGF-1) (Daughaday, 1989), most of the available evidence does not favour a role for pituitary GH in fetal growth and development. For instance, anencephalic human fetuses (Chard, 1989) and hypophysectomised fetuses of numerous other species (Heggestard and Wells,

1965; Jost, 1966; Gluckman et al., 1981) have little or no circulating immunoreactive GH, yet appear to grow almost normally. In addition, fetal tissues, with the exception of liver (Hill et al., 1988), were not thought to possess the GH receptor. In the human fetus, GH can be detected after 10 weeks gestation and reaches a peak at 20-24 weeks (Chard, 1989) and, in fetal lambs, plasma GH can reach 10-12 times postnatal levels (Bassett et al., 1970). These high levels of fetal GH have been regarded as of little functional significance and the result of immaturity in secretory control mechanisms (Gluckman et al., 1981). Nevertheless, a number of fetal tissues have been shown to respond to GH in vitro (Strain et al., 1987; Swenne et al., 1987; Slootweg et al., 1986; Stracke et al., 1984) implying the

presence of a functional GH receptor during fetal development.

Because the role of GH in fetal growth and maturation is poorly understood, we decided to define the location of expression and ontogeny of the GH receptor/BP in the fetus. In this study, we have used immunohistochemistry to localise GH receptor/BP expression in the E12 to E18 rat embryo and the placenta. Northern blot analysis, reverse transcriptase-polymerase chain reaction and solution hybridization techniques were also used to demonstrate or quantify the GH receptor/BP mRNA in the E12-E18 fetus.

Materials and methods

Production and characterisation of GH receptor/BP monoclonal antibodies

Monoclonal antibodies (mAbs 1,5,7,43) to the rabbit GH receptor were produced by application of hybridoma technology to splenic lymphocytes from mice immunised against a human (h) GH affinity purified rabbit liver GH receptor (Barnard et al., 1984). These antibodies recognise independent epitopes on the extracellular portion of the receptor, do not cross react with insulin or prolactin receptors in the appropriate receptor assays and react specifically with GH receptors in immunoblots (Leung et al., 1987). mAb 263 recognises a cross-species determinant with high affinity (Barnard et al., 1985) and was prepared by immunization of mice with purified rat GH receptor. mAb 263 is reactive against the GH receptor of a number of species and does not react with insulin or prolactin receptors in rabbit or rat liver. Under certain conditions, mAb 263 precipitates rat and rabbit GH receptor, although it can also compete for hormone binding to subtypes of the GH receptor, as does mAb 7 (Barnard et al., 1985).

Tissue preparation for immunohistochemistry

Twelve female Sprague Dawley rats from the Herston Medical School animal House (Herston, Qld.) which had undergone timed matings were killed at 12,14,16 and 18 days of gestation by CO_2 asphyxiation. The uterine horns were excised, embryos were dissected from maternal tissues and then immersion fixed in Bouin's solution (0.9% picric acid, 9% (vol/vol) formaldehyde and 5% acetic acid) for 4 hours at 4°C. Tissues were subsequently dehydrated and processed for wax embedding.

Immunohistochemistry

Paraffin was removed from the sections and they were subjected to immunohistochemical staining according to Lobie et al. (1990b). This involved (a) elimination of endogenous hydrogen peroxidase activity with 0.5% H_2O_2 in phosphate-buffered saline (PBS, pH 7.4) for 15 minutes at room temperature (25°C); (b) elimination of non-specific protein binding by incubation in 10% normal sheep serum for 1 hour at room temperature; (c) incubation overnight at 4°C with mouse anti-GH receptor monoclonal antibody (mAb 7 at 100 μg/ml, mAb 43 at 50 μg/ml and mAb 263 at 25 μg/ml) or anti-Brucella mAb (50 μg/ml in PBS containing 1% bovine serum albumin (BSA); (d) incubation with goat anti-mouse or sheep anti-mouse biotinylated IgG (Amersham: diluted 1:100 in PBS containing 1% BSA) for 2 hours at room temperature; (e) incubation with streptavidin horseradish peroxidase complex (Amersham: diluted 1:100 in PBS containing 1%

BSA) for 1 hour at room temperature; (f) treatment with 0.05 mg/ml of diaminobenzidine in PBS containing 0.5% H_2O_2 for 3 minutes. Between each step, sections were washed 3 times in PBS and once in PBS containing 1% BSA. All incubations were performed in a humidified chamber. Sections were left uncounterstained, or were counterstained in Mayers' haematoxylin, dehydrated and mounted. Controls included: (a) omission of the primary antibody; (b) replacement of the anti-GH receptor/BP mouse IgG (mAb 263 or 43) by unrelated monoclonals (anti-Brucella abortus and mAb 7) of the same isotype (IgGk1) and concentration and (c) preincubation (24 hours at 4°C) of mAb 263 with a 10 molar excess of a recombinant truncated (1-238) version of the human GH receptor before application to the section.

Recombinant human GH receptor, extracellular region (1-238) produced in E. coli (Fuh et al., 1990) was kindly provided by Genentech, Inc., S. San Francisco CA.

RNA extraction

Rats were killed by cervical dislocation under CO_2 anaesthesia. 12-, 14-, 16- and 18-day-old embryos (n=8-12 per age group), adult liver (n=3) and liver from pregnant rats (n=3) were immediately removed and snap-frozen in liquid nitrogen.

Total cellular RNA was prepared by the method of Brooker et al. (1980). Briefly, tissues were homogenized in 6 M guanidinium HCl/0.2 M sodium acetate/1 mM β-mercaptoethanol (pH 5.2) followed by precipitation with two volumes ethanol. The pellets were resuspended in 6 M guanidine HCl/0.2 M sodium acetate/10 mM EDTA (pH 5.2) and precipitated with 2 vols ethanol. The resultant pellets were then resuspended in 7 M urea/10 mM Tris-HCl/0.1 mM EDTA/0.1% (v/v) SDS, pH 8.5 and a phenol/chloroform, chloroform/ether extraction was performed. The aqueous layer was adjusted to 0.1 M potassium acetate and precipitated by overnight incubation with ethanol. Remaining DNA and tRNA were extracted by washing the pellets with 2 M LiCl. RNA was spun down and washed with 70% ethanol/30% (v/v) 0.1 M potassium acetate (pH 5), recovered by centrifugation, dried under vacuum and resuspended in TE buffer (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA). RNA concentration was determined by spectrophotometry at 260 nm (Sambrook et al., 1989).

Riboprobe synthesis

The GH receptor cRNA probe used for both northern analysis and solution hybridization was a 560-bp BamHI fragment (Mathews et al., 1989) corresponding to the signal peptide and the first 167 amino acids of the GH receptor. The plasmid DNA was linearised with EcoRI to allow for transcription of antisense RNA using T3RNA polymerase with ^{35}S-labelled UTP as per Zeller and Rogers (1987). Briefly, linearized DNA (1 μg) was incubated for 30 minutes at 37°C with 4 μl of 5× transcription buffer, 0.2 μl of 1 M DTT, 60 U RNasin (Promega). 1 μl of 10 mM ATP, CTP and GTP, 100 μCi ^{35}S-UTP and 20 U T3RNA polymerase. The DNA template was then digested with RNAase-free DNAase (Promega) in the presence of RNasin and 20 μg carrier tRNA for 10 minutes at 37°C. Subsequently, 1 μl 1 M DTT, 10 μl 3 M sodium acetate (pH 5.2) and H_2O were added to a total volume of 100 μl, the labelled probe was precipitated with ethanol, centrifuged, dried and resuspended in 10 mM DTT.

Northern blot analysis

This was performed according to Selden (1987). 20 μg of total RNA was denatured in the presence of 50% formamide and electrophoresed in an agarose gel with 2.2 M formaldehyde

and 1×MOPS (0.02 M N-morpholinopropane sulphonic acid/0.05 M sodium acetate pH 7.0 with 0.001 M sodium EDTA). RNA was transferred to Hybond-N nylon membranes (Amersham) using a Vacugene apparatus (Pharmacia-LKB) and 20×SSC (3 M NaCl, 0.3 M sodium citrate). The membranes were baked at 80°C for 2 hours, prehybridized for 1 hour at 65°C in 50% formamide 2×SSC 1% SDS 20 mM DTT, with denatured salmon sperm DNA, and subsequently hybridized overnight in the prehybridization buffer following addition of the ^{35}S-labelled cRNA probe (2×10^6 cpm/ml). Membranes were then washed twice with 2×SSC, 0.1% SDS, 20 mM DTT at 65°C for 15 minutes, dried and finally autoradiographed at $-80°C$ using Kodak AR5 film with intensifying screens.

Polymerase chain reaction

This was performed as per Wang et al. (1989) in two phases as follows:

(a) Reverse transcriptase - cDNA synthesis

In a final volume of 20 µl, the reaction contained 1 µg of cytoplasmic RNA, 1 mm of each dNTP, 20 U RNAsin, 100 pmoles of random hexamer, 50 U reverse transcriptase in 10 mm Tris-HCl, pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 0.01% Tween 20 and 0.01% NP40. The sample was incubated for 10 minutes at room temperature, then 30-60 minutes at 42°C. The reaction was heated at 95°C for 5 minutes then quickly chilled on ice and PCR components added.

(b) PCR amplification

To the 20 µl cDNA reaction mixture 80 µl containing 50 pmoles of the two primers, 1 U of AmpliTaq DNA polymerase in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween 20 and 0.01% NP40 were added.

The target sequence was amplified by 30 cycles of the following thermal program: 95°C denaturation for 45 seconds, 60 seconds cooling to 45°C, annealing of primers at 45°C then 47°C for 30 seconds at each temperature, extension of the primers at 60°C for 2 minutes.

Solution hybridization/RNAse protection assays

To quantify GH receptor/BP mRNA, the ^{35}S-labelled rat cRNA probe was hybridized overnight at 70°C to RNA samples from whole embryos (E12-E18), adult liver and liver from pregnant rats as described previously (Mathews et al., 1989). Briefly the incubation (volume 40 µl) contained 0.06 M NaCl, 20 mM Tris-HCl (pH 7.5), 4 mM EDTA, 0.1% SDS, 10 mN dithiothreitol, and 25% formamide. After incubation, RNA samples were treated with 40 µg RNAase-A (Sigma) in the presence of 100 µg herring sperm DNA for 45 minutes at 37°C in a volume of 1 ml. Protected probe was precipitated by addition of 100 µl 6 M trichloroacetic acid and incubation for 20 minutes at 4°C. Precipitates were collected on glass fibre filters (GF/C, Whatman) and counted in a 1900 CA Packard liquid scintillation counter. The hybridization signal obtained with the rat GH receptor cRNA riboprobe in E12-E18 samples was expressed as percentage hybridization relative to adult rat liver RNA. RNA concentration was standardized based on absorbance at 260 nm (Sambrook et al., 1989).

Results

Specificity of immunohistochemical staining

Incubation of sections with 10% donkey serum, an unrelated primary antibody (anti-Brucella) or a non-cross-reacting rabbit GH receptor specific antibody (mAb 7) at the same or greater concentration as the test mAb did not produce a histochemical reaction (Figs 2B,3B). Both mAbs 263 and 43 (which recognise independent epitopes) were intensely immunoreactive in identical locations. Further, mAb 263 immunoreactivity was abolished by preincubation with a 10 molar excess of recombinant truncated (1-238) human GH receptor (Fig. 1).

Localisation of the GH receptor/BP

General

The GH receptor/BP displayed surface membrane, cytoplasmic and nuclear localisation (Fig. 2H), consistent with localisation of the receptor by immunogold electron microscopy in the adult to the rough endoplasmic reticulum, cytoplasm, nuclear membranes, heterochromatin and nucleolus (Lobie et al., 1991). Weak immunoreactivity was detected in the E12 embryo, and a general increase of GH receptor/BP immunoreactivity was observed from E12 to E18, with a marked increase occurring between E16 and E18. Further description of the GH receptor/BP distribution will be based on the E18 embryo. For comparison of immunoreactivity between E12 and E18 the following terms will be used; intense (4+), strong (3+), moderate (2+) and weak (1+).

Embryonic ectoderm

General body ectoderm

The cutaneous epidermal cells and their derivatives, the respiratory nasal epithelium, the epithelia lining the labia, cheeks, gingiva and palates and their respective duct-lining cells, the epithelia of the cornea and conjunctiva, secretory and duct lining cells of the lacrimal gland, the embryonic enamel organ and ameloblasts, and the epithelium of the lower anal canal of the E18 embryo were strongly to intensely immunoreactive (Fig. 1). The analogous structures in the E16 embryo were weakly immunoreactive and in E14 and E12 were immunonegative.

Neural plate ectoderm

The neurones of the central nervous system, (telencephalon, diencephalon, metencephalon and myelenchephlon) and spinal cord, of macroglial cells and ependymal cells were all immunoreactive (Fig. 2G). In the E18 embryo, the ependymal cells were intensely immunoreactive (especially those of the choroid plexus) followed by neurones (strong), whereas the developing fibre tracts were only moderately immunoreactive. All retinal cells of the E18 embryo derived from neural plate ectoderm were strongly immunoreactive. In the E16 embryo, weak to moderate immunoreactivity was observed in neurones of the central nervous system and spinal chord, with the exception of the medullary plate, which was moderately immunoreactive. The E16 ependymal cells of the choroid plexi and ventricular lining were strongly immunoreactive. Derivatives of the neural plate ectoderm in E14 and E12 embryos were weakly immunoreactive or negative.

Neural crest ectoderm

The neurones of the dorsal root and cranial ganglia were intensely immunoreactive in the E18 fetus (Fig. 2H). These were strongly immunoreactive in E16 and weak to moderately immunoreactive in E14 and E12. Schwann (ANS) cells, dentine-producing osteoblasts of the tooth germs and osteogenic elements of the cranium were all strongly immunoreactive whereas chromaffin cells of the adrenal medulla were weakly stained or immunonegative.

Ectodermal placodes

The receptor and sustentacular cells of the E18 olfactory epithelium were strongly immunoreactive, E16 moderately immunoreactive and E14 and E12 weakly immunoreactive. The epithelial walls of the E18 inner ear were intensely immunoreactive (Fig. 2). The developing lens fibres and the superficial epithelium of the lens were strong to intensely immunoreactive. Rathke's pouch and the adenohypophysis was moderately immunoreactive in the E18 embryo.

Embryonic mesoderm

Paraxial mesoderm

The developing striated musculature of the trunk (as well as limb muscles and lingual muscles that develop *in situ*) was intensely immunoreactive by E18 (Figs 1A, 2B), strongly immunoreactive (E16) or weakly immunoreactive (E14,E12). The chondroprogenitor cells of the vertebrae and other skeletal components (invertebral disks, ribs, costal cartilages) were intensely immunoreactive, whereas chondrocytes of the same structures displayed heterogeneous immunoreactivity from strong to negative at E18 (Fig. 2K). The analogous structures of younger embryos were essentially immunonegative.

Intermediate mesoderm

The renal capsules and nephric tubules were strongly immunoreactive in the E18 embryo. Epithelial components of the mesonephric (Wolffian) duct, ureter, collecting tubules, epididymis, vas deferens, seminal vesicles and paramesonephric (Mullerian) ducts (uterus) were immunoreactive. Gonadal tissues were strongly immunoreactive and cells of the adrenal cortex and mesothelium were intensely immunoreactive. The analogous structures of younger embryos followed the general age-dependent increments.

Lateral plate mesoderm

The lateral plate mesoderm becomes separated into the somatic and splanchnic layers. Mesenchymal condensations transform the somatopleuric mesenchyme into the osseous, cartilaginous, muscular, vascular, lymphatic and connective tissue of the body wall (Fig. 2). Vascular elements (endothelium and haemopoietic cells) were intensely immunoreactive at all stages of development (E12-E18) in contrast to the weak or immunonegative non-haemangioblastic tissues of E12 and E14. E18 myoblasts were intensely immunoreactive (Fig. 2J) and fibroblasts and osteo and chondro

progenitor cells were strongly immunoreactive whereas chondrocytes displayed heterogeneous immunoreactivity (Fig. 2G) (immunoreactivity, when present, was mainly associated with hypertrophic chondrocytes). The splanchopleuric mesenchyme clothes the endodermal gut tube (Fig. 2C), its subdivisions and glandular derivatives and the walls of the tracheobronchial tree (Fig. 2D) and the urinary bladder. Non-striated muscle and vascular derivatives of these structures were intensely immunoreactive (E18), as were fibroblasts.

Embryonic endoderm

All derivatives of the embryonic endoderm (salivary glands, glandular and duct lining epithelia of the esophagus, stomach, small intestine, colon, hepatocytes, pancreatic acinar and duct-lining cells, pharyngeal epithelium, tracheobronchial alveolar respiratory tree, transitional epithelium lining the urinary bladder, parenchyma of prostate) of the E18 embryo were intensely or strongly immunoreactive (Fig. 1A). The analogous structures of the E12 to E16 embryos displayed weak (E12, E14) to moderate (E16) immunoreactivity.

Placenta

Decidual cells of the E12 placenta were strongly immunoreactive (E12) and a slight gradation in intensity was observed to E18, where they were intensely immunoreactive. Trophoblastic cells and epithelial cells of the amniotic membrane were weak and moderately immunoreactive respectively at E12 and similarly progressed to display strong immunoreactivity at E18.

Northern blot analysis

Northern blots of the embryonic RNA hybridized with the rat GH receptor ^{35}S-cRNA probe revealed 3.9 and a 1.2 kb transcripts, coding for the full-length receptor and the putative BP respectively (Smith et al., 1989; Baumbach et al., 1989) (Fig. 4). In addition, variable times of exposure of X-ray film to the hybridized membranes (overnight, 3 days, 3 weeks) revealed a graded intensity of hybridization from E14 to E18, adult liver and liver from pregnant rats (Fig. 4A).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Since GH receptor mRNA was not readily detectable at E12 and E14 by conventional northern blot analysis, RT-PCR was used to demonstrate GH receptor gene expression during this period. The scheme of the experiment with the predicted size of the amplified cytoplasmic fragment is shown in Fig. 5. GH receptor mRNA is unequivocally present at E12 and E14 (Fig. 5).

Solution hybridization

Solution hybridization (nuclease protection assay) was used to quantify the changes in GH receptor/BP mRNA from E12 to E18 in whole embryos. As seen in Fig. 6,
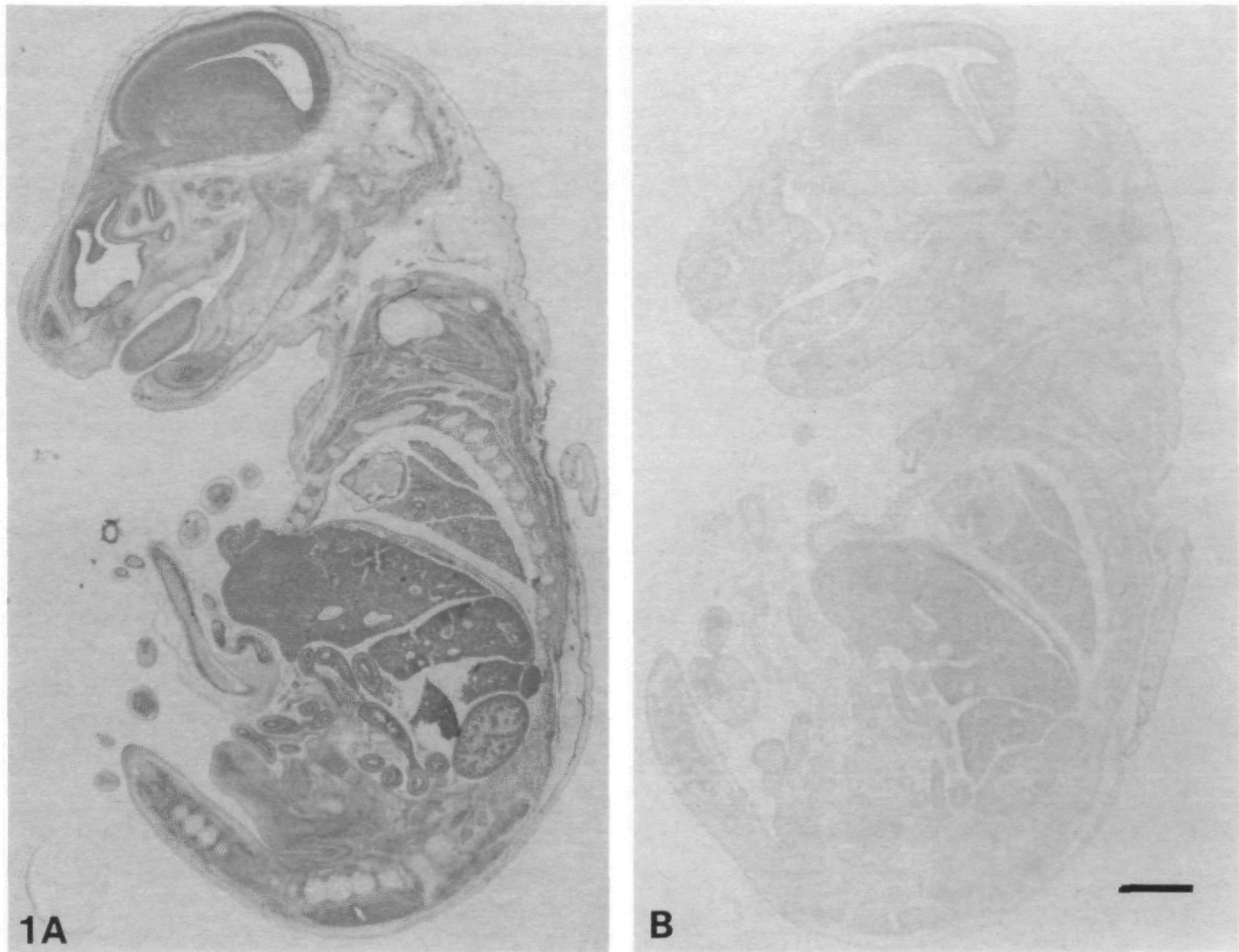
Fig. 1. Low-power photomicrograph of the E18 embryo. Magnification bar is 1000 μm. Both sections have been counterstained with haematoxylin to show nuclei. (A) mAb 263 immunoreactivity in the E18 rat fetus. Note that the immunoreactivity is associated with many organ systems. (B) Adjacent section to that of Fig. 1A when mAb 263 was preincubated (24 hours at 4°C) with a recombinant soluble form of the human GH receptor (1-238) before application to the section.
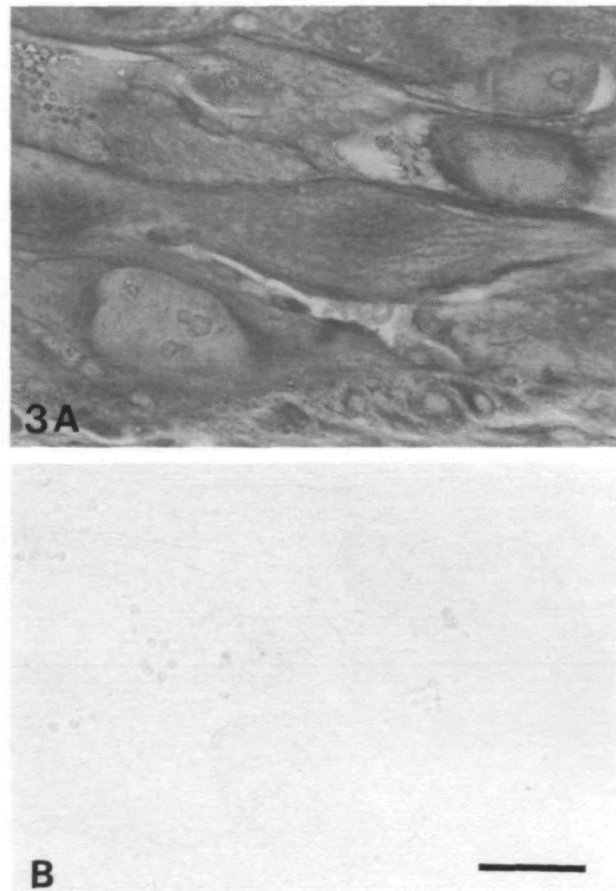
Fig. 3. (A) GH receptor/BP immunoreactivity (mAb 263) in the decidual cells of the placenta at 14 days gestation. (B) Adjacent control section (mAb 7) showing lack of immunoreactivity. The magnification bar is 100 μm.
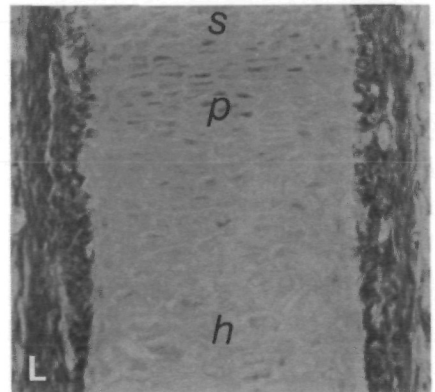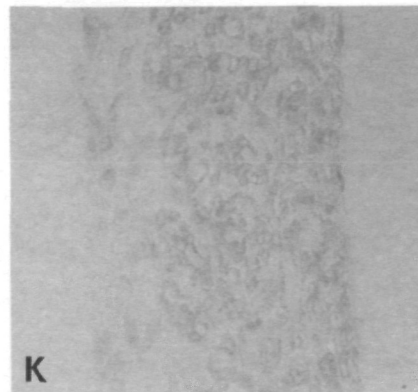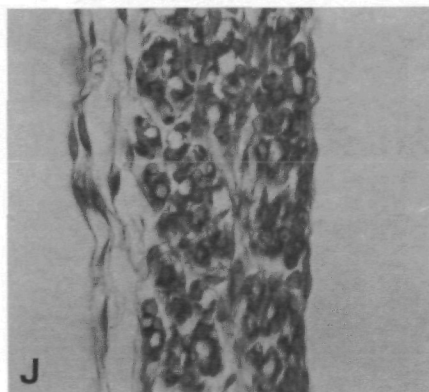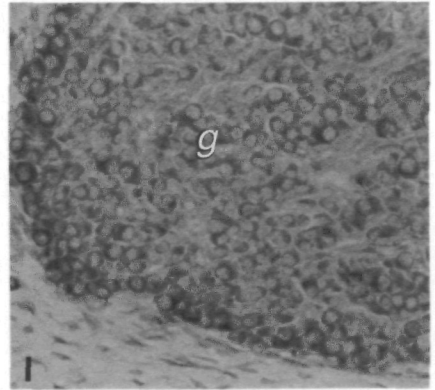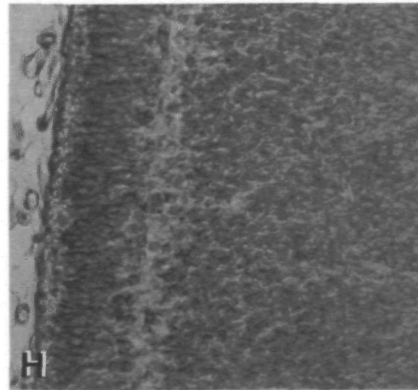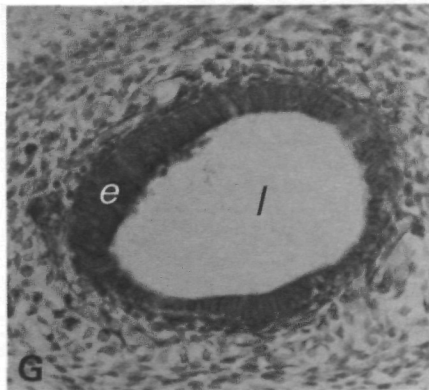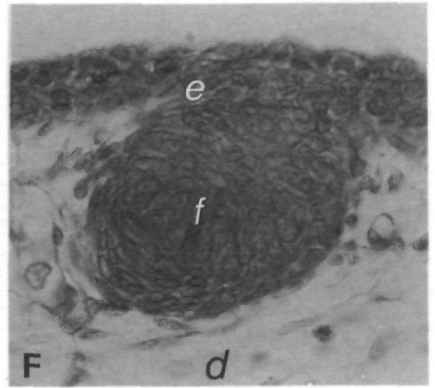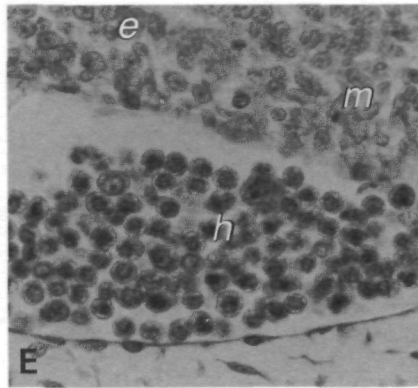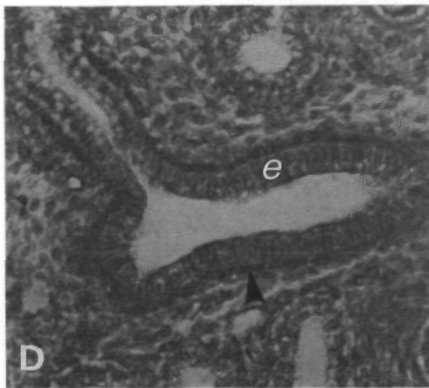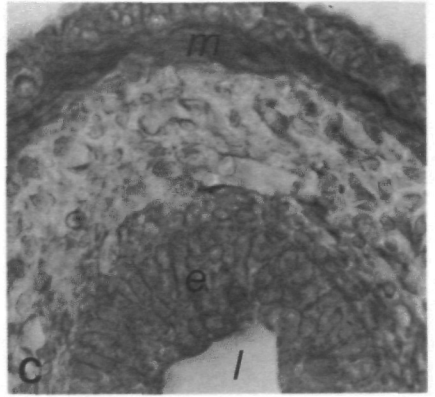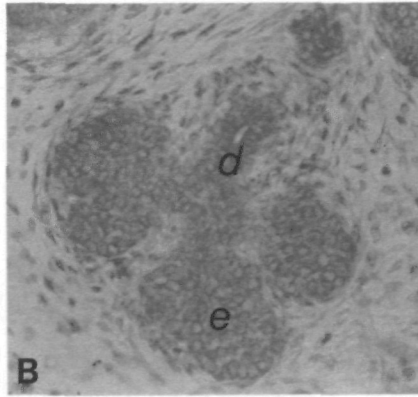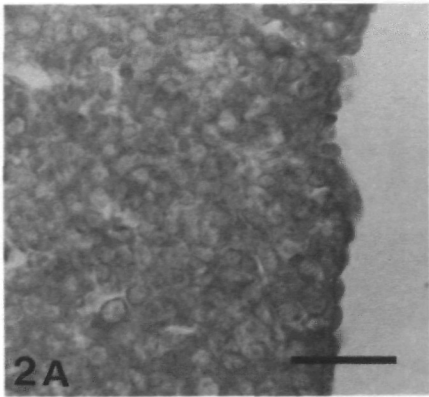
Fig. 2. GH receptor/BP immunoreactivity in the rat embryo/fetus. Magnification bar is 100 μm. (A) mAb 263 immunoreactivity in the E18 liver. (B) mAb 43 immunoreactivity in the developing submandibular gland of an E18 embryo (d=duct, e=epithelial component). (C) mAb 263 immunoreactivity in the midgut of the E18 embryo. Note the prominent immunoreactivity associated with the epithelial (e) and smooth muscular (m) components (l=lumen). (D) mAb 263 immunoreactivity in the lung of an E18 embryo. Note the prominent immunoreactivity associated with the epithelial (e) and smooth muscular (arrow) components of the developing bronchioles. (E) mAb 263 immunoreactivity in the E14 embryo. Note the prominent immunoreactivity in the haemopoietic cells (h) and vascular endothelial lining (arrow), and the weak immunoreactivity of the surrounding mesenchymal (m) and epithelial (e) tissue. (F) mAb 263 immunoreactivity in the E18 epidermis (e) and the developing hair follicle (f). Note also the prominent nuclear immunoreactivity present in some cells (d=dermis). (G) mAb 43 immunoreactivity in the developing semicircular canals of the inner ear. Note the intense epithelial (e) immunoreactivity and the moderate immunoreactivity in the underlying mesenchyme. Note also the heterogeneous immunoreactivity present in chondrocytes (c) (l=lumen). (H) mAb 43 immunoreactivity in the metencephalon of the E18 embryo. (I) mAb 263 immunoreactivity in the trigeminal ganglion (g) of the E18 embryo. Note the intense immunoreactivity associated with the ganglionic neurones. (J) mAb 263 immunoreactivity in myoblasts of the E18 embryo. (K) Lack of immunoreactivity in the E18 muscle with mAb 7 in the adjacent section to that of Fig. 2J. (L) mAb 263 immunoreactivity in the E18 epiphysial region of the femur bone. Note the absence of immunoreactivity on most of the hypertrophic chondrocytes (h) and heterogenous staining of the stem (s) and proliferative (p) zone. Note also the prominent immunoreactivity of the myotubules on both sides.
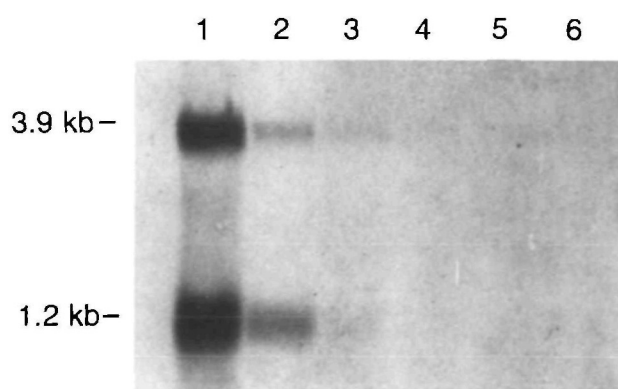

Fig. 4. Northern blot analysis of E12-E18 rat embryo and adult rat liver RNA with a rat GH receptor riboprobe (as described in Materials and methods). With a ³⁵S-labelled riboprobe the 3.9 and 1.2 kb transcripts are present from at least E16 (3 days exposure) and become more obvious with prolonged exposure (lane 1=liver from pregnant rat; 2=adult liver; 3=E18; 4=E16; 5=E14; 6=E12).

GH receptor/BP mRNA concentration, (expressed as a percentage of adult liver RNA) increased exponentially from E12 to E18.
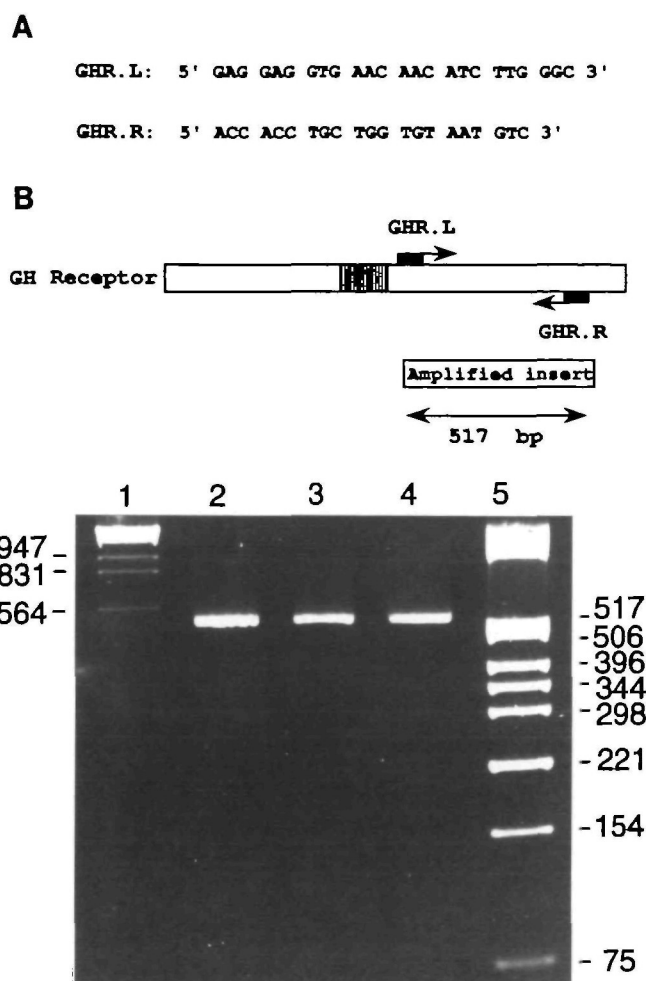

Fig. 5. (A) primer sequences. (B) Diagrammatic representation of the amplified insert taken from position 961 bp to 1478 bp in the intracellular region of the rat GH receptor (Baumbach et al., 1989). (C) The photograph shows the amplified fragment for the different age groups flanked by molecular weight markers as follows: 1. Molecular weight markers (HinfI digest of pBR.322). 2. E18. 3. E14. 4. E12. 5. Molecular weight markers (EcoRI and HindIII digest of λDNA).

Discussion

We have demonstrated a widespread distribution of the GH receptor/BP in the rat embryo, analogous to the adult (Barnard et al., 1988; Mathews et al., 1989; Lobie et al., 1990a-c; Waters et al., 1990) where GH has actions on many postnatal tissues (Isaksson et al., 1985). In the fetus the most prominent immunoreactivity was associated with epithelial and endothelial cell types, and with myoblasts, ganglion cells and haemangioblastic tissues. In support of these findings, in vitro responses of several fetal tissues to GH have been reported. These responses include; proliferation of 12-16 week fetal human hepatocytes in response to human GH (Strain et al., 1987), proliferation of day 18 fetal mouse osteoblasts (Slootweg et al., 1988), increased IGF-I and alkaline phosphatase secretion from day 18
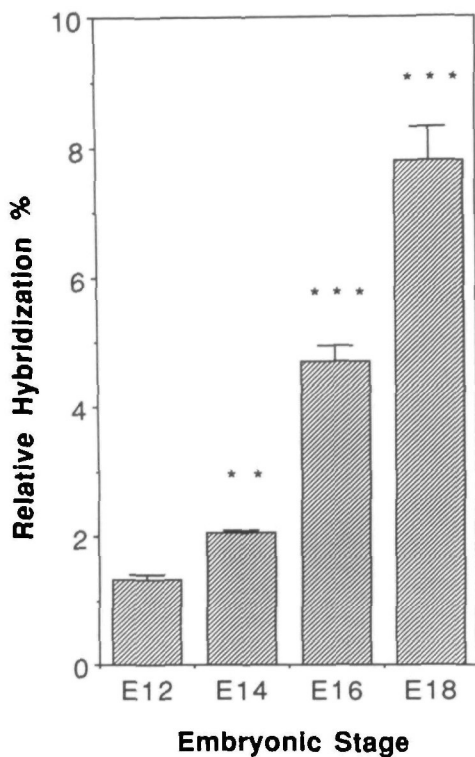
Fig. 6. Ontogeny of GH receptor mRNA during embryogenesis as determined by solution hybridization assay. The GH receptor/BP mRNA concentration is expressed as a percentage of adult rat liver mRNA hybridization to the rat GH receptor/BP cRNA probe where adult liver RNA gave 2060 cpm/μg RNA±274. Solution hybridization was performed as described in materials and methods. **$P<0.01$ between E12 and E14. ***$P<0.001$ between E16,18 and E12,14.

rat tibia (Stracke et al., 1984), increased β-islet cell proliferation in fetal rat islets (Dudek et al., 1984), elevated insulin release in human islet cell cultures (Sandler et al., 1987) and increased myelin synthesis in fetal rat brain cultures (Almazan et al., 1985).

Comparatively low levels of GH receptor/BP mRNA were detected throughout embryogenesis. However, even very low levels of GH receptor are able to mediate GH effects; for example, adult rat tibial growth plate chondrocytes possess only 5% of the GH receptor of hepatocytes yet respond to GH with enhanced IGF-I mRNA synthesis (Isgaard et al., 1988) and RIN5 pancreatic cells (Billestrup and Martin, 1985) express less than 5% of the receptor number of rat hepatocytes (Ranke et al., 1976) or adipocytes (Fagin et al., 1980) but are able to respond to GH.

Classical decapitation and hypophysectomy experiments have generally found that pituitary GH contributes less than 20% to fetal growth (see Gluckman, 1986). Therefore placental lactogen has been proposed as a growth hormone able to substitute for pituitary GH (Daughaday et al., 1979). However, in receptor assays, purified rat placental lactogen is not somatogenic (Robertson and Friesen, 1975). It is possible that a placental growth hormone (Ogilvie et al., 1990) is

responsible for fetal growth. This hormone could be analogous to the human placental GH variant of Frankenne et al. (1988), but present in fetal plasma. In preliminary experiments, we have found quite high levels of GH-like activity (>400 ng/ml) in E17 fetal plasma using a homologous rat radioreceptor assay. We propose that such a factor would act via the fetal GH receptor/BP before pituitary GH appears on E19 (Strasser and Mialhe, 1975).

Since both long and short forms of the GH receptor mRNA are present in the fetus, it seems likely that full-length receptor (Leung et al., 1987) and binding protein (Smith et al., 1989; Baumbach et al., 1989) are present. Ymer and Herington (1990) have recently reported short and long transcripts in fetal rabbit liver, skeletal muscle, heart, kidney and lung. It is intriguing to speculate on the role of the BP in a milieu lacking pituitary GH (E18), particularly in the light of our demonstration that the GH BP is present in the nucleus (Lobie et al., 1991). The role of the BP may be to interact with a placental GH to mediate its actions during embryogenesis. Since the circulating level of GH BP is low at birth (Daughaday et al., 1987) it would seem likely that any modulatory action would occur at the tissue level.

The increment in GH receptor/BP immunoreactivity and mRNA observed from E12 to E18 suggests that GH-like effects on prenatal development, are likely to occur during the later fetal period, coinciding with a state of maximal organogenesis. This suggests a differentiative as well as proliferative role for GH during fetal morphogenesis. Such a proposal is supported by the lack of villar development of fetal small intestinal transplants in hypophysectomised hosts, and subsequent normal villar morphogenesis following GH replacement therapy (Cooke et al., 1986). GH is also necessary for normal differentiation of fetal paw transplants in hypophysectomised hosts (Cooke et al., 1983).

The role of the fetal rat GH receptor/BP would be best addressed with a receptor-deficient mutant. Such a condition exists in the Laron dwarf (Godowski et al., 1989), and most newborn Laron dwarfs are greater than 2 standard deviations shorter than normal (Laron et al., 1971). This finding is consistent with the ovine hypophysectomy studies of Mesiano et al. (1987), and supports a role for GH in fetal skeletal growth. It is relevant that we found intense GH receptor immunoreactivity in skeletal chondroprogenitor cells and osteoblasts in bone undergoing intramembranous ossification, in agreement with previously mentioned in vitro studies on murine fetal bone cells (Slootweg et al., 1988) and our studies on the postnatal ontogeny of the GH receptor in the tibial growth plate (Barnard et al., 1988)

Intense GH receptor/BP immunoreactivity was seen in hemangioblastic tissue of the day 12 embryo before any other organ system exhibits prominent receptor/BP reactivity. It may be relevant that the circulatory system is the first to become functional during intrauterine development and the first to exhibit strong GH receptor expression. Golde et al. (1978) demonstrated that

nanogram concentrations of GH stimulate erythropoiesis of human erythroblasts, and GH is known to modulate lymphocyte function postnatally (Kiess, 1983). Intense immunoreactivity of E18 endothelial cells lining blood vessels in all embryonic tissues is consistent with observations (Hausman, 1989) that the number of small capillaries in muscle sections from decapitated or hypophysectomized pigs is reduced compared with muscle from control fetuses, as is the capillary/fibre ratio.

Prominent immunoreactivity was observed in both skeletal and smooth muscle precusors in our study. This finding is supported by a recent report that GH receptor mRNA is present in fetal rabbit muscle (Ymer and Herington, 1990). Mathews et al. (1989) also observed substantial levels of GH receptor/BP mRNA in postnatal skeletal muscle. Although it has been difficult to show an *in vitro* effect of GH on myoblast replication, Isgaard et al. (1989) have reported that pulsatile GH administration markedly increases IGF-I mRNA in skeletal muscle. During myogenic differentiation, the levels of IGF-I increase by 6-10 fold within 48-72 hours concurrent with an increase in IGF-I receptor and binding protein (Tolleffsen, 1989).

Our localisation of GH receptor/BP to nervous tissue beginning with the neuroepithelium is in accord with a considerable body of data supporting the concept of a GH axis in the brain, and forms the subject of a separate study (Lobie et al., 1989).

The distribution of fetal IGF-I immunoreactivity observed by Hill et al. (1989) is very similar to the GH receptor immunoreactivity that we report here. It is of interest that rat fetal IGF-I mRNA levels are higher than adult levels in all tissues examined (intestine, lung, brain) except for the liver (Lund et al., 1986) and that fetal IGF-I mRNA rises from day 11 (Rotwein et al., 1987), when GH receptor immunoreactivity and mRNA are first detected. IGF-II mRNA, on the other hand, appears somewhat earlier in fetal development and does not correlate temporally with GH receptor expression, although spatially there are many similarities (Beck et al., 1987; Stylianopoulou et al., 1988).

The observation that GH receptor/BP immunoreactivity is widely distributed in the placenta in both fetal and maternal compartments suggests that the GH receptor/BP may play an integrated role in fetal development both directly at the fetus and indirectly at the placental/nutrient supply level. Ymer et al. (1989) recently reported GH binding protein at high levels in both maternal and fetal compartments of rabbit placenta, with short and full-length GH receptor mRNA being present in the fetal placenta. These authors suggested that GH may play a role in placental metabolism. Alternatively the placental GH receptor/BP could mediate GH-dependent IGF-I synthesis as the human placenta is capable of synthesis of IGF-I (Mills et al., 1986).

In conclusion, we have demonstrated the presence of the GH receptor in the rat embryo/fetus with maximum expression of the receptor during organogenesis. The results presented here provide a basis for understanding the role of GH or GH-like hormones in fetal growth and development.

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