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Histochemical localization of IGF-I and -II mRNA in the developing rat embryo

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Summary

We describe the histological localization of embryonic and fetal tissues whose cells express the genes coding for insulin-like growth factors I and II (IGF-I and IGF-II) in the developing rat. Our studies span the period between early somite stages and full term. We have used oligodeoxyribonucleotide probes and obtained results which are both topographically precise and highly reproducible. The gene coding for IGF-II is predominant throughout development. It is strongly expressed in the liver and yolk sac. A variety of other tissues also expresses the IGF-II gene, especially many mesodermally derived structures in the process of differentiation. Many tissues do not express IGF genes. Thus no IGF mRNA was demonstrable in ectodermally derived structures, including the central and peripheral nervous systems as well as the skin and its derivatives.

Key words: *in situ* hybridization, IGF-I and IGF-II, hybridization histochemistry, growth factors.

Introduction

The nature and role of growth factors in normal mammalian development is a subject of increasing interest to developmental biologists. It has become clear that humoral factors play a significant part in prenatal growth and differentiation but, in certain species, some agents (e.g. somatotrophins) which are of prime importance after birth, seem to play a relatively small part in regulating embryonic and fetal development (Jost, 1960; Kaplan, 1983) whilst others, such as insulin, appear to have an important role at least during fetal maturation (Underwood & d'Ercole, 1984). When rat embryos are cultured in serum it can be demonstrated that a number of macromolecules passes across the extraembryonic membranes from the maternal to the embryonic surface (Huxham & Beck, 1984) and circumstantial evidence is available that some of these act as growth factors (Calvert, Pratten & Beck, 1986). In addition, embryonic tissues undoubtedly produce a variety of polypeptides which regulate cell growth, differentiation and functional maturation (see Adamson, 1983, for review).

In this paper, we identify the embryonic and fetal tissues whose cells express the genes coding for insulin-like growth factors I and II (IGF-I and IGF-II) in the developing rat.

Many of the major observations concerning the actions of IGFs have been made on normal and abnormal cells in tissue culture (Froesch, Schmid, Schwander & Zapf, 1985; Heath & Rees, 1985) and their role *in vivo* has yet to be fully elucidated. Under physiological conditions, circulating IGFs are almost entirely bound to specific carrier proteins and, in this state, they may not interact with their specific receptors (Zapf *et al.* 1979). Serum protein-bound IGFs thus seem to act as a storage pool (Froesch *et al.* 1985). Where data is available, IGF-I and -II account for over 90 % of the body somatomedins (Froesch *et al.* 1985) and it has been suggested that they are important in the growth, differentiation and regeneration of mesodermally derived tissues (Hall & Sara, 1983). It has been calculated that, in adult rats, the liver secretes more than 90 % of the total IGFs as well as being responsible for the secretion of their carrier proteins (Binoux, Hossenlop, Lassare & Seurin, 1980). The chief factors regulating IGF concentrations in adult serum are growth hormone levels

and insulin secretion but observations of postnatal animals have shown that only IGF-I levels are entirely dependent upon growth hormone. Pituitary dwarfs have very low IGF-I levels but their IGF-II is about one third normal (Zapf, Walter & Froesch, 1981). Nevertheless, these levels of IGF-II are insufficient to stimulate normal growth processes. IGF-II is less active in stimulating DNA synthesis in cultured human fibroblasts and rat osteoblasts than IGF-I (Schmid, Steiner & Froesch, 1983).

Recently, suggestions have been made that IGF-II sustains adult tissue repair and regenerative processes and also that it is of importance in prenatal development (Moses *et al.* 1980; Daughaday *et al.* 1982; Daughaday, Yanow & Kapadia, 1986; Gluckman & Butler, 1983; Brown *et al.* 1986; Lund *et al.* 1986) and in regulating placental growth and development (Sheng *et al.* 1986).

Shortly after the submission of this work, Han *et al.* (1987) published an elegant study of somatomedin mRNA localization in the human fetus. The present study confirms that many of their findings are applicable to the rat; it is also the first report of a demonstration of these growth factors in embryonic tissues as opposed to later (fetal) stages of development. Our histochemical observations (supported by Northern blot analysis) shows that IGF-II is predominant prenatally. On the other hand, we were able to show only small quantities of IGF-I mRNA in embryonic tissues and in the yolk sac.

The variety of cells and tissues in which we have demonstrated mRNA for IGF-II leads us to postulate that the proteins coded for take part in autocrine and/or paracrine processes concerned with differentiation in many (though not all) tissues of mesodermal origin as well as in the endodermally derived epithelium of the gastrointestinal and tracheobronchial systems at specific stages of development. In addition, we suggest that the embryonic liver and yolk sac may be the principal sources of circulating embryonic IGF-II.

Materials and methods

(A) *Northern blotting*

RNA preparation

Total tissue RNA was prepared using a modification of the method of Auffray & Rougeon (1980). Tissue was homogenized with a polytron in a buffer ($10\,\mathrm{ml\,g^{-1}}$) containing 6 M-urea, 3 M-lithium chloride (LiCl), 0·1 % sodium dodecyl sulphate (SDS), 10 mM-sodium acetate pH 5·6 and $20\,\mu\mathrm{g\,ml^{-1}}$ heparin. RNA was allowed to precipitate at 4 °C overnight and pelleted by spinning at $10000\,\mathrm{revs\,min^{-1}}$ at 4 °C for 30 min. The pellet was resuspended in 6 M-urea: 3 M-LiCl and pelleted again. The RNA was then suspended in 10 mM-Tris–HCl pH 7·6, 0·5 % SDS, phenol-/chloroform-extracted twice, chloroform-extracted once and then ethanol precipitated, washed with 70 % ethanol, dried and resuspended in water. The concentration of the RNA solution was calculated from the optical density value at 260 nm and the integrity checked on an ethidium-bromide-stained agarose gel.

Northern blotting and hybridization

$100\,\mu\mathrm{g}$ of total RNA from maternal liver and kidney, 18·5-day yolk sacs and 18·5-day whole embryos were electrophoresed on a 1·2 % agarose gel containing formaldehyde (Maniatis, Fritsch & Sambrook, 1982). Transfer to Hybond, N. (Amersham, UK) was carried out as described by the manufacturer.

The oligonucleotide probes for IGF-I and -II were 5′-end-labelled as described below for *in situ* hybridization. Probes were heated at 70 °C for 2 min immediately prior to use.

Prehybridization was carried out for 4 h at 45 °C in a buffer containing 5 × SSPE (20 × SSPE = 3·6 M-NaCl, 0·2 M-sodium phosphate, pH 7·7, 0·002 M Na_2EDTA), 50 % (V/V) formamide, 5 × Denhardt's solution, 0·5 % SDS and $200\,\mu\mathrm{g\,ml^{-1}}$ denatured salmon-sperm DNA. Hybridization was carried out overnight (16–20 h) at 45 °C in freshly prepared buffer containing $5\,\mathrm{ng\,ml^{-1}}$ of probe labelled to greater than $2\times10^{8}\,\mathrm{cts\,min^{-1}\,\mu g^{-1}}$. Post-hybridization washes were at 45 °C with 3 × SSPE, 0·1 % SDS for 30 min, repeated three times, followed by 1 × SSPE, 0·1 % SDS for 15 min, repeated once. Autoradiography was carried out at −70 °C using intensifying screens and hyperfilm-MP (Amersham, UK).

Molecular weights were estimated by incorporating in the gel tracts containing both denatured λ/*Hin*dIII, stained subsequently with ethidium bromide (Maniatis *et al.* 1982) as well as an RNA molecular weight ladder (BRL, UK) which was stained with methylene blue after transfer to Hybond N. Both gave almost identical weight estimates.

(B) In situ *hybridization*

Preparation of tissues

Rat embryos were collected after timed matings on every day of gestation between the 10th and full term. The morning on which vaginal plugs were observed was counted as the first day of pregnancy. Embryos were placed in aluminium foil moulds, embedded in OCT compound (Tissue Tek) and rapidly frozen at −70 °C in a mixture of hexane and dry ice. $5\,\mu\mathrm{m}$ cryostat sections were then thawed onto slides which had previously been coated with 1 % gelatin, hardened with 0·25 % formaldehyde. Slides were placed on dry ice for about 30 min to facilitate adherence of the sections whilst inhibiting ribonuclease activity. Following this, the sectioned tissues were fixed at 0 °C for 5 min in 4 % glutaraldehyde and 20 % ethylene glycol in 0·1 M-phosphate buffer at pH 7·3. Subsequently, the slides were prehybridized in hybridization buffer made up of 600 mM-sodium chloride, 50 mM-sodium phosphate (pH 7·0), 5·0 mM-EDTA, 0·2 % Ficoll, 0·02 % bovine serum albumin, 0·02 % polyvinylpyrrolidone, 0·1 % herring sperm DNA (Sigma) and 40 % formamide which had previously been deionized by mixing with $3\,\mathrm{g\,l^{-1}}$ of ion exchange resin (Bio-Rad, Richmond, CA, Cat. No. 142-

6425) and filtered. The prehybridization was done at 40°C for about 1 h, after which the slides were thoroughly rinsed in absolute alcohol, air dried and stored in ethanol vapour at 4°C for up to one week before use.

Sections of adult rat liver, kidney and pancreas were similarly prepared as tissue controls.

Synthesis of DNA probes

In order to locate IGF-II message, we used a 30 mer probe complementary to a part of the mRNA for rat IGF-II; IGF-I was investigated by means of a 30 mer probe complementary to a part of mouse IGF-I mRNA. The sequences selected for the probes were chosen to eliminate cross-reactivity. The probes were synthesized chemically by the solid-phase phosphoramidite procedure (Caruthers *et al.* 1982). The rat IGF-I DNA sequence has not been described but we are reasonably confident that the mouse probe gives acceptable results because, first, the gene is highly conserved (only three mismatches are present when the mouse probe is compared with the relevant sequence of the human IGF-I gene) and, second, the mouse IGF-I probe produced strong labelling in adult rat liver where IGF-I is known to be produced in large quantities (Schwander, Hauri, Zapf & Froesch, 1983) but not in adult rat kidney and pancreas which do not manufacture the protein to any great extent. The sequence of the IGF-II 30 mer oligonucleotide probe (5′-CTG ATG GTT GCT GGA CAT CTC CGA AGA GGC-3′) is complementary to the mRNA for the amino acid sequence 147 to 156 of the rat IGF-II precursor protein reported by Whitfield *et al.* (1984). The sequence of the IGF-I 30 mer oligonucleotide probe (5′-CTC AGC CCC GCA AAG GGT CTC TGG TCC AGC-3′) is complementary to the mRNA for the amino acid sequence −1 to +9 of the mouse IGF-I precursor protein (Bell, Stampien, Fong & Rall, 1986).

100 ng aliquots of each probe were 5′-end-labelled with 20 pmol of γ-[^{32}P]ATP using 20 units of T_4 polynucleotide kinase for 1 h. The labelled probe was purified on a Sephadex G-25 column, precipitated in ethanol with tRNA carrier, dried under vacuum and diluted to 400 ng ml^{-1} in hybridization buffer. The specific activity obtained is in the region of $6–9\times10^8$ cts min^{-1} μg^{-1} ($2–3\times10^6$ cts min^{-1} pmol^{-1}).

Hybridization

Selfcomplementary interactions were minimized by heat denaturation of the probes at 90°C immediately before application to coverslips over which the sections were then inverted. Hybridization was performed at 40°C in a humidified chamber for about 60 h. Subsequently slides were immersed and agitated in 2 × standard saline citrate (0·3 M-sodium chloride and 0·03 M-sodium citrate) to dislodge the cover slips and then washed at 40°C in single-strength standard saline citrate for 30 min, rinsed in absolute ethanol and allowed to dry.

At each stage of embryonic development, additional sections were hybridized with control probes. A 30 mer rat insulin and a 30 mer rat arginine vasopressin probe (Coghlan *et al.* 1985) were used for this purpose.

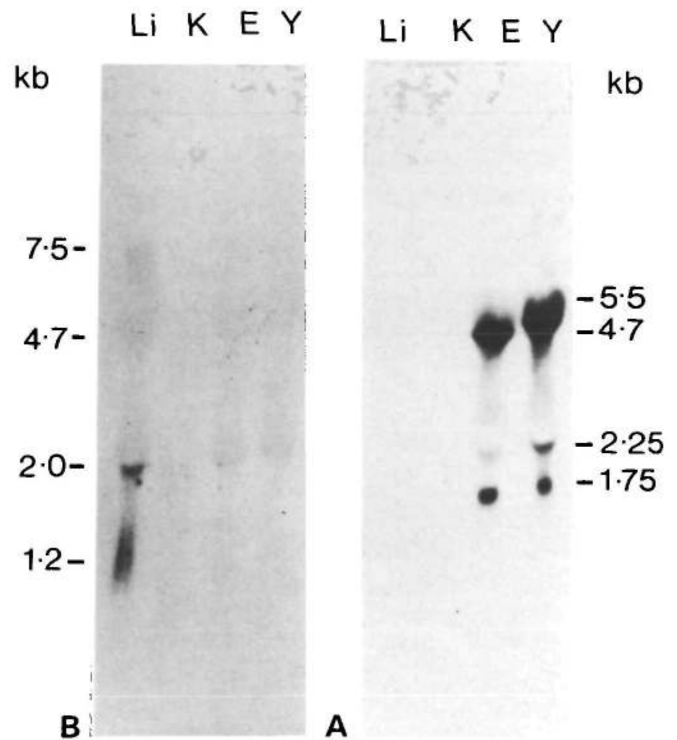

Fig. 1. Northern blots of 100 μg total RNA from adult rat liver (Li) and kidney (K), 19·5 embryo (E) and yolk sac (Y) probed with (A) IGF-II oligonucleotide probe and (B) IGF-I oligonucleotide probe.

Autoradiography

Slides were taped to a backing sheet and exposed to Kodak X-Omat AR film for 18 h in an X-ray cassette from which the intensification screen had been removed. The signal obtained by this method served as a guide for subsequent autoradiography. The slides were then dipped using Ilford K5 emulsion diluted to one-third strength, exposed on average for 4 days, developed, fixed and stained with haematoxylin and eosin. Dark-field photography was done using an Olympus BHS photomicroscope with matching bright-field exposures for orientation.

Results

(A) *Northern blot analysis*

The IGF-II probe hybridized with three distinct mRNA species (4·7 kb, 2·25 kb and 1·75 kb) in the RNA from the yolk sac and embryo (Fig. 1A). In addition, 5·5 kb RNA was detected in the yolk sac. No specific hybridization was seen in the RNA from maternal liver and kidney in the time allowed for exposure, although on dot-blot analysis (results not shown) a very weak signal was present in adult liver.

The IGF-I probe hybridized with several RNA species, strongly in maternal liver RNA and very weakly in RNA from yolk sac and embryo (Fig. 1B).

Table 1. *The distribution of IGF-II mRNA in rat embryos during somite stages (days 10·5–14·5)*

Strong labelling	Distinctly above background	Background only
Yolk sac* (Fig. 2A)	Postpharyngeal foregut and midgut endoderm	Neural groove, neural tube and neural crest
Hepatic bud		Developing brain and spinal cord (Fig. 2B)
Dermato-myotome	Cardiovascular endothelium (Fig. 2B)	Optic cup
Sclerotome (Fig. 2B)	Myocardium	Surface ectoderm, olfactory epithelium, otic vesicles
Branchial arch mesoderm		Stomatodeum and proctodeum Thyroid primordium
Septum transversum		Notochord (Fig. 2B)

* Labelling particularly associated with mesodermal component.

7·5 kb, 4·7 kb, 2·0 kb and 1·2 kb bands were identified, the 2·0 kb being the most abundant.

(B) In situ *hybridization (hybridization histochemistry)*

(1) *IGF-II*

Tables 1 and 2 show the levels of labelling obtained after probing for IGF-II message in embryonic tissues and developing fetal organs. They give an overview of its distribution but every embryonic and fetal system has not been detailed because of limitation in the quantity of sectioned material and the necessary constraints of a general article. Localization is given principally at the tissue and organ level. Papers giving detailed histological and temporal analyses by systems are in preparation.

Levels of IGF-II mRNA synthesis vary greatly between different embryonic tissues and within individual tissues at various stages of development. Control sections illustrating the absence of demonstrable hybridization with a rat insulin probe in tissue that gives positive results for IGF-II is shown in

Table 2. *The distribution of IGF-II mRNA in rat embryos during late embryonic and fetal stages (days 15·5–21·5)*

Strong labelling	Distinctly above background	Background only
Yolk sac*	Foregut and midgut epithelium	Central nervous system (Fig. 2D,E; Fig. 4) including retina. Membranous labyrinth
Liver (Fig. 2C)	Epithelium of major bronchi (Fig. 2C)	Peripheral nervous system (Fig. 2D)
Myoblasts, myotubes and skeletal muscle fibres (Fig. 2C,D)	Endothelium of proliferating blood vessels	Epithelium of skin and derivatives Epithelium of mouth and nose (including olfactory epithelium) Anal epithelium
Pre-Cartilaginous mesenchymal condensations (Fig. 3) Perichondrium and immature chrondrocytes (Fig. 2E) including branchial arch cartilage	⟶	Chondrocytes of mature cartilage (Fig. 2E)
Periosteum	⟶	Osteocytes (Fig. 2E)
Centres of intramembranous ossification		Epithelium of postbronchial respiratory tree (Figs 2C,E)
Developing sclera	Choroid plexus of 3rd (Fig. 4) and 4th ventricles	Nucleus pulposus
		Viscera after completion of histogenesis – including pancreas, thymus, kidney and ureter Developing and mature gonads

* Labelling particularly associated with mesodermal component.

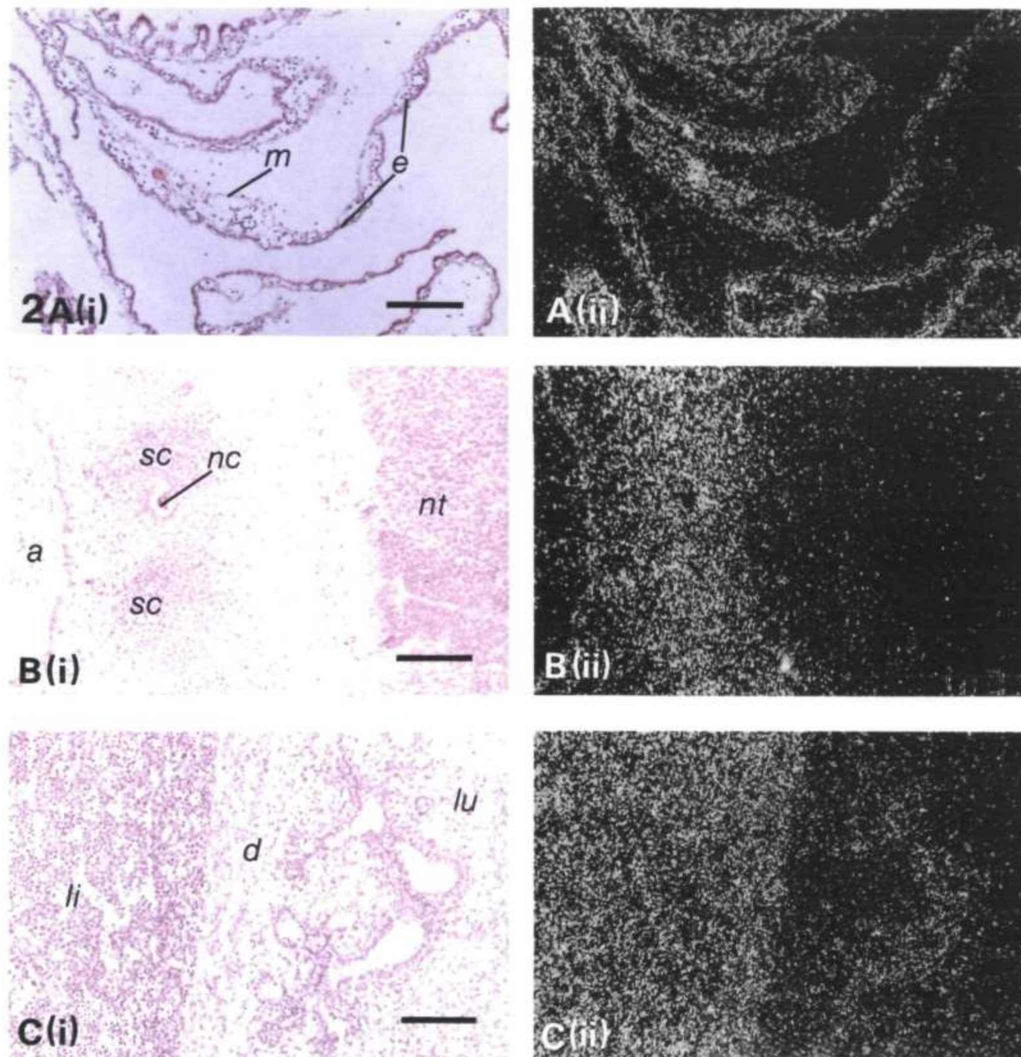

Fig. 2A–C. (A) Autoradiograph of visceral yolk sac from 13·5-day rat embryo following *in situ* hybridization with IGF-II probe. Bright field (i) shows endodermal epithelium (*e*) with underlying mesoderm (*m*) containing vitelline vessels. Dark field (ii) indicates that labelling is principally mesodermal in location. Bar for all parts, 50 μm.

(B) Autoradiograph from 14·5-day rat embryo following *in situ* hybridization with IGF-II probe. Bright field (i) shows neural tube (*nt*), including an oblique section entering the neural canal. Successive sclerotomic condensations (*sc*) are seen together with a sectioned portion of the notochord (*nc*). The aorta (*a*) lies anteriorly. Dark field (ii) shows the sclerotome heavily labelled. There is above background labelling in the aortic endothelium. Labelling of the neural tube and notochord is of background intensity only.

(C) Autoradiograph from a 17·5-day rat embryo following *in situ* hybridization with IGF-II probe. Bright field (i) shows developing lung (*lu*), diaphragm (*d*) and liver (*li*). Dark field (ii) shows strong labelling in developing diaphragmatic musculature and in the liver. The epithelial lining of major bronchi is moderately labelled but postbronchial lung tissue shows background labelling only.

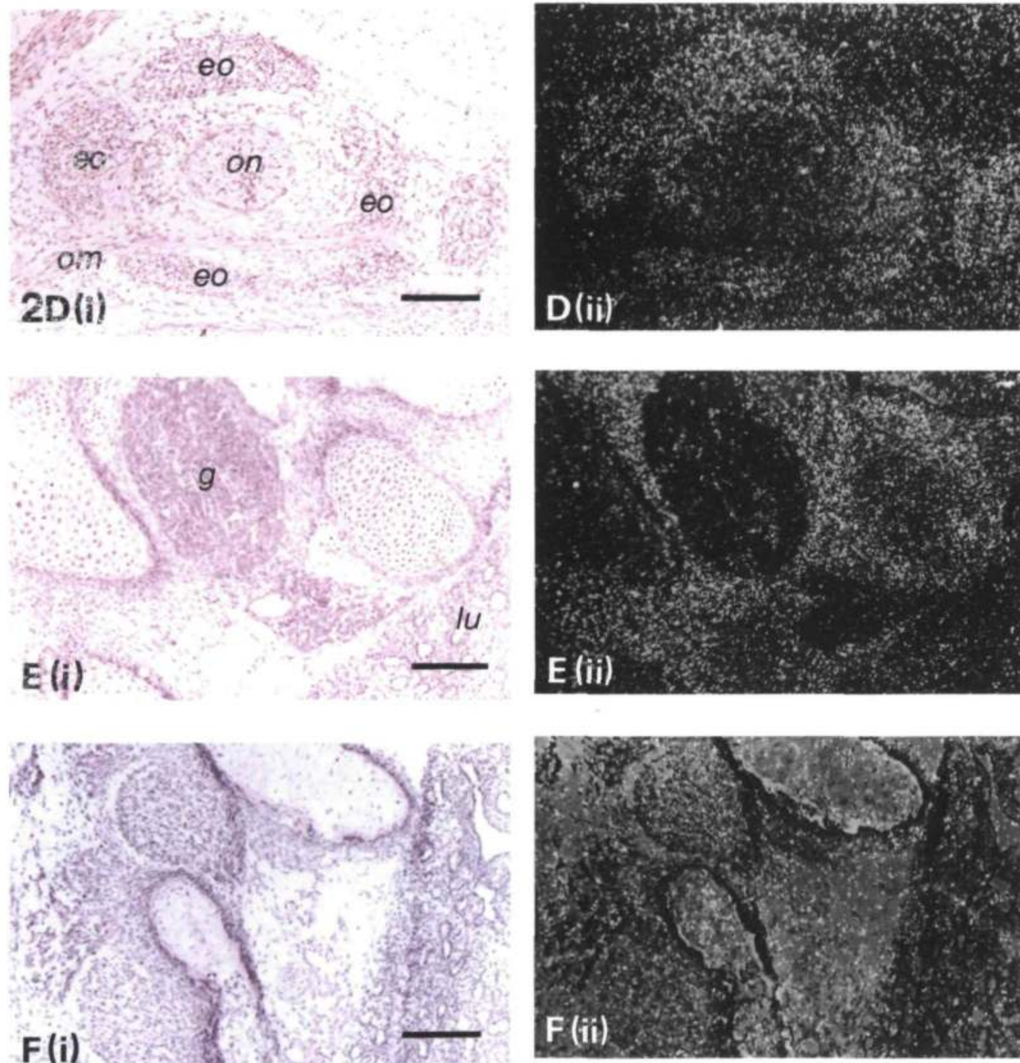

Fig. 2D–F. (D) Autoradiograph from a 17·5-day rat embryo following *in situ* hybridization with IGF-II probe. The section is cut at right angles to the optic nerve (*on*). The extraocular muscles (*eo*) surround the nerve and the oculomotor nerve (*om*) has been sectioned. Dark field (ii) shows labelling in the muscles but background labelling only in the optic and oculomotor nerves.

(E) Autoradiograph from an 18·5-day rat embryo in vertebral region at the thoracic level following *in situ* hybridization with IGF-II probe. Bright field (i) shows cartilage of vertebrae and vertebral processes. Ossification is just beginning. A developing dorsal root ganglion (*g*) and lung tissue (*lu*) are clearly visible. Dark field (ii) shows labelling of perichondrium and of proliferating chondrocytes but background labelling only of mature chondrocytes, bone, nervous tissue and lung.

(F) Autoradiograph from an 18·5-day rat embryo in a similar region to that shown in E following hybridization with an IGF-I probe. Labelling above background levels is not apparent.

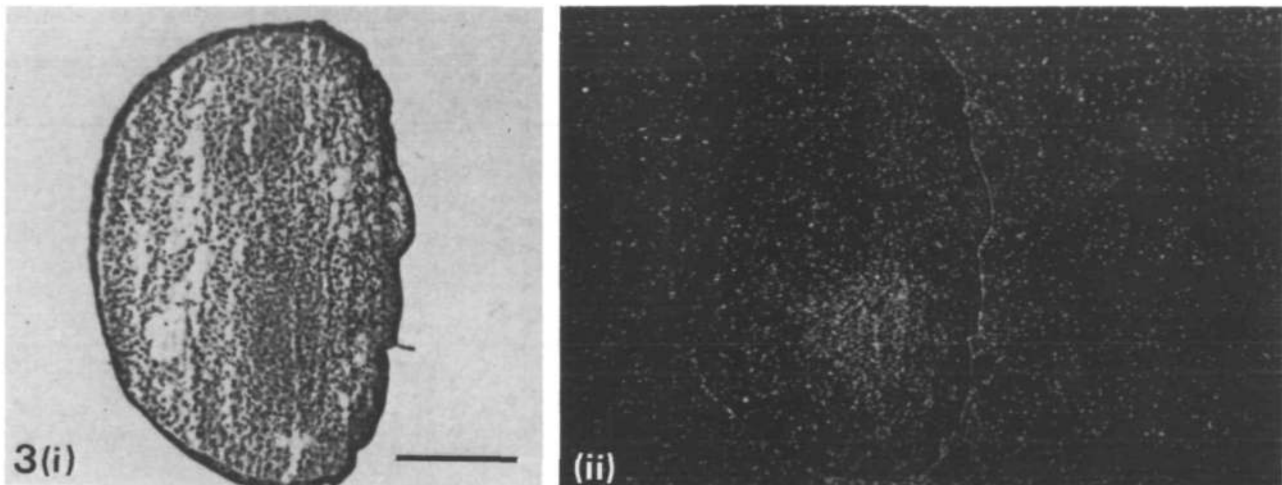

Fig. 3. Section through digit of 17·5-day embryo (i) bright field (ii) dark field showing presence of IGF-II mRNA in a region of precartilaginous mesenchyme. Bar, 50 μm.

Fig. 6A,B and the specificity of the insulin probe is confirmed by demonstrating its hybridization with fetal and adult pancreas (Fig. 6C,D).

(a) *Visceral yolk sac.* High levels of IGF-II mRNA were found in the visceral yolk sac throughout gestation (Fig. 2A). Though much of this activity appeared to be localized in the mesoderm, the endodermal epithelial cell contents also hybridized with the probe. There was no apparent alteration of distribution or density of reaction as pregnancy progressed.

(b) *Embryonic stages.* Activity in the general embryonic mesenchyme was above background but particularly high levels of probe hybridization were demonstrable in regions of the branchial arch mesoderm, the septum transversum, the sclerotomes (Fig. 2B) and the dermatomyotomes. No activity was demonstrable in the surface ectoderm, the neural tube and its derivatives, the neural crest or in the notochord (Fig. 2B).

(c) *Musculoskeletal system.* High levels of IGF-II mRNA were present in structures developing from the dermatomyotome and the sclerotome (Fig. 2B) of early embryos. At later stages, activity was clearly demonstrable in precartilaginous mesenchymal condensations (Fig. 3) and in developing cartilage, irrespective of its embryological origin. As chondrocytes assumed a differentiated appearance in lacunae of the cartilaginous matrix, they appeared to lose most of their activity (Fig. 2E). However, high activity was maintained in the perichondrium particularly where active growth was taking place. In regions of cartilaginous or membranous ossification, osteoblasts were highly active and periosteum maintained its activity throughout gestation. Osteocytes were without activity.

High activity was present throughout gestation in areas of developing striated muscle (Fig. 2C,D). Given the resolving power of the system it proved impossible at early stages of gestation to determine whether hybridization had occurred in myoblasts and myotubes or in associated fibroblasts. At later stages, there was clearly strong activity in the connective tissue cells surrounding individual muscle fibres but even at full term hybridization seems to have occurred in the muscle fibres also. It is interesting to observe that in the first branchial arch the mesoderm in the region in which the tongue will develop is strongly positive before there is evidence of muscle formation.

(d) *Gut and derivatives.* Distinct IGF-II mRNA was detectable in foregut and midgut epithelium but low levels only were demonstrable in the hindgut and none in the stomatodeum and proctodeum. The developing gut (smooth) musculature was without activity. No IGF-II mRNA was detectable in the thyroid diverticulum at early stages of development nor was any present in the thymus at 20 days or at term. Developing pancreas was without activity. The liver at all stages of development hybridized with the probe for IGF-II mRNA (Fig. 2C). Much of the activity appeared to be localized to the cells concerned with haemopoiesis and low levels only were demonstrable in the hepatocytes. In the developing respiratory system, some activity was detectable in the epithelium of the major bronchi (Fig. 2C) and in the perichondrium of cartilage but none was apparent in the smooth musculature or in the developing alveoli.

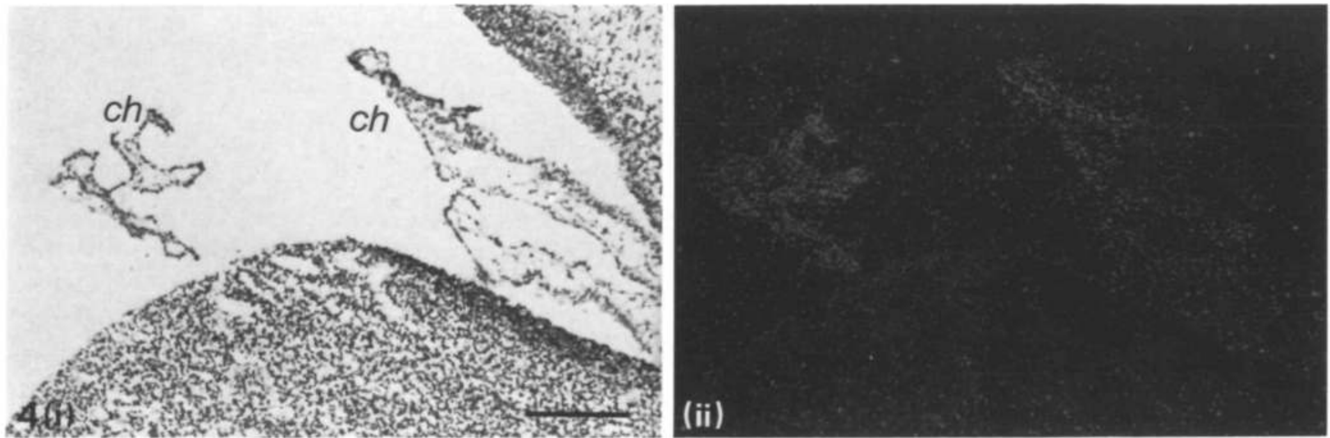

Fig. 4. Section through ventricular system of the brain in a 17·5-day embryo in the region of the choroid plexus (*ch*). Dark field (ii) shows IGF-II mRNA activity in the choroid plexus but not in neural tissue. Bar, 50 μm.

(e) *Cardiovascular system.* The mesothelium of the heart and proliferating vessels (Fig. 2B) showed activity as did the myocardium. The material available was insufficient to judge whether hybridization had occurred in the vascular coats of the major vessels.

(f) *Genitourinary system.* No activity was apparent in the mesonephric or metanephric kidney, nor was any apparent in the mesonephric duct and ureter or ureteric bud. The bladder and allantois were not sectioned. No activity was detectable in the gonadal ridge.

(g) *The nervous system.* No IGF-II mRNA was demonstrable in any part of the central or peripheral nervous systems (Fig. 2B,D,E, Fig. 4). There was no detectable activity in the nasal or olfactory epithelium or in the membranous labyrinth. The suprarenal medulla was not examined. Hybridization had occurred in the mesenchyme surrounding the optic cup in the region of the developing sclera. There was also evidence throughout gestation of activity in the choroid plexuses developing in the lateral and third ventricles (Fig. 4).

(h) *Skin.* No IGF-II mRNA was demonstrated in the epidermis or in developing hair follicles. There appeared to be above background activity in the dermis when the dematome grew into the somatopleure but this may have been an effect caused by the greater density of cells in the area compared to surrounding regions.

(2) *IGF-I and control probes*

Probing for IGF-I mRNA failed to reveal evidence of its production at most of the sites that gave a strong positive signal with the probe for IGF-II (Fig. 2F). From day 16·5 of gestation onwards extremely faint hybridization with the IGF-I mRNA was just detectable in some regions of perichondrium, skeletal muscle and in the sclera but not in other areas for which hybridization with the IGF-II probe is described above. By contrast maternal liver labelled strongly with the IGF-I directed probe (Fig. 5).

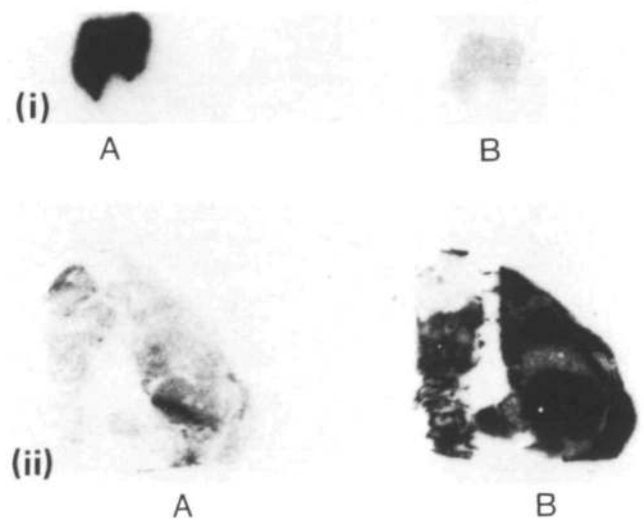

Fig. 5. Kodak X-Omat AR5 film exposed for 23 h to sections hybridized with probes for IGF-I (A) and IGF-II (B). Sections of adult liver are strongly labelled after exposure to IGF-I probe but only weakly labelled following hybridization with IGF-II probe. Sagittal sections of 18-day rat embryos are strongly labelled by the IGF-II probe (particularly over the liver) but are practically unlabelled following exposure to the IGF-I probe.

In situ hybridization with a probe for rat insulin showed specific hybridization to insulin-producing cells in the adult and 16·5-day fetal pancreas (Fig. 6C,D) but no hybridization to perichondrium in a 16·5 day embryo to which the probe for IGF-II mRNA bound strongly (Fig. 6A,B). This illustrates, formally, the specificity of the IGF-II mRNA probe.

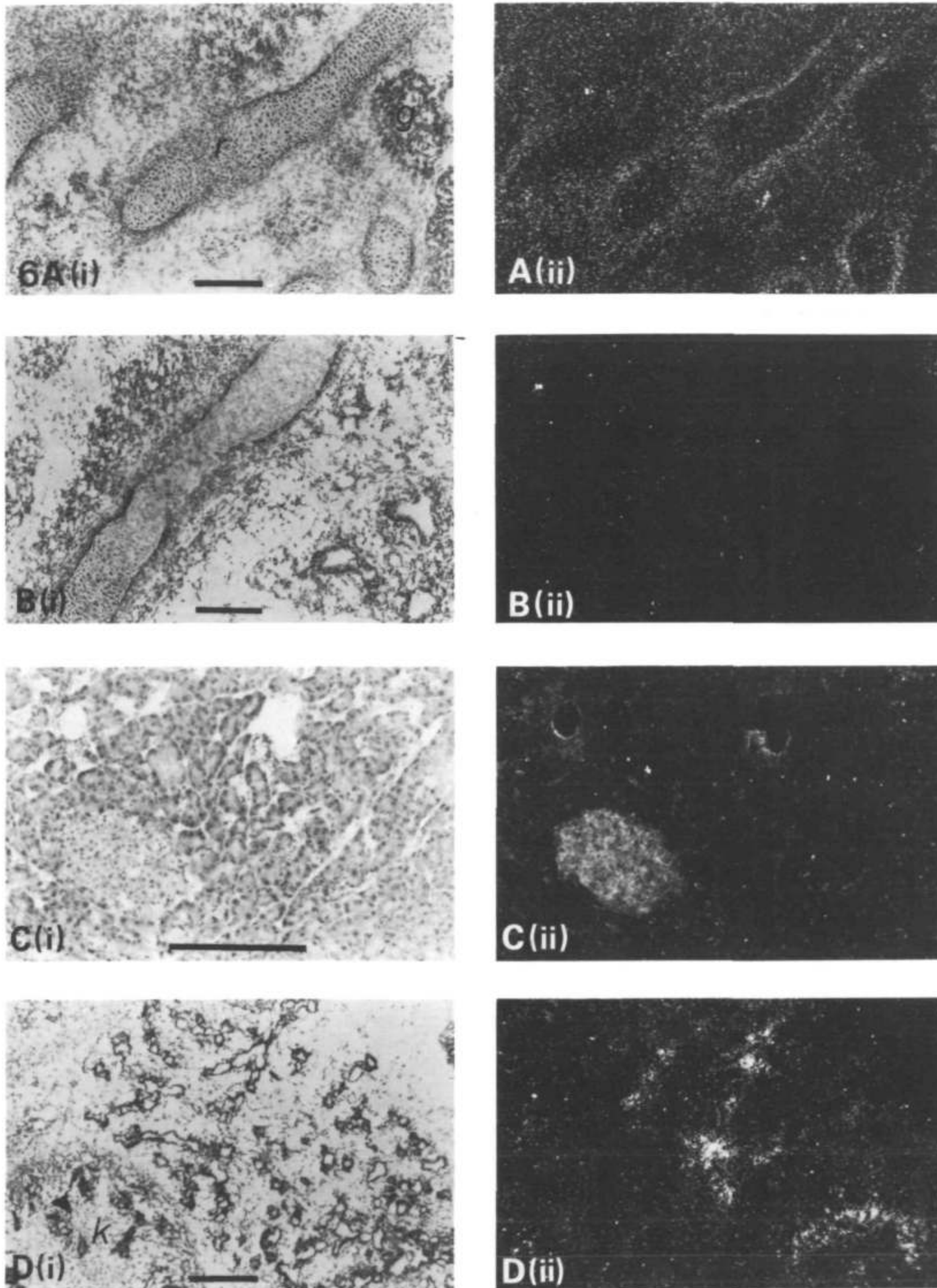

Fig. 6. (A) Autoradiograph of a section through a rib of a 16·5-day fetus following *in situ* hybridization with IGF-II probe. Bright field (i) shows ossifying rib (*r*) surrounded by developing muscle with a nearby sympathetic ganglion (*g*). Dark field (ii) shows intense labelling of perichondrium and muscle but none in the sympathetic ganglion. Bars, 50 μm for all parts.

(B) Autoradiograph from a 16·5-day fetus in a similar region to that shown in (A) following hybridization with a probe for rat insulin. Background labelling only is seen.

(C) Section through pancreas of an adult rat following *in situ* hybridization with an insulin probe. (i) Bright field and (ii) dark field showing intense labelling of an islet of Langhans.

(D) Section through the pancreas of a 16·5-day fetus following *in situ* hybridization with a probe for rat insulin. The fetal kidney (*k*) is also shown. Dark field (ii) clearly shows labelling in the developing islet region; none is present in the exocrine pancreas or in the kidney.

Discussion

Several different mRNA species have been detected by both Lund *et al.* (1986) and Brown *et al.* (1986) on probing fetal rat tissues with IGF-II cDNA probes. Despite using a 30 mer oligonucleotide probe we were able to pick up four transcripts indicating perhaps the importance of the region of the IGF-II precursor protein coding for amino acids 147 to 156. However, the exact significance of the multiple RNA species remains to be determined. Our molecular weight estimates agree with both Lund *et al.* and Brown *et al.* for the 2·25 and 1·75 kb species and with the former for the 4·7 kb species. Neither detected a 5·5 kb species although it is conceivable that this is the same as the 6 kb species detected by Brown *et al.* As with Lund *et al.* the major hybridizing species detected both in the embryo and the yolk sac was the 4·7 kb mRNA.

The IGF-I oligonucleotide probe hybridized with RNA species of 7·5 kb, 4·7 kb, 2·0 kb and 1·2 kb sizes in maternal liver RNA. These agree closely with the species detected by Lund *et al.* using a cDNA probe. The 2·0 kb species was the predominant species.

The unequivocal demonstration of IGF-II gene transcripts in certain embryonic and fetal tissues and its apparent absence from others, provides a framework on which a hypothesis for the role of somatomedins in mammalian development may be based. The high levels of labelling in both the embryonic liver and the yolk sac suggest that these organs could be the principal sources of circulating embryonic IGF-II. Alternatively, the IGF-II message in these structures may be associated with haematopoiesis (Kurtz, Jelkmann & Baner, 1982). In addition, high levels of IGF-II mRNA are present in a variety (though not all) of the differentiating tissues of mesodermal origin as well as in endodermally derived epithelium of the gastrointestinal and tracheobronchial systems at specific stages of development. Fully differentiated tissues of mesodermal origin (e.g. mature chondrocytes as opposed to the chondroblasts of the perichondrium) lose much of their ability to produce IGF-II mRNA. Therefore, it would seem possible that, in contrast to the IGF-II produced by the liver and yolk sac, the IGF-II produced by differentiating tissues is part of an autocrine or paracrine process concerned with differentiation. A study of the sites of synthesis of IGF carrier proteins and receptor sites may help to support this hypothesis.

Most areas of skeletal muscle continue to express the IGF-II gene strongly at full term when histological differentiation has occurred; this is possibly connected with the differentiation of slow twitch fibres which occurs postnatally in the rat (Rubinstein & Kelly, 1981; Kelly, Lewis, Anderson & Goldspink, 1984; Whalen *et al.* 1981). The organization of skeletal muscle at this stage of development is such that the question of whether IGF-II mRNA is principally produced by muscle fibres or by closely adjacent mesodermal cells (or both) cannot be resolved by the use of ^{32}P-labelled probes.

Numerous tissues and organs, including virtually all those of ectodermal origin (e.g. the epithelium of the skin and skin appendages, the central and peripheral nervous systems, and otocyst and membraneous labyrinth) as well as other structures of mesodermal and endodermal origin (e.g. the gonad, the mesonephric and metanephric kidney, the ureter, the thymus, the pancreas, the postbronchial respiratory tree and areas of developing smooth muscle and connective tissues associated with the gut) do not produce histochemically demonstrable quantities of IGF-II mRNA and other growth factors are probably concerned in the histodifferentiation of these structures.

The correlation between IGF-II mRNA levels and the incidence of mitosis *in vivo* is not striking. It seems unlikely, therefore, that the role of IGF-II in normal mammalian development is exclusively concerned with cell division, even though this growth factor has been shown to stimulate mitosis in a variety of cultured cells (Zapf *et al.* 1978).

The observations that choroid plexus expresses the IGF-II gene strongly is of some interest. It may provide an explanation for the presence of IGF-II in the cerebrospinal fluid of some adult primates (L. Rall, personal communication) and the demonstration by Northern blotting of IGF-II in rat brain (Lund *et al.* 1986), in spite of the fact that none appears to be produced by the cells of the central nervous system.

Our findings should be considered in relation to those reported by others. Lund *et al.* (1986) were able to detect somatomedin C/insulin-like growth factor-I related mRNAs in fetal rat intestine liver, lung and brain on Northern analysis using ^{32}P-labelled human and mouse cDNA probes. Han *et al.* (1987), on the other hand, found that hybridization signals on Northern blotting of human fetal tissue using IGF-I oligomers were 'barely detectable' and obtained extremely weak *in situ* hybridization using this probe. Our oligonucleotide probe, like that used by Han and his co-workers gave only a very weak signal for IGF-I mRNA activity in fetal tissues as well as on Northern blotting. Han *et al.* (1987) have described the cellular localization of IGF-II mRNA in human aborted fetuses between 16 and 20 weeks of gestation. Their results indicate both similarities and differences when compared with our own. Han *et al.* (1987) found IGF-II mRNA to be localized exclusively to connective

tissue or cells of mesenchymal origin but, whereas our findings confirm its presence in perichondrium, the sclera and the connective tissue associated with skeletal muscle, we also obtained hybridization in gut epithelium, bronchial epithelium, myocardium and probably in skeletal muscle fibre themselves.

Brown *et al.* (1986) have described Northern and slot blot studies in which RNAs from various rat tissues at different stages of gestation and postnatal development were hybridized with a rat IGF-II cDNA probe. The results are reasonably consistent with those reported here if it is assumed that those tissues that hybridized at low levels only in Brown *et al.*'s studies failed to produce a positive *in situ* signal in our studies.

Our findings are in broad agreement with those of Scott *et al.* (1985) who reviewed the expression of the gene coding for IGF-II based on dot blot and Northern blot analysis of RNA from human first trimester therapeutic abortions. The major discrepancy between our findings and those of Scott *et al.* (1985) is the complete absence of demonstrable IGF-II in rat kidney in our material. A more extensive study of both human and rat tissue at critically timed stages of gestation is necessary before it can be stated that a real difference in gene expression between the two species exists.

No attempt at quantification in various fetal tissues has been made in the present study. Such an approach would be feasible by serially diluting the labelled probe used for hybridization with increasing quantities of 'cold' probe. An 'extinction' level for demonstrable hybridization under controlled conditions could thus be established and used to compare levels of mRNA in various tissues. The methodology is tedious and clearly not appropriate for the general study reported here; it should, however, have a place in a detailed study of changing levels of mRNA in specific tissues at various stages of development.

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References

ADAMSON, E. D. (1983). Growth factors in development. In *The Biological Basis of Reproductive & Developmental Medicine* (ed. J. B. Warshaw), pp. 307–336. London: Edward Arnold.

AUFFRAY, C. & ROUGEON, F. (1980). Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumour RNA. *Eur. J. Biochem.* **107**, 303–314.

BELL, G. I., STAMPIEN, M. M., FONG, N. M. & RALL, L. B. (1986). The sequences of cDNAs encoding two different mouse insulin-like growth factor/precursors. *Nucleic Acids Research* **14**, 7873–7882.

BINOUX, M., HOSSENLOP, P., LASSARE, C. & SEURIN, D. (1980). Somatomedin production by rat liver in organ culture. I: Validity of the technique. Influence of the released material on cartilage sulphation. Effects of growth hormone and insulin. *Acta Endocrinol.* **93**, 73–82.

BROWN, A. L., GRAHAM, D. E., NISSLEY, S. P., HILL, D. J., STRAIN, A. J. & RECHLER, M. M. (1986). Developmental regulation of insulin like growth factor II mRNA in different rat tissues. *J. biol. Chem.* **261**, 13 144–13 150.

CALVERT, N. I. P., PRATTEN, M. K. & BECK, F. (1986). Trophic factors in rat serum and embryonic development. *Biochem. Soc. Trans.* **14**, 980–981.

CARUTHERS, M. H., BEAUCAGE, S. L., EFCAVITCH, J. W., FISHER, E. F., GOLDMAN, R. A., DE HASETH, P. L., MANDECKI, W., MATTEUCCI, M. D., ROSENDAHL, M. S. & STABINSKY, Y. (1982). Chemical synthesis and biological studies on mutated gene-control regions. *Cold Spring Harbor Symp. quant. Biol.* **47**, 411–418.

COGHLAN, J. P., ALDRED, P., HARALAMBIDIS, J., NIALL, H. D., PENSCHOW, J. D. & TREGEAR, G. W. (1985). Hybridization histochemistry. *Analyt. Biochem.* **149**, 1–28.

DAUGHADAY, W. H., PARKER, K. A., BOROWSKY, S., TRIVEDI, B. & KAPADIA, M. (1982). Measurement of somatomedin-related peptides in fetal, neonatal and maternal rat serum by IGF-II RIA, IGF-II RRA and MSB-RRA after acid-ethanol extraction. *Endocrinology* **110**, 575–581.

DAUGHADAY, W. H., YANOW, C. E. & KAPADIA, M. (1986). Insulin-like growth factors I and II in maternal and fetal guinea pig serum. *Endocrinology* **119**, 490–494.

FROESCH, E. R., SCHMID, CH., SCHWANDER, J. & ZAPF, J. (1985). Actions of insulin-like growth factors. *A. Rev. Physiol.* **47**, 443–467.

GLUCKMAN, P. D. & BUTLER, J. H. (1983). Parturition-related changes in insulin-like growth factor-I and factor-II in the perinatal limb. *J. Endocrinol.* **99**, 223–000.

HALL, K. & SARA, V. R. (1983). Growth and somatomedins. In *Vitamins and Hormones*, vol. 40, pp. 175–233. New York: Academic Press.

HAN, V. K. M., D'ERCOLE, A. J. & LUND, P. K. (1987). Cellular localisation of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science* **236**, 193–197.

HEATH, J. K. & REES, A. R. (1985). Growth factors in mammalian embryogenesis. In *Growth Factors in Biology & Medicine, Ciba Foundation Symposium*, **116**, pp. 3–14. London: Pitman.

HUXHAM, I. M. & BECK, F. (1984). Characterisation of exocoelomic fluid protein from rat conceptuses cultured in rat and human sera: a measure of yolk sac activity

during organogenesis. *J. Embryol. exp. Morph.* **84**, 203–215.

JOST, A. (1960). The role of fetal hormones in prenatal development. *Harvey Lect.* **55**, 201–226.

KAPLAN, S. A. (1983). Ontogenesis of hormone action. In *The Biological Basis of Reproductive & Developmental Medicine* (ed. J. B. Warshaw), pp. 291–305. London: Edward Arnold.

KELLY, F. J., LEWIS, S. E. M., ANDERSON, P. & GOLDSPINK, D. F. (1984). Pre- and post-natal growth and protein turnover in four muscles of the rat. *Muscle & Nerve* **7**, 235–242.

KURTZ, A., JELKMANN, W. & BANER, CH. (1982). A new candidate for the regulation of erythropoiesis. *FEBS Lett.* **149**, 105–108.

LUND, P. K., MOATS-STAATS, M., HYNES, M. A., SIMMONS, J. G., JANSEN, M., D'ERCOLE, A. J. & VAN WYK, J. J. (1986). Somatomedin C/insulin like growth factor I and insulin like growth factor II mRNAs in rat fetal and adult tissues. *J. biol. Chem.* **261**, 14539–14544.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (ed.) (1982). *Molecular Cloning, a Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

MOSES, A. C., NISSLEY, S. P., SHORT, P. A., RECHLER, M. W., WHITE, R. M., KNIGHT, A. B. & HIGA, O. Z. (1980). Increased levels of multiplication-stimulating activity, and insulin-like growth factor, in fetal rat serum. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3649–3653.

PENSCHOW, J. D., HARALAMBIDIS, J., ALDRED, P., TREGEAR, G. W. & COGHLAN, J. P. (1986). Localisation of gene expression using C.N.S. hybridization histochemistry. *Methods in Enzymology* **124**, 534–548.

RUBINSTEIN, N. A. & KELLY, A. M. (1981). Development of muscle fibre specialization in the rat hind limb. *J. Cell Biol.* **90**, 128–144.

SCHMID, CH., STEINER, TH. & FROESCH, E. R. (1983). In *Insulin-like Growth Factors/Somatomedins*, pp. 421–429. New York: de Gruyter.

SCHWANDER, J., HAURI, C., ZAPF, J. & FROESCH, E. R. (1983). Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. *Endocrinology* **113**, 297–305.

SCOTT, J., COWELL, J., ROBERTSON, M. E., PRIESTLEY, J. M., WADEY, R., HOPKINS, B., PRITCHARD, J., BELL, G. I., RALL, L. B., GRAHAM, C. F. & KNOTT, T. J. (1985). Insulin-like growth factor-II gene expression in Wilms tumour and embryonic tissues. *Nature, Lond.* **317**, 260–262.

SHEN, S.-J., WANG, C. Y., NELSON, K. K., JANSEN, M. & ILAN, J. (1986). Expression of insulin-like growth factor II in human placentas from normal and diabetic pregnancies. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9179–9182.

UNDERWOOD, L. E. & D'ERCOLE, A. J. (1984). Insulin and insulin-like growth factors/somatomedins in fetal and neonatal development. *Clinics in Endocrinology and Metabolism* **13**, 69–89.

WHALEN, R. G., SELL, S. M., BUTLER-BROWN, G. S., SCHWARTZ, K., BOUVERET, P. & PINSET HARSTROM, I. (1981). Three myosin heavy-chain isozymes appear sequentially in rat muscle development. *Nature, Lond.* **292**, 805–809.

WHITFIELD, H. J., BRUNI, C. B., FRUNZIO, R., TERRELL, J. E., NISSLEY, S. P. & RECHLER, M. M. (1984). Isolation of cDNA clone encoding rat insulin-like growth factor-II precursor. *Nature, Lond.* **312**, 277–280.

ZAPF, J., SCHOENLE, E., JAGARS, G., SAND, I., GRUNWALD, J. & FROESCH, E. R. (1979). Inhibition of the action of non-supressible insulin-like activity on isolated rat fat cells by binding to its carrier protein. *J. Clin. Invest.* **63**, 1077–1084.

ZAPF, J., WALTER, H. & FROESCH, E. R. (1981). Radioimmunological determination of insulin-like growth factors I and II in normal subjects and in patients with growth hormone disorders and extra-pancreatic tumour hypoglycaemia. *J. Clin. Invest.* **68**, 1321–1330.

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